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Effects of Anti-Ethylene Compounds on Vitrification and Genome Fidelity of *Stevia rebaudiana* Bertoni

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SUBCULTURE repeats was essential prerequisite for mass micropropagation in *stevia* but it accompanied by vitrification of the cultured shoots. Increase the number of shoots/explant without vitrification was detected when nodal segments of *in vitro* obtained shoots were subcultured twice, three weeks each, on Murashige and Skoog (MS) medium with 0.25mg/L BAP. From the third subculture, shoot multiplication decreased and vitrification increased as the number of subcultures increased; where 40% of the formed shoots of 10th subculture were vitrified. Decrease of water potential by polyethylene glycol (PEG) increased the incidence of shoot vitrification but shortened the period of subculture from three to two weeks delayed its appearance up to sixth subcultures. Also, application of anti-ethylene agents [$AgNO_3$, $CoCl_2$ or salicylic acid (SA)] decreased vitrification problems up to six subcultures. The best results were obtained by 1.7mg/L $AgNO_3$ where verification was reduced without significant reduction in the number of shoots/explant and shoot growth. The equivalent concentration of $AgNO_3$ in nano-particles form (AgNPs) showed a drastic increase in shoot vitrification (20%).When shoots of the 1st, 4th, 7th and 10th subcultures were subjected to genome amplification using RAPD or ISSR primers, the registered polymorphism under the influence of 1.7mg/L $AgNO_3$ was lower than that of 1.7mg/L AgNPs; both of them were lower than that of shoots cultured on anti-ethylene free medium (control).

Keywords: Anti-ethylene compounds, Micropropagation, Molecular markers, Stevia, Vitrification.

Introduction

The interest of researchers is increased to get sugar alternatives to cover the prevalence of obesity and diabetes among human kind all over the world. *Stevia rebaudiana* Bertoni is a sweet perennial herb originated in Paraguay and belongs to the family Asteraceae. Increases human interest in *stevia* has led to the spread of plant cultivation in many tropical and sub-tropical countries. The sweet taste of the plant is due to the presence of specific chemical compounds namely glycosides (steviosides and rebaudioside). For its sweet taste, steviosides are used in many food and pharmaceutical industries (Yu et al., 2009; Liu et al., 2010). Steviosides sweetness is 300 times higher than sucrose (Crammer & Ikan, 2003). Rebaudioside sweetness is lower than that of steviosides where it is 200 times higher than sucrose (Geuns Jan, 2003). Steviosides and rebaudioside are characterized as non-toxic, non-mutagenic, low calorie and stable chemical compounds. They are better than old sugar substitutes such as xylitol or sorbitol (Yamazaki & Flores, 1991; Lyakhovkin et al., 1993; Toyoda et al., 1997).

Propagation of stevia via seeds is not recommended because germination of *stevia* seeds is very low (Carneiro et al., 1997). Also, propagating through vegetative procedure is limited where low number of individuals can be obtained from each donor plant (Sakaguchi &

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Kan, 1982). Consequently, *in vitro* techniques are recommended for large scale sativa propagation. Generally, microprpagation is the fastest and most efficient procedure to obtain high number of plants in short time all year (Badr-Elden, 2017). In addition, *in vitro* obtained plants are pathogens-free and homogenous in terms of glycoside contents (Sivaram & Mukundan, 2003). Genetically stable and breeding lines were obtained when plant micropropagation was executed using axillary buds or shoot tips (George & Sherrington, 1984). *In vitro* clonal propagation of stevia was also established using other explant types such as leaf, nodal and internodal segments (Akita et al., 1994; Salim et al., 2006; Attaya, 2017).

Hyperhyricity or vitrification is a type of morphological and physiological disorders that are commonly detected under tissue culture conditions. Culture conditions require a sterile culture environment that may result in increase of the relative humidity, lack of oxygen and increased ethylene in the plant growth container (Leshem et al., 1988; Chen & Ziv, 2001; Rojas-Martínez et al., 2010; Salem, 2016; Hassanein et al., 2018). Vitrification of the *in vitro* cultured shoots leads to reduction of shoot multiplication, necrosis of shoot tips and loss of apical dominance, retardation of plant acclimatization and limitation of *in vitro* technique potential for mass propagation (Kevers et al., 2004; Ivanova & van Staden, 2008; Salem, 2016; Hassanein et al., 2018). Also, vitrification reduces cell cycle, increases water content, impairs stomatal function and results in abnormal cell wall lignifications (Kevers et al*.,* 2004).

Medium containing polyethylene glycol (PEG) was used to obtain culture environment with varying water potentials for controlling vitrification in *Petunia* (Thomas et al., 1991). In addition, improving aeration or reducing the relative humidity within culture containers and increasing the concentration of agar in cultured medium was exercised to control vitrification (Debergh et al., 1981; Saez et al., 2012; Salem, 2016). Recently, application of anti-ethylene signaling compounds such as $CoCl_2$, $AgNO_3$, silver nanoparticles or salicylic acid (SA) decreased vitrification phenomenon on *in vitro* induced shoots (Syu et al., 2014; Isah, 2015; Hassanein et al*.,* 2018) but expressed different values of genome un-stability especially under long term cultures (Kaeppler et al., 2000; Hassanein et al*.,* 2018). Many events cause genome instability such as chromosomal changes in number or structure, transposable elements and genetic events pre-exist in somatic cells of the donor plant (Isah, 2015). Many molecular techniques have been proposed to detect somaclonal variation, such as Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR). (Isah, 2015; Hassanein et al., 2018).

Mass micropropagation requires several subcultures but it may be accompanied by problems such as vitrification and somaclonal variation. Several strategies using different plant species were investigated to understand these problems (Debergh et al*.,* 1981; Ivanova et al*.,* 2006; Ivanova & Van Staden, 2010; Salem, 2016; Hassanein et al., 2018). Since these aspects were not extensively studied in *stevia* especially under long term culture, the aim of this research was to know how the implementation of anti-ethylene signaling compounds such as $AgNO_3$, in its ionic- or nano- form, $CoCl_2$ and salicylic acid can affect vitrification under long-term culture. The study also extended to know the effect of $AgNO_3$, as anti-ethylene signaling agent, in its ionic-or nano-form on genome stability of stevia plants under long-term culture.

Materials and Methods

Plant material and cultures establishment:

Stevia rebaudiana plants were supplied from Sugary Plants Department, Agricultural Research Center, Cairo, Egypt. Leaves were discarded and shoot segments (2-3cm length) with two or three nodes were sterilized in EtOH (70% v/v) for 30 seconds, sodium hypochlorite (commercial bleach; 5 % v/v) for 5min and $HgCl_2$ (0.2% w/v) for 2min. To remove disinfectant agents, shoot segments were rinsed three times in sterilized deionized H_2O. Sterilized segments were cut into 1 cm explants (each with one node) and cultured on MS (Murashige & Skoog, 1962) medium fortified with 0.5mg/L BAP for three weeks. Cultures were incubated under tissue culture room conditions (24 ± 2ºC, a photoperiod of 16hrs light and 8hrs dark and irradiated by 100µmol m^{-2} s^{-1}). *In vitro* obtained plant materials were subcultured to study the effect of several factors on stevia micropropagation.

In all experiments of multiplication, four replicates per each treatment have been taken, each with ten explants and the following parameters were determined: percentage of shoots survival, number of shoots/explant, length of shoot (cm), number of

nodes/shoot, number of leaves/shoot, fresh mass/shoot cluster (g) and percentage of vitrified shoots.

Effects of different BAP concentrations on stevia multiplication

Nodal segments of *in vitro* obtained shoots were cultured on MS medium fortified with different BAP concentrations (0, 0.25, 0.5, 1, 1.5, 2 or 3mg/L) for two weeks.

Mass multiplication through subcultures

Nodal segments of *in vitro* obtained shoots were subcultured for three times on MS medium supplemented with 0.25mg/L BAP for three weeks.

Effect of polyethylene glycol on shoot multiplication and growth

Nodal segments of *in vitro* grown shoots were cultured on MS medium supplemented with 0.25mg/L BAP and different concentrations of polyethylene glycol (0, 5, 10, 15, 20, 25 or 30g/L) for three successive subcultures, three weeks each.

Shoot multiplication and growth as influenced by different concentrations of different anti-ethylene compounds

Nodal segments of the second subculture were transferred to MS medium contained 0.25mg/L BAP and different concentrations of $AgNO_3$ or AgNP (1.7, 4.25 or 12.74mg/L), $CoCl_2$ (11.9, 23.8 or 47.6mg/L), or salicylic acid (6.9, 13.8 or 27.6mg/L) for three weeks. Solution of silver nano particles and salicylic acid were added to media after autoclaving. Silver nano particles were biologically synthesized and characterized by El-Deeb et al. (2013).

Shoot multiplication and growth as influenced by selected concentrations of anti-ethylene compounds under long term culture

Nodal segments of third subculture were subcultured on MS medium supplemented with 0.25mg/L BAP and selected concentrations of $AgNO_3$ or AgNPs (1.7mg/L), $CoCl_2$ (47.6mg/L), or salicylic acid (6.9mg/L) for two weeks.

Induction of root formation on shoot cuttings

Shoot segments (2cm length) from *in vitro* grown shoots were cultured on MS medium enriched with different indole butyric acid (IBA) concentrations (0, 0.5, 1 or 2mg/L). Four replicates with ten shoots each were rooted for two weeks under tissue culture conditions. Frequency of root formation, number of roots/shoot, length of root system and length of shoot were determined.

DNA extraction for molecular analysis

Microshoots of first (on MS + 0.25mg/L BAP), fourth, seventh and tenth subcultures on MS supplemented with 0.25mg/L BAP + 1.7mg/L $AgNO_3$ or AgNPs were subjected to DNA extraction for RAPD and ISSR analysis. Extraction was carried out using modified cetyltrimethyl ammonium bromide (CTAB) protocol according to Porebski et al. (1997).

RAPD-PCR analysis

For RAPD analysis, DNA amplification was performed in PCR tube with 25µl total volume containing 12.5µl master mix, 6.5µl deionized H_2O, 3µl primer and 3µl template DNA. Nine RAPD primers (OPA-03, OPA-05, OPA-07, OPA-13, OPA-17, OPC-02, OPC-05, OPD-01 and OPD-18) were used to amplify the genomic DNA to detect its genome stability under the impact of silver ions or silver nanoparticles. Amplification was accomplished in a Perkin-Elmer/GeneAmp® PCR system 9700 (PE Applied Biosystems, Foster City, USA). Amplification program was accomplished through 40 cycles after an initial denaturation cycle at 94ºC for 5min. Each cycle consisted of a denaturation step (45sec at 94ºC), an annealing step (50sec at 36ºC), and an elongation step (1min at 72ºC). The primer extension was fulfilled for 7min at 72ºC in the final cycle.

ISSR-PCR analysis

Five ISSR [ISSR1 $(AC)_8CTG$, ISSR2 $(CA)_8AAGG$, ISSR3 $(GA)_8CTG$, ISSR4 $(GA)_8CTC$ and ISSR5 $(GTG)_3GC$] primers were used to amplify the genomic DNA templates extracted from microshoots grown on the previous conditions. The ISSR-PCR technique was performed as described by Nagaoka & Ogihara (1997). DNA amplification reactions were carried out in 25µl end volumes containing the same components of RAPD reactions except that primers were exchanged by ISSR primers. The amplification was accomplished by 40 cycles of 1min at 95°C (denaturation), 1min at 48°C (annealing), and 2min at 72°C (extension), and final incubation step at 72°C for 10min to insure that the primer extension reaction goes on completion.

Visualization of DNA bands

The amplified PCR products were visualized by electrophoresis using 1.5% (w/v) agarose gel

supplemented with 0.5 µg/ml ethidium bromide in 1x TBE (750mM Tris-HCl, 900mM boric acid and 2mM Na_2-EDTA) buffer for RAPD products. For detection of ISSR products, 2% (w/v) agarose gels were used. Electrophoresis was carried out for 1.5hrs using 90V for detection of RAPD products and 70V for ISSR products. The obtained DNA banding patterns were analyzed by the MVSP computer software program of Nei & Li (1979).

Statistical analysis

In a completely randomized design, experiments were planned. Data were analyzed statistically as means ± standard deviations (SDs) according to the method described by Snedecor & Cochran (1980). Analysis of variance (ANOVA) was performed using the software SPSS 16. The level of significance was measured running a Tukey test; $P \leq 0.05$ was considered as significant.

Results

Successful establishment of stevia shoot culture was obtained when nodal segments of field grown plants were cultured on MS medium supplemented with 0.5mg/L BAP for three weeks. The obtained shoots were used for further experiments to study the effect of BAP concentration, long term culture and anti-ethylene compounds on stevia multiplication and genome stability.

To study the effect of different BAP concentrations on shoot multiplication of stevia, nodal segments of the *in vitro* obtained shoots were cultured on MS medium supplemented with 0, 0.25, 0.5, 1, 1.5, 2 or 3mg/L BAP (Table 1). Medium supplemented with the lowest BAP concentration (0.25mg/L) resulted in the highest shoot number/explant (5.7), the highest number of leaves/shoot (7.7) and the highest fresh weight/ shoot cluster (0.141g). On the other side, the highest BAP concentration (3mg/L) resulted in the lowest frequency of shoot formation and the lowest growth values. When nodal segments of *in vitro* obtained plants were cultured on BAP free medium, they expressed low number of shoots/explant but their growth was better than that grown on MS medium containing BAP. Plants of the first subculture did not show any sign of vitrification irrespective the used BAP concentration.

Multiplication of stevia shoots was estimated under the influence of successive three subcultures on MS medium containing 0.25mg/L BAP for three weeks each (Table 2). Fresh weight/shoot cluster increased as the number of subcultures increased and it was associated with the increase of shoot number/explant, the best results were detected on explants of the third subculture (Fig. 1A), it was associated with death of some shoot tips as a sign of vitrification (Fig. 1B, arrows).

The influence of low water potential created by polyethylene glycol (PEG) on sativa shoots was studied as a trial to overcome vitrification (Table 3). When the increase in PEG concentration was accompanied by increase in the number of subcultures, vitrification was highly increased. In addition, shoot multiplication and shoot growth decreased as the concentration of PEG or the number of subcultures increased. Under the influence of PEG, shoots were translucent with glassy appearance and their color was pale green especially at the higher concentrations of PEG. Also, the length of internode and leaf size of the formed shoots were reduced when shoots were subcultured on medium containing different PEG levels in comparison to that of control.

To know the best concentration and the best anti-ethylene compound to overcome the appearance of vitrification (Table 4), nodal segments of the second subculture were subcultured on MS medium containing 0.25mg/L BAP and different concentrations of $AgNO_3$, AgNPs, $CoCl_2$ or SA for 3 weeks. Shoot multiplication and growth of stevia shoots decreased with the use of multiplication media containing anti-ethylene compounds. In comparison to that of control plants, low concentration of $AgNO_3$ (1.7mg/L) reduced the development of verification on cultured stevia shoots without significant reduction in the number of shoots/explant. The equivalent concentration of $AgNO_3$ (1.7mg/L) in nano-particles form (AgNPs) showed also non-significant decrease in shoot number/explant but it was associated with a drastic increase in shoot vitrification (20%). In comparison to that of control, progressive decrease in shoot vitrification was registered with the increase of $CoCl_2$ concentrations; it led to a complete disappearance of shoot vitrification when 47.6mg/L $CoCl_2$ was used. All the applied concentrations of SA resulted in a disappearance of shoot vitrification but it was associated with significant decrease in shoot number/explant. Growth of formed shoots was negatively influenced by the application of all types of anti-ethylene compounds even in low concentrations.

TABLE 1. Effect of MS medium supplemented with different BAP concentrations on shoot multiplication and growth of stevia after two weeks culture.

BAP conc. (mg/L)	Frequency of shoot formation (%)	No. of shoots/ explant	Length of shoot (cm)	No. of nodes/ shoot	No. of leaves/ shoot	F.w./ shoot cluster (g)
0	93.33	2 ± 0	3.5 ± 0.15	4.3 ± 0.58	7.7 ± 0.58	0.04 ± 0.01
0.25	100	5.7 ± 0.58*	3.4 ± 0.06	4 ± 0	7.7 ± 0.58	0.141 ± 0.01*
0.5	100	3.3 ± 0.58	2.6 ± 0.06*	3.7 ± 0.58	7.3 ± 0.58	0.136 ± 0.02*
1	100	3 ± 0	2.6 ± 0.1*	3.7 ± 0.58	7 ± 1	0.132 ± 0.02*
1.5	100	3 ± 0	2.4 ± 0.31*	4 ± 0	7 ± 1	0.126 ± 0.02*
2	96.67	3 ± 1	2.4 ± 0.1*	3.7 ± 0.58	6.7 ± 0.58	0.095 ± 0.01*
3	83.33	2 ± 0	1.5 ± 0.15*	2.7 ± 0.58*	6.3 ± 0.58	0.077 ± 0.01

* Indicates to a significant difference between BAP-free MS medium and media with different BAP concentrations at P≤ 05.

Fig. 1. Stevia micropropagation: (A) Microshoots at the 3rd subculture on MS medium + 0.25mg/L BAP, (B) Multiplied shoots were accompanied with vitrification, (C) Roots formation on microshoots grown on media containing high concentrations of IBA, (D) Vitrification symptoms developed on rooted microshoots grown on media containing low IBA concentration, (E) Rooted plantlet after shifted in plastic pots to be acclimatized to *ex vitro* conditions, [Arrows refer to the vitrified shoots].

TABLE 2. Multiplication of stevia shoots on MS medium with 0.25mg/L BAP for three successive subcultures, three weeks each.

Subculture No.	No. of shoots/ explant	Length of shoot (cm)	No. of nodes/ shoot	No. of leaves/ shoot	F.w./shoot cluster (g)	% of vitrified shoots
1th subculture	4.7±0.6	4.5±0.2	4±0	8 ± 0	0.192 ± 0.02	0
2th subculture	5.3±0.6	4.9±0.2	4.7±1.2	8 ± 0	0.217 ± 0.03	0
3th subculture	9.3±1.2*	5 ± 0.2*	4 ± 0	8 ± 0	1.346 ± 0.08*	10

* Means a significant difference between values at the 1st subculture and those at the 2nd and 3rd subcultures at P≤ 0.05.

TABLE 3. Shoot multiplication and growth of stevia during three successive subcultures, three weeks/each, on MS medium with 0.25mg/L BAP and different PEG concentrations.

Subculture No.	PEG conc. (g/L)	Frequency of shoot formation (%)	No. of shoots/ explant	Length of shoot (cm)	No. of nodes/ shoot	No. of leaves/ shoot	F.w./ shoot cluster (g)	% of vitrified shoots
1th subculture	0 (control)	100	8 ± 0	6.8 ± 0.3	5 ± 0	11.3 ± 1.2	0.35 ± 0.04	10
	5	100	4.7 ± 0.6*	3.6 ± 0.1*	3.3± 0.6*	6.7 ± 1.2*	0.11 ± 0.01*	23.3
	10	100	4.7 ± 0.6*	3.3 ± 0.3*	3 ± 0*	6.7 ± 1.2*	0.10 ± 0.01*	46.7
	15	100	4.3 ± 1.2*	3.4 ± 0.2*	3 ± 0*	6.7 ± 0.6*	0.08 ± 0.01*	66.7
	20	100	3.3 ± 0.6*	3.3 ± 0.1*	3 ± 0*	6.3 ± 0.6*	0.07 ± 0.02*	80
	25	100	3 ± 0*	2.6 ± 0.3*	2.7±0.6*	6.3 ± 0.6*	0.06 ± 0.01*	100
	30	100	2.7 ± 0.6*	2.4 ± 0.1*	2 ± 0*	6 ± 0*	0.05 ± 0.01*	100
2th subculture	0 (control)	100	8 ± 1	6.3 ± 0.2	4.7 ± 0.6	11.7 ± 1.5	0.37 ± 0.09	10
	5	93.3	4 ± 1*	4.7 ± 0.1*	4 ± 0	9 ± 0	0.10 ± 0.003*	36.7
	10	66.7	2.3 ± 0.6*	3 ± 0.4*	4 ± 1	8.3 ± 1.2*	0.09 ± 0.02*	43.3
	15	50	2.3 ± 0.6*	2.4 ± 0.2*	3.3 ±0.6*	8.3 ± 0.6*	0.07 ± 0.01*	70
	20	50	2.3 ± 0.6*	2.3 ± 0.2*	3.3±0.6*	7.7 ± 0.6*	0.06 ± 0.01*	83.3
	25	40	2 ± 1*	1.7 ± 0.1*	3.3±0.6*	7.3 ± 0.6*	0.05 ± 0.005*	100
	30	36.7	1.7 ± 0.6*	1.7 ± 0.2*	2.7±0.6*	6.7 ± 1.2*	0.03 ± 0.001*	100
3th subculture	0 (control)	100	7.3± 1.2	5.6 ± 0.1*	4.7±0.6	10.7 ± 0.6	0.26 ± .04*	13.3
	5	90	3 ± 0*	3.8 ± 0.1*	4 ± 0	9.3 ± 0.6	0.09± 0.01*	40
	10	60	2 ± 0*	2.4 ± 0.2*	3.3±0.6*	6 ± 1*	0.08 ±0.01*	70
	15	43.3	2 ± 0*	2.3 ± 0.1 *	3 ± 0*	7.7 ± 1.2*	0.05±0.001*	76
	20	40	1.7 ± 0.6*	2.2 ± 0.1*	3 ± 0*	6 ±0*	0.04±0.002*	100
	25	36.7	1.3 ± 0.6*	1.5 ±0.06*	2.7±0.6*	5.3 ± 1.21*	0.02±0.002*	100
	30	20	1 ± 0*	1.1± 0.2*	2 ± 0*	4 ± 0*	0.01±0.0004*	100

* Implies a significant difference at P≤ 0.05 between values of control at 1st subculture and those determined at other treatments in each subculture.

TABLE 4. *In vitro* multiplication and growth of stevia microshoots obtained from the third subculture on MS medium with 0.25mg/L BAP and different concentrations of $AgNO_3$, Ag NPs, $CoCl_2$ and SA for three weeks.

Treatment (mg/L)	Frequency of shoot formation (%)	No. of shoots/ explant	Length of shoot (cm)	No. of nodes/ shoot	No. of leaves/ shoot	F.w./shoot cluster (g)	% of vitrified shoots
Control (0.25mg/L BAP)	100	8 ± 0	6.8 ± 0.3	5 ± 0	11.3 ± 1.2	0.35 ± 0.04	10
1.7 $AgNO_3$	100	7.7 ± 0.6	6.5 ± 0.2	4.7 ±0.6	9.7 ±0.6	0.32 ± 0.01	3.3
4.25 $AgNO_3$	100	3.7 ±0.6*	3.4 ± 0.3*	4 ± 0	10 ± 0	0.2 ± 0.02*	3.3
12.7 $AgNO_3$	100	3 ± 0*	3.2 ±0.06*	3 ± 0*	5.7 ±0.6*	0.14 ± 0.002	10
1.7 Ag NP	100	7.7 ±0.6	4.8 ± 0.2	4 ± 0	8.7 ±0.6*	0.15 ± 0.01	20
4.25 Ag NP	100	5 ± 0*	3.4 ± 0.1*	3.3 ±0.6*	7 ± 0*	0.17 ± 0.02	20
12.7 Ag NP	100	3 ± 0*	2.6 ±0.06*	2 ± 0*	6 ± 0*	0.11 ± 0.003*	20
11.9 $CoCl_2$	100	7.3 ±0.6	4.7 ± 0.4	4 ± 0	8.7 ±0.6*	0.17 ± 0.01	26.7
23.8 $CoCl_2$	93.3	7 ± 0*	4.7 ± 0.06	5 ± 0	9.3 ±0.6*	0.19 ± 0.02	10
47.6 $CoCl_2$	100	7.3 ± 1.2	5.4 ± 0.2*	3.3 ±0.6*	8 ± 0*	0.2 ± 0.01*	0
6.9 SA	83.3	3.7 ±0.6*	3.1± 0.2*	3.3 ±0.6*	7.7 ±0.6*	0.06 ± 0.01*	0
13.8 SA	60	2 ± 0*	1.3 ± 0.2*	3 ± 0*	6.7 ±0.6*	0.04 ± 0.002*	0
27.6 SA	10	1 ± 0*	0.4 ± 0.1*	1 ± 0*	2 ± 0*	0.01 ± 0.001*	0

* Points to a significant difference between control values and the corresponding values of other treatments at P≤ 0.05.

TABLE 5. Shoot multiplication and growth of stevia under the influence of long term culture conditions (4th to 10th subcultures), 15 days each, on MS medium containing 0.25mg/L BAP and selected concentrations of anti-ethylene compounds.

Sub-culture No.	Treatment (mg/L)	Frequency of shoot formation (%)	No. of shoots/ explant	Length of shoot (cm)	No. of nodes/ shoot	No. of leaves/ shoot	F.w./shoot cluster (g)	% of vitrified shoots
4th subcult.	Control	100	5.7 ± 1.2	2.6±0.1	4 ± 0	8 ± 0	0.09 ±0.02	0
	1.7 $AgNO_3$	100	5.3 ± 0.6*	2.5±0.1	3.7±0.6	8 ± 0	0.065 ±0.01	0
	1.7 Ag NP	100	3.3±0.6	3.4±0.1*	3.7±0.6	8 ± 0	0.104 ±0.01	0
	47.6 $CoCl_2$	93.3	3.3±0.6	3.3±0.1*	3.7±0.6	7.7±0.6	0.065 ± 0.01	0
	6.9 SA	83.3	3.3±0.6	3.1 ± 0.2	3.3±0.6	6.3±0.6	0.042±0.01	0
5th subcult.	Control	100	5.7±0.6	4.6 ± 0.1*	4 ± 0	8.7±0.6	0.16 ±0.05*	0
	1.7 $AgNO_3$	100	3 ± 0	3.3 ± 0.3	3.3±0.6	8 ± 1	0.075 ±0.003	0
	1.7 Ag NP	100	3 ± 0*	4.6 ± 0.1*	3.3±0.6	7.3±0.5	0.152 ±0.02	23.3
	47.6 $CoCl_2$	80	3.3±0.6	2.5 ± 0.2	2 ± 0*	5.7±0.6*	0.055± 0.001	0
	6.9 SA	70	3 ± 0*	2.6± 0.5	3.3±0.6	7.7±0.6	0.025±0.001	0
6th subcult.	Control	100	5 ± 0	5.5 ± 0.1*	4 ± 1	8 ± 0	0.14 ±0.01	0
	1.7 $AgNO_3$	100	3 ± 0*	3.7±0.06*	3 ± 0	6 ± 0	0.173 ±0.03*	0
	1.7 Ag NP	100	2.3±0.6*	3.6 ± 0.1*	3 ± 0	5 ± 0*	0.104 ±0.02	33.3
	47.6 $CoCl_2$	96.7	3 ± 0*	4.1 ± 0.4*	4 ± 0	9 ± 0	0.08 ± 0.01	0
	6.9 SA	70	3 ± 0*	3.2 ± 0.2	4 ± 0	8 ± 0	0.031±0.003	0
7th subcult.	Control	100	5 ± 1	7.1 ± 0.2*	2.7±0.6	7.3±1.2	0.186 ± 0.03*	16.7
	1.7 $AgNO_3$	100	3 ± 1*	6.3 ± 0.4*	2.7±0.6	6.7±0.6	0.116 ±0.02	40
	1.7 Ag NP	100	3 ± 0*	5.1 ± 0.2*	2.3±0.6*	5.3±1.2	0.144 ±0.005	43.3
	47.6 $CoCl_2$	23.3	1.3±0.6*	1.6 ± 0.3*	2 ± 0*	6 ± 0	0.04 ± 0.004	0
	6.9 SA	63.3	2 ± 1*	2.2 ± 0.2	3 ± 0	7 ± 0	0.023 ±0.01	0
8th subcult.	Control	100	4.3±0.6	4.4 ± 0.2*	4±0	9 ±1	0.09 ± 0.003	40
	1.7 $AgNO_3$	100	2.7±0.6*	4.8 ± 0.4*	2.7±0.6	4.3±0.6*	0.121 ±0.03	60
	1.7 Ag NP	100	2 ± 0*	3.9 ± 0.2*	2.3±0.6*	6 ± 1	0.100 ±0.01	80
9th subcult.	Control	100	4 ± 0	4.7 ± 0.3*	4±0	9 ±0	0.182± 0.01	40
	1.7 $AgNO_3$	100	2 ± 0*	3.4 ± 0.1*	2 ± 0*	5 ± 0*	0.19 ±0.05	63.3
	1.7 Ag NP	96.7	2 ± 0*	3.7 ± 0.2*	2 ± 0*	5.7±0.6*	0.181 ±0.01	93.3
10th subcult.	Control	100	3.7±0.2	4.7 ± 0.3	3.7±0.6	9 ±1	0.165± 0.01	40
	1.7 $AgNO_3$	96.7	2 ± 0*	3.2 ± 0.1	2 ± 0*	4.3±0.6*	0.15 ±0.04	66.7
	1.7 Ag NP	93.3	1.7±0.6*	2.6 ± 0.1	2 ± 0*	5.3±1.2*	0.122 ±0.03	93.3

* Points out to a significant difference between values of the control at the 4th subculture and the corresponding values in the other subcultures and treatments at P≤ 0.05.

When subculture period was shortened from three (Table 4) to two weeks (Table 5), the appearance of vitrification delayed up to sixth subculture. Percentage of vitrified shoots on anti-ethylene free medium reached 16.7% on plants of the seventh subculture and 40% thereafter. Vitrification was detected on shoots of the fifth subculture (23.3%) when shoots were subcultured on MS medium supplemented with 0.25mg/L BAP and 1.7mg/L AgNPs. Then, the percentage of vitrification increased by increasing the number of subcultures until it reached 93.3% by the tenth subculture (Table 5). Under the influence of selected concentrations of $CoCl_2$ (47.6mg/L) or

SA (6.9mg/L), no evidence of vitrification was detected until the seventh subculture, but this was accompanied by decreasing the frequency of shoot formation. While shoots of eighth subculture did not show any ability to form shoots on medium containing 47.6mg/L $CoCl_2$or 6.9mg/L SA, shoots subcultured on medium containing 1.7mg/L $AgNO_3$ or Ag NPs showed shoot formation up to tenth subculture, but shoot vitrification was very high. Generally, shoot length and number of nodes/ shoot of anti-ethylene treated culture were lower than that of control (Table 5 and Fig. 2A & B).

Fig. 2. Stevia microshoots grown on MS + 0.25mg/L BAP + 1.7mg/L AgNPs (A) or $AgNO_3$ (B) at the 10th subculture, shoots showing vitrification symptoms (display low number of shoots, died shoot tip that referred to by the arrow, translucent and glassy appearance, a pale green–brown color and abnormal shoot growth).

When stevia shoot cuttings were subjected for root formation on MS medium supplemented with different concentrations of IBA, 1mg/L was the best where it expressed the highest percentage of root formation/shoot, number of roots/microshoot and length of root system. In addition, medium containing IBA expressed higher shoot length than that of control (Table 6 and Fig. 1C). Necrosis of apical shoot tips and loss of apical dominance of some shoot cuttings were detected when they were cultured on MS medium containing a low concentration of IBA (Fig. 1D). Plantlets with comprehensive root system were implanted to *ex vitro* conditions (Fig. 1E) and they continued growth upon their transfer to the field conditions.

In this work, nine RAPD and five ISSR primers were used to screen the genetic stability of stevia shoots subjected for ten subcultures on MS medium containing 0.25mg/L BAP only or 0.25mg/L BAP with 1.7mg/L $AgNO_3$ or AgNPs. Using nine RAPD primers, the amplified genomes of the 1st, 4th, 7th and 10th subcultures (Fig. 3 and Tables 7) on MS medium with 0.25mg/L BAP expressed 94 fragments. Data analysis indicated the DNA polymorphism ranging from 16.7% (OPC-02) to 62.5% (OPA-03) with an average of 47.9%. Amplification of DNA extracted from regenerated shoots at the 1st, 4th, 7th and 10th subcultures on MS medium supplemented with 1.7mg/L $AgNO_3$ revealed DNA polymorphism ranging from 0.0% using OPC-02 to 45.5% using OPA-05 with an average of 30.1% (Table 7). In the case of AgNPs, RAPD primers gave DNA polymorphism ranging from 16.7% (OPC-02) to 64.3% (OPC-05) with an average of 40.2% (Table 7 and Fig. 3).

When stevia shoots of the 1th, 4th, 7th and 10th subcultures were grown on MS medium supplemented with 0.25mg/L PAB and subjected to genome amplification using ISSR primers, the registered polymorphism ranging from 0% (ISSR5) to 60% (ISSR2) with an average of 39.6% (Table 8 and Fig. 4). Shoot polymorphism decreased when shoots were cultured on MS medium with anti-ethylene $AgNO_3$, it ranged from 0% (ISSR5) to 30.8% (ISSR4) with an average of 21.6%. Application of AgNPs increased polymorphism among stevia microshoots more than $AgNO_3$ and anti-ethylene free medium (control); polymorphism ranged from 0% (ISSR5) to 42.9% (ISSR4) with an average of 30% (Table 8 and Fig. 4).

Discussion

To establish *in vitro* plant materials, nodal segments were cultured on MS medium supplemented with 0.5 mg/l BAP for three weeks. Generally, *in vitro* grown plant materials were preferred for micropropagation and related studies in stevia and other plant species (Hassanein et al., 2010, 2019; Salem & Hassanein, 2017).

TABLE 6. Root induction and growth on stevia microshoots after two weeks culture on MS medium enriched with different IBA concentrations.

IBA conc. (mg/L)	Percentage of rooted shoots (%)	No. of roots/shoot	Length of root system (cm)	Length of shoot (cm)
0	70	2.7 ± 0.58	1.8 ± 0.1	5.47 ± 0.25
0.5	96.67	7.7 ± 0.58*	2.3 ± 0.27*	8.1 ± 0.36
1	96.67	8 ± 0*	2.8 ± 0.14*	9.73 ± 1.94*
2	76.67	7.3 ± 0.58*	1.7 ± 0.09	7.17 ± 0.29

* Implies a significant difference between values registered at IBA-free MS medium and those at MS medium with different IBA concentrations at P≤ 0.05.

TABLE 7. RAPD analysis of stevia shoots of the 1st, 4th, 7th and 10th subcultures, two weeks each, on MS medium containing 0.25mg/L BAP without or with 1.7mg/L of $AgNO_3$ or AgNPs.

Treatment	Primer code	No. of total scorable fragments	Size of amplified fragments (bp)	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphism (%)	Similarity (%)
MS + 0.25mg/L BAP	OPA-03	8	450-1500	1	3	4	62.5	37.5
	OPA-05	11	200-1490	1	6	4	45.5	54.5
	OPA-07	12	280-1850	5	7	0	41.7	58.3
	OPA-13	9	670-1800	4	4	1	55.6	44.4
	OPA-17	7	540-2000	2	5	0	28.6	71.4
	OPC-02	12	230-1875	0	10	2	16.7	83.3
	OPC-05	15	330-3000	9	3	3	80	20
	OPD-01	7	500-1500	1	5	1	28.6	71.4
	OPD-18	13	400-1815	3	6	4	53.8	46.2
	Total	**94**		**26**	**49**	**19**	**47.9**	**52.1**
MS + 0.25mg/L BAP + $AgNO_3$	OPA-03	7	450-1100	1	4	2	42.9	57.1
	OPA-05	11	200-1490	4	6	1	45.5	54.5
	OPA-07	12	280-1850	5	7	0	41.7	58.3
	OPA-13	8	670-1800	2	6	0	25	75
	OPA-17	7	540-2000	2	5	0	28.6	71.4
	OPC-02	10	230-1300	0	10	0	0	100
	OPC-05	12	330-1785	4	8	0	33.3	66.7
	OPD-01	8	500-1690	2	6	0	25	75
	OPD-18	8	400-1500	2	6	0	25	75
	Total	**83**		**22**	**58**	**3**	**30.1**	**69.9**
MS + 0.25mg/L BAP + AgNPs	OPA-03	8	450-1250	3	4	1	50	50
	OPA-05	8	200-1490	2	5	1	37.5	62.5
	OPA-07	13	280-1850	4	7	2	46.2	53.8
	OPA-13	8	670-1800	2	6	0	25	75
	OPA-17	7	540-2000	2	5	0	28.6	71.4
	OPC-02	12	230-1875	0	10	2	16.7	83.3
	OPC-05	14	330-1785	6	5	3	64.3	35.7
	OPD-01	8	500-1500	3	5	0	37.5	62.5
	OPD-18	9	400-1500	4	5	0	44.4	55.6
	Total	**87**		**26**	**52**	**9**	**40.2**	**59.8**

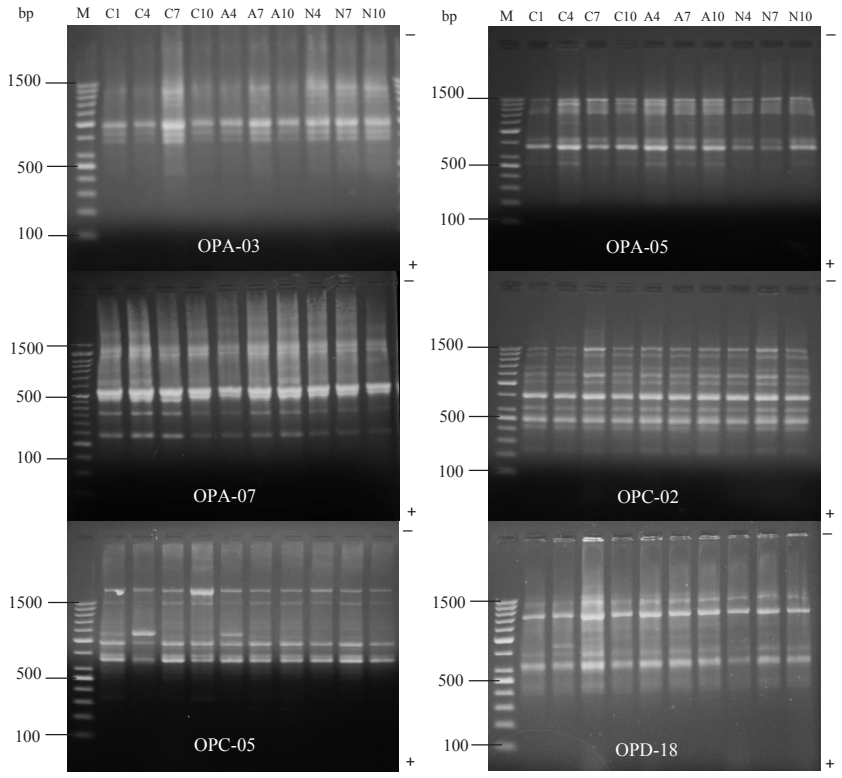

Fig. 3. RAPD-PCR profiles generated by the shown primers for genomic DNA of stevia shoots grown on: multiplication medium (control) at the 1st, 4th, 7th and 10th subcultures (lanes: C1, C4, C7 and C10, respectively); multiplication medium + 1.7mg/L $AgNO_3$ or AgNPs at the 4th, 7th and 10th sucultures (lanes: A4, A7 and A10, or N4, N7 and N10, respectively), [M: DNA ladder].

TABLE 8. ISSR analysis of stevia shoots of the 1st, 4th, 7th and 10th subcultures, two weeks each, on MS medium containing 0.25mg/L BAP without or with 1.7mg/L of $AgNO_3$ or AgNPs

Treatment	Primer code	No. of total scorable fragments	Size of amplified fragments (bp)	No. of polymorphic bands	No. of monomorphic bands	No. of unique bands	Polymorphisim (%)	Similarity (%)
Control	ISSR1	11	400-1570	3	7	1	36.4	63.6
	ISSR2	10	300-2000	4	4	2	60	40
	ISSR3	12	550-1200	4	5	3	58.3	41.7
	ISSR4	14	600-3270	3	8	3	42.9	57.1
	ISSR5	7	390-850	0	7	0	0	100
	Total	**54**		**14**	**31**	**9**	**39.6**	**60.4**
$AgNO_3$	ISSR1	11	400-1570	3	8	0	27.3	72.7
	ISSR2	9	300-2000	1	8	0	11.1	88.9
	ISSR3	11	500-1100	3	8	0	27.3	72.7
	ISSR4	13	600-3270	3	9	1	30.8	69.2
	ISSR5	7	390-850	0	7	0	0	100
	Total	**51**		**10**	**40**	**1**	**21.6**	**78.4**
AgNPs	ISSR1	10	460-1570	3	6	1	40	60
	ISSR2	10	300-2000	3	7	0	30	70
	ISSR3	9	500-1100	2	7	0	22.2	77.8
	ISSR4	14	600-3270	4	8	2	42.9	57.1
	ISSR5	7	390-850	0	7	0	0	100
	Total	**50**		**12**	**35**	**3**	**30**	**70**

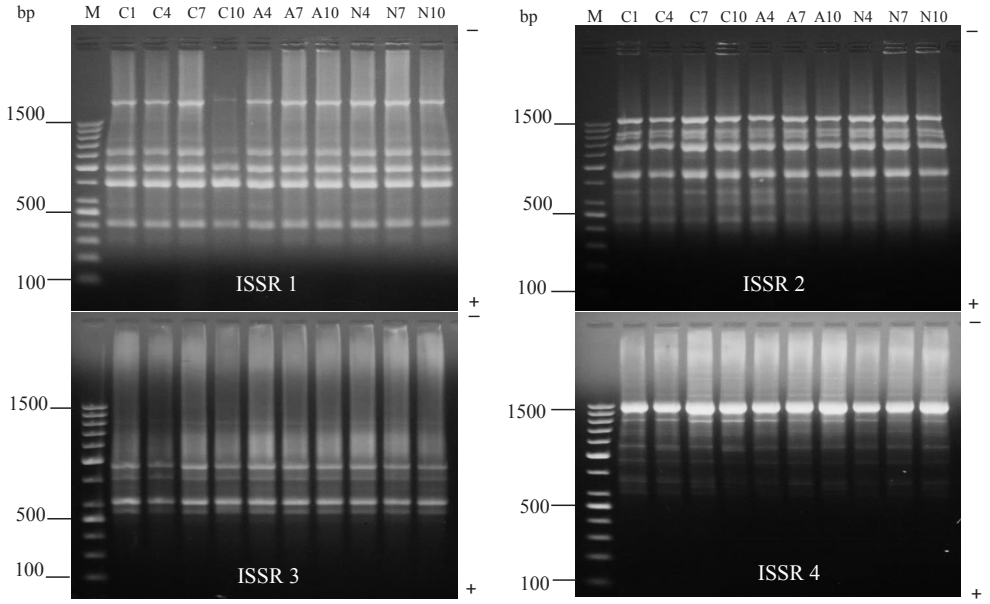

Fig. 4. ISSR-PCR profiles generated by the shown primers for genomic DNA of stevia shoots grown on: multiplication medium (control) at the 1st, 4th, 7th and 10th subcultures (lanes: C1, C4, C7 and C10, respectively); multiplication medium + 1.7mg/L $AgNO_3$ or AgNPs at the 4th, 7th and 10th subcultures (lanes: A4, A7 and A10, or N4, N7 and N10, respectively), [M: DNA ladder].

When the *in vitro* obtained shoots were subculture for the first time, shoot multiplication and growth on MS medium containing relatively low concentrations of BAP were better than others subcultured on MS medium with relatively high BAP, as was previously reported (Sivaram & Mukudan, 2003; Tadhani et al., 2006). Nodal segments subcultured on MS medium fortified with 0.25mg/L BAP expressed the best results (Table 1). In contrast to our data, nodal or shoot tip explants of stevia cultured on MS medium enriched with 2mg/L BAP expressed the highest number of regenerated shoots (Jahan et al., 2014). In this work, while the highest concentration of BAP (2-3mg/L) retarded shoots multiplication, no sign of vitrification was detected. These relatively high concentrations of BAP caused vitrification in several plant species (Constantine, 1986; Hussey, 1986; Gürel & Gülsen, 1998). Consequently, the appearance of vitrification under the influence of BAP concentration is species dependent.

In stevia, shoot multiplication increased as the number of subcultures increased up to the third one (Table 2); it was also reported in cherry and apple, and attributed to rejuvenation of mature explant tissues under the influence of *in vitro* culture conditions (Grant & Hammat, 1999). Successive subcultures for a long period, up to ten times, led to a reduction of shoot proliferation and increasing of hyperhydricity in stevia and other plant species (Vieitez et al., 1985; Gómez et al., 2007) even in a low concentration of the BAP (0.25mg/L) where it reached 40% by the tenth subculture (Table 5). The association between vitrification and subculture number was reported in *Castanea sativa* Mill (Vieitez et al., 1985; Quiala et al., 2014) and moringa (Hassanein et al., 2018). Under tissue culture conditions, divided cells are not sufficiently protected where their cell walls lack sufficient lignin and cellulose (Kevers & Gaspar, 1986). Deficiency of both components especially in atmosphere of high relative humidity enhances water diffusion into the cultured plant tissues leading to the appearance of highly vacuolated cells (Vieitez et al., 1985; Safrazbekyan et al., 1990). Under atmosphere with high humidity in closed container, endogenous level of BAP in cultured shoots increased as the number of subcultures increased in stevia and other plant species. Excess in this type of cytokinin led to induction of rapid cell divisions in shoot tip region leading to necrosis of shoot tips and other vitrification symptoms. While the number of vitrified shoots reached 40% under long term culture in stevia, it reached 50-80% in others (Leshem et al., 1988; Safrazbekyan et al., 1990).

Increase agar concentration in the medium was accompanied by decrease in water availability and incidence of vitrification (Debergh et al., 1992; Whitehouse et al., 2002). It was expected that decreasing of media water potential due to the application of PEG may led to reduction of water availability and vitrification of the cultured stevia shoots. In addition, there isn't evidence about induction of hyperhydricity in *in vitro* cultured plant shoots due to PEG application (Sen & Alikamanoglu, 2013). In this study, increase the concentrations of PEG 6000 stimulated hyperhydricity in stevia, which increased progressively over time and with higher PEG concentrations. Zimmerman et al. (1991) reported that application of PEG did not avoid vitreous development.The detected vitrification symptom may be due to increase in water content or accumulation of PEG 6000 in cultured tissues (Sen & Alikamanoglu, 2013). Excessive water accumulation in the vitrified shoots in stevia and other plant species resulted in oxygen depletion leading to hypoxia (Gaspar et al., 2002). Direct (osmotic stress) and indirect (hypoxia) effects of PEG resulted in accumulation of ROS leading to death of plant cells (Bowler et al., 1992) or retardation of stevia growth parameters. In stevia, increase mannitol concentration in the medium resulted in decrease of shoot growth parameters (Ghaheri et al., 2018).

Generally, anti-ethylene containing media reduced shoot multiplication and the incidence of vitrification in a dose-dependent way. Number of vitrified shoots was reduced without significant reduction in shoot multiplcation when a low concentration of $AgNO_3$ (1.7mg/L) was used. On the other side, 1.7mg/L AgNPs showed a drastic increase in shoot vitrification (20%). On the other side, Castro-González et al. (2019) detected that low concentration of AgNPs increased growth parameters in stevia. The toxicity of AgNPs was explained by the release of free Ag inside plant cells and triggering cellular oxidative stress (Oukarroum et al., 2013). In addition, AgNPs express deteriorating effects on the physiological state of organism may be due to increase ROS formation and lipids peroxidation (Oukarroum et al., 2012) leading to a decrease of plant growth in stevia. In $CoCl_2$ treated shoots, vitrification was progressively decreased in dose-dependent manner; it was absent when salicylic acid was used. Avoidance of vitrification was associated with a significant decrease in shoot multiplication. Although there was an appropriate concentration of each anti-ethylene compound that led to avoid vitrification, they couldn't avoid the negative effects of these compounds on shoot growth which may be due to the accumulation of ROS (Cho & Park, 2000).

Appearance of vitrification was delayed when stevia shoots were subjected to long term culture in combination with shortening of the subculture period and application of selected concentrations of $AgNO_3$ or AgNPs. Decrease the time of subculture from three to two weeks prevented the appearance of vitrification up to sixth subculture. Vitrified shoots was detected only on shoots of the fifth subculture (23.3%) when 1.7mg/L AgNPs was used; their number increased by increasing the number of subcultures until it reached 93.3% by the tenth subculture (Table 5 and Fig. 2). These conditions decreased shoot multiplication and growth may be due to increase of total Ag^+ content in the plant tissues (Vinković et al., 2017; Tripathi et al., 2017). No evidence of vitrification was detected up to the seventh subculture using the selected concentrations of $CoCl_2$ (47.6mg/L) or SA (6.9mg/L). In moringa, SA was the best ethylene inhibitor where it enhanced healthy shoot formation that facilitated root formation and plantlets acclimatization (Hassanein et al., 2018). Application of $AgNO_3$ was better than AgNPs. Yin et al. (2011) reported that AgNPs were more toxic than $AgNO_3$.

Shoot cuttings of stevia were rooted on MS medium supplemented with different concentrations of IBA; 1mg/L was the best (Table 6 and Fig. 1C). Necrosis of apical shoot tips and loss of apical dominance of some shoot cuttings were detected on control (MS without IBA) or under the influence of 0.5mg/L IBA as was reported in other plant species (Wang & Hu, 1984; Kataeva, 1986). Cytokinin deficiency during root formation led to stop cell divisions in the apical meristem and reduction of endogenous IAA and ABA synthesis. With time on root formation medium, endogenous cytokinins increased due to their de novo synthesis by formed roots. Then, cytokinins moved to and accumulated in the shoot apex (Tamas, 1987), thus preventing the arrest of the shoot apexes growth and stimulating their growth in stevia on IBA containing medium. In addition, this situation led to re-continue shoot growth and appearance of necrotic shoot tips as a symptom of vitrification.

In this work, nine RAPD primers were used to screen the genetic stability of stevia microshoots that subjected for 1st, 4th, 7th and 10th subcultures on MS medium supplemented with 0.25mg/L BAP (Fig. 3 and Table 7). The highest number of polymorphic bands (9) was obtained by primer OPC-05 but the lowest number (0) was obtained by OPC-02 (Table 7). The used RAPD primers gave DNA polymorphism ranging from 16.7% (OPC-02) to 62.5% (OPA-03) with an average of 47.9%.

Under long term cultures, the detected polymorphisms were 47.9%. and 39.6% using RAPD and ISSR, respectively (Tables 7 and 8). Genetic variations under long term culture in stevia and other plant species may be due to DNA hypermethylation (Rival et al., 2013), changes in chromosomal number and structure, pre-existed genetic changes in the donor plant or other reasons (Isah, 2015; Khatab & Youssef, 2018). The combination between shorten of subculture period and application of anti-ethylene improved shoot health through the disappearance of the vitrification phenomenon up to the sixth subculture. Further increase in the number of subcultures increased the percentage of vitrification and genetic polymorphism (Hassanein et al., 2018). The genetic polymorphism of plants exposed to $AgNO_3$ as an anti-ethylene was lower than those grown in the absence of anti-ethylene. The calculated polymorphisms of RAPD primers were 30.1% and 47.9% but those of ISSR (Table 8) were 21.6% and 39.6% for plants subcultured on MS with or without $AgNO_3$, respectively. In this work, the use of AgNPs increased the incidence of vitrification in comparison with plants grown on media with or without $AgNO_3$. On the other hand, genetic polymorphism of plants treated with AgNPs was higher than that of $AgNO_3$ as was detected by RAPD or ISSR.

Conclusion

Shortening the period of subculture from three to two weeks, reduced the vitrification during *in vitro* multiplication of stevia. $AgNO_3$ genotoxicity was previously detected leading to chromosome aberration and DNA damage in different plant species but it was lower than its nanoparticles form (Ghosh et al., 2012; Patlolla et al., 2012). Consequently, the phytotoxicity of AgNPs is primarily due to their physiochemical characteristics, and not only due to release of Ag^+ (Vannini et al., 2013).

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تأثيرات المركبات المضادة للإثيلين علي التزجيج والثبات الجيني لنبات الإستيفيا

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يعتبر تكرار عملية إعادة الزراعة علي نفس البيئة الغذائية في مزارع الأنسجة النباتية (تجديد المزارع) متطلب أساسي لعملية الإكثار المعملي الدقيق لنبات الإستيفيا، ولكنه كان مصحوباً بحدوث التزجيج (الظاهرة الزجاجية) علي الأفرع الخضرية النامية علي المستقطعات العقدية المنزرعة. لوحظ زيادة عدد الأفرع الناتجة علي كل مستقطع نباتي بدون تزجيج عندما تم إعادة زراعة المستقطعات العقدية للأفرع الناتجة من الزارعة النسيجية الأولي لمرتين متتاليتين بعد ذلك (الجيل الثاني والثالث) علي الوسط المغذي موراشيجي وسكوج المضاف إليها 0.25 مجم/لتر من البنزيل أمينو بيورين، وكانت مدة التحضين لكل فترة زراعة ثلاثة أسابيع. إبتداءاً من الجيل الرابع ، بدأ تضاعف الأفرع يتناقص والتزجيج يزداد كلما زاد عدد مرات إعادة الزراعة (تجديد الزراعة علي بيئة جديدة) ، حيث أنه عند الوصول إلي الجيل العاشر كانت نسبة تزجيج الأفرع (للعينة الضابطة) هو 40%. أدي إنخفاض إمكانية إتاحة الماء باستخدام البولي إيثيلين جليكول (PEG) إلي زيادة التزجيج بالأفرع الخضرية، ولكن أدي تقصير فترة التحضين لكل مرة إعادة الزراعة (تجديد المزارع) من ثلاثة أسابيع إلي أسبوعين إلي تأخير حدوث التزجيج وذلك حتي الجيل السادس. أيضاً، أدي استخدام المركبات المضادة للإيثيلين (نترات الفضة، كلوريد الكوبلت أو حمض السالسليك) إلي انخفاض مشاكل الزجيج حتي الجيل السادس. تم الحصول علي أفضل النتائج عندما استخدمت نترات الفضة بتركيز 1.7 مجم/لتر حيث انخفض التزجيج بدون حدوث إنخفاض معنوي في عدد الأفرع الخضرية النامية / مستقطع نباتي وكذلك بدون حدوث انخفاض معنوي في نمو الأفرع الخضرية. استخدام الفضة في شكل دقائق نانوية (AgNPs) بتركيز مكافئ لتركيزها (1.7 مجم/لتر) في شكل ايونات ($AgNO_3$)، أدي إلي زيادة شديدة في حدوث تزجيج الأفرع الخضرية (20%). عند تكبير جينوم الأفرع الخضرية الناتجة من الجيل الأول والرابع والسابع والعاشر باستخدام البادئات العشوائية (RAPD) أو بادئات بين التتابعات البسيطة (ISSR)، كانت تعددية الأشكال الناتجة تحت تأثير تركيز 1.7 مجم/لتر من نترات الفضة أقل من تلك الناتجة تحت تأثير تركيز 1.7 مجم/لتر من دقائق الفضة النانوية، وكل منهما كانت أقل من تلك (تعددية الأشكال) الناتجة من الأفرع الخضرية المنماة علي الوسط المغذي الخالي من مضادات الإيثيلين (العينة الضابطة).