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Article

In vitro cloning of *stevia rebaudiana* Bertoni to avoid verification and production of synthetic seeds

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Abstract

Stevia rebaudiana Bertoni is an important medicinal plant; plant leaves are used as a source of non-caloric sweetener-stevioside, which is estimated to be 300 times sweeter than cane sugar. Seed germination of stevia is very low, so micropropagation is an essential prerequisite for stevia mass production and modification. Determined plant tissue culture conditions were applied to establish shoot cloning and synthetic seeds (synseeds) formation. Efficient shoot multiplication was established on MS medium with 0.25 mg/l BAP. While the best shoot formation frequency was obtained when nodal or shoot cuttings were sub cultured for the fifth time, addition of 1.7 mg/l of $AgNO_3$ increased the values of shoot formation frequency and reduced verification. For production of artificial seeds, small nodal cuttings, about 3 mm long each, were coated using sodium alginate (4%) and $CaCl_2$ (75 mM) solution as a gel matrix and a complexing agent, respectively. Shoot cuttings resulting from the conversion of synseeds or shoot culture were used to establish root formation on MS medium containing 1 mg/l IBA. Plantlets with extensive root systems were hardened and successfully established in the soil.

Keywords: Artificial seeds, Micropropagation, Stevia, Verification, Silver nitrate

1. Introduction

To gain customer satisfaction, manufacturers try to replace artificial sweeteners with other natural sugars. Stevia (*S. rebaudiana* Bertoni) leaves have been used as a low-calorie sweetener for centuries worldwide. Stevia sweetness is due to the bio-accumulation of natural compounds such as diterpenoid steviol glycosides, which are up to 30% of the leaf's dry weight; and 300 times sweeter than common table sugar (Crammer and Ikan 2003). Additional advantages were reported about steviosides, where they have a long shelf life, zero carbohydrates, zero calories,

make blood sugar levels more stable, characterized by non-fermentation ability, and thermal stability at 100 °C. Steviosides are better than old sugar substitutes such as xylitol or sorbitol (Singh and Rao 2005). Therefore, stevia has found great interest among researchers, manufacturers, and consumers because it is a valuable medicinal plant of the Asteraceae family with wide application in the pharmaceutical and food industries.

Conventional propagation of stevia is limited due to poor seed viability, prolonged and low germination rate, poor rooting ability of vegetative plant materials, and the low number of individuals that can be obtained from each donor plant (Sakaguchi and Kan 1982; Carneiro et al., 1997). Therefore, plant tissue culture technique such as micropropagation is recommended for large-scale sativa propagation. Micropropagation can be applied to obtain a high number of plants in a short time all over the year, somaclonal variation could be controlled, pathogens-free propagules could be obtained and a high success rate homogenous in terms of glycoside contents could be established (Sivaram and Mukundan 2003). It has been found that various conditions can affect the success of micropropagation such as explant type, genotype, chemical composition of the medium, and type and concentration of growth regulators. During in vitro culture, Plant growth regulators play an important role in morphogenesis (Hassanein, 2004) and regulation of metabolic pathways (Wang *et al.*, 2023).

To conserve sterile conditions, in vitro, culture was established in the vessel; conditions distinctly different from those of the natural environment. Closed vessel increases the relative humidity, decreases the available oxygen, and increases the ethylene, as well as accumulates CO2, acetaldehyde, and ethanol (Rojas-Martínez *et al.*, 2010, Salem 2016, Hassanein *et al*., 2018). These conditions stimulate the appearance of hyperhydricity, also known as vitrification, which results in the appearance of morphological, anatomical, and physiological malformations, and makes the plant tissue water-swollen (Paˆques and Boxus 1987). Abdalla *et al.* (2022) describe factors are responsible for the appearance of vitrification: (1) explant factors including physiological age, genotype, organ type, and size; (2) medium content including the chemical composition of the basal medium, gelling agents, and growth regulators; (3) culture conditions including ventilation and light intensity; and (4) existence of certain substances including salicylic acid, polyethylene glycol, and antiethylene compounds.

Vitrification leads to a reduction of in vitro shoot multiplication, necrosis of shoot tips and loss of apical dominance, a decrease of the plant multiplication and growth, and retardation of plantlets acclimatization leading to limit the potential for mass propagation (Kevers *et al*., 2004; Salem 2016, Hassanein *et al.*, 2018). In addition, vitrified plant materials show a reduction in cell cycle, enhancement of plant water content, impairing stomatal function, and formation of abnormal cell wall lignification (Kevers *et al*., 2004). To avoid vitrification, several antiethylenes in different concentrations were used (Hassanein *et al.*, 2018, Salem 2020). Genome analysis of long-term cultured in moringa using RAPD, ISSR, and SSR indicated that AgNO3 was the optimal anti-ethylene compound to avoid vitrification but it led to high somaclonal variation. On the other side, salicylic acid could be used to decrease vitrification without a severe increase in somaclonal variation (Hassanein *et al*., 2018). In stevia, Salem (2020) reported that the lowest vitrification rate was obtained when 1.7 mg/L AgNO3 was used, it was associated with a non-significant reduction in the number of shoots/explant and shoot growth. Also, molecular studies indicated that the registered polymorphism under the influence of 1.7 mg/L AgNO3 was lower than that of shoots cultured on an anti-ethylene-free medium. In addition to avoidance of vitrification by silver ions, in the form of silver nitrate (AgNO3), it plays a major role in several in vitro culture

techniques including efficient shoot and root formations (Salem 2020, Hassanein *et al*., 2018, Abdalla *et al*., 2022). Avoidance of vitrification is the essential prerequisite of the effective application of in vitro culture in several techniques such as artificial seed technology (synseeds).

Artificial seed technology is considered an attractive alternative to traditional in vitro multiplication (micropropagation) where it avoids the problems that may result due to difficulties in conservation and transportation of the in vitro obtained plant materials, root formation, acclimatization, and delivery of in vitro obtained plantlets to the soil under ex vitro conditions. Artificial seeds were obtained by encapsulating small explants (embryo or nodal segment or shoot tip) within calcium alginate beads to protect them from dehydration and mechanical damage. Encapsulation of plant materials within artificial endosperm makes them able to resist preservation, transport, and sowing. Several potential advantages can be obtained using synseed technology such as: 1) simplify the handling of plant materials between labs, 2) creation of a low-cost technique, 3) production of uniform and true-to-type propagules (5) avoidance of rooting and acclimatization due to the availability of direct delivery to the soil, (6) shorten and completion of the breeding cycle at any time of the year, and (7) reduction of the storage space (Maruyama *et al.*1977) and 8) creation germplasm conservation (Maruyama *et al.*1997).

Encapsulation of *in vitro*-derived plant materials such as axillary buds or shoot tips could be used for the production of artificial seeds in mass quantity but it needs the establishment of fast regeneration protocols, decrease somaclonal variation and save enough plant materials. Therefore, plant material that can be used to produce artificial seeds must be multiplied in abundance, and what results from this multiplication is free from manifestations of disruption of the process of seed formation or conversion later on. Therefore, the aim of this work was to define a protocol for producing a sufficient amount of vitrified-free plant materials for the quantitative production of artificial seeds.

2. Materials and Methods

Plant material and cultures establishment: One-year *S. rebaudiana* plants were obtained from Sugary Plants Department, Agriculture Research Center, Cairo, Egypt. Leaves were discarded and shoot segments (2-3 cm length) with two or three nodes were subjected to sterilization using sodium hypochlorite (commercial bleach; 5 % v/v) for 5 min as well as HgCl2 (0.2% w/v) for 2 min. To remove residues of the used disinfectant agents, stevia shoot segments were rinsed three times for 5, 10, and 15 minutes with sterilized deionized water. Segments were cut into 1-1.5 cm explants, each one had one node. For induction of shoot formation, nodal explants were cultured on MS (Murashige and Skoog, 1962) medium fortified with 0.25 mg/l BAP (Benzyl aminopurine) for three weeks. Stevia shoot cultures were incubated under tissue culture room conditions at 24 ± 2 ºC, a photoperiod of 16 hr. light and 8 hr. dark, and irradiated by 100 µmol m-2 s-1. For shoot multiplication, in vitro obtained shoots of the initial culture were sub-cultured on the same medium to save mass materials to study the effect of several factors on stevia micropropagation and production of artificial seeds. In all investigated experiments, three replicates per treatment were prepared, each with ten explants. The following parameters were determined: percentage of shoot survival, number of shoots/explant, length of the shoot (cm), number of nodes/shoot, number of leaves/shoot, and fresh mass/shoot cluster. The appearance of vitrification was followed and registered.

Enrichment of multiplication medium with silver nitrate: Under the described condition of the tissue culture room, nodal segments of the third subculture were transferred to MS medium containing 0.25 mg/l BAP and 1.7 mg/l $AgNO_3$ during 4^{th}, 5^{th}, and 6^{th} subcultures for three weeks each. Also, vitrification frequency as well as the percentage of shoots survival, the number of

shoots/explant, length of the shoot (cm), number of nodes/shoot, number of leaves/shoot, and fresh mass/ shoot cluster were determined.

Production of artificial seeds: Small nodal segments of *in* vitro-grown plant materials were excised and encapsulated in single-layer beads as described by Hassanein *et al.* (2005). Liquid MS with 0.25 mg/l BAP was used for synthetic endosperm synthesis. For encapsulation, nodal cuttings were immersed in MS medium containing 0.25 mg/l BAP and 4% (w/v) of sodium alginate (gelling matrix). The complexing agent was 75 mM $CaCl_2$. The reaction between gelling matrix and complexing agent to form synseeds was carried out for 30 min. Synseeds were conserved in a closed glass jar containing a thin layer of a liquid MS medium with 0.25 mg/l BAP at 4 °C in the refrigerator for one month. For conversion, synseeds were transferred directly or after conservation on solid MS with 0.25 mg/l BAP or MS with 0.25 mg/l BAP + 1.7 mg/l $AgNO_3$, and incubated under tissue culture room conditions for 3 weeks.

Induction of root formation on shoot cuttings: Healthy shoot segments (2 cm length) were cut and sub-cultured on MS medium supplemented with 15 g/l sucrose and different concentrations of IBA (Indole butyric acid). Cultures were incubated under tissue culture room conditions. Three replicates with ten shoots each were subjected to root formation for two weeks. Shoot and root growth parameters were determined

Plantlets acclimatization: Plantlets with well-formed root systems were transferred individually to small plastic pots containing a mixture of sand and peat moss (1:2 v/v). Plantlets were covered by small transparent polyethylene bags to guarantee high relative humidity under tissue culture room conditions and watered every 3 days using tap water. After five days, a one-centimeter diameter pore was made in each bag and its diameter was doubled every five days for a gradual reduction in relative humidity for three weeks, where bags were completely removed. Then, they were transferred outdoors under full sunlight.

Statistical analysis: Experiments were planned to be in a completely randomized design. The obtained data were analyzed statistically as means ± standard deviations (SDs) according to the reported method of Snedecor & Cochran (1980). In addition, analysis of variance (ANOVA) was carried out using the software SPSS 16. The significance level was measured; $P< 0.05$ was considered significant.

3. Results

An efficient sterilization procedure was established when stevia shoot segments with two or three nodes were sterilized using commercial bleach (5 % v/v) for 5 min and $HgCl_2$ (0.2% w/v) for 2 min. To remove disinfectant agents, shoot segments were rinsed three times in sterilized deionized water. The described protocol resulted in better survival (85%) of stevia explants. Sterilized shoot segments were subjected to induction of shoot formation and multiplication on MS medium containing 0.25mg/l BAP for three weeks (Table 1). The shoot multiplication was 100% during the first three subcultures. The increase in the number of subcultures was accompanied by a decrease in shoot multiplication until it reached 50% by the tenth subculture. On the contrary, by increasing the number of subcultures, the number of shoots/explant increased significantly, as the best results were obtained at the tenth subculture. The length of shoots increased and was accompanied by an increase in the number of leaves/shoot on the first subculture of the *in vitro* obtained shoots, and it continued during the successive subcultures.

No.of sub-culture (week)	Shoot formation frequency (%)	No. shoots/ Explant	Length of shoot (cm)	No.nodes/shoot	No.of leaves /shoot	F.W/shoot (g)	F.W/shoot cluster (g)
1	100	4.7±0.6	2.9±0.1	3.3±0.6	6.7±1	0.03±0.007	0.3±0.25
3	100	7.3±1*	3.2±0.15*	3.6±0.6	7.7±0.6	0.03±0.003	0.3±0.4

4	84.1	8.7±0.6*	4.3±0.25*	3.7±0.6	9±1	0.04±0.005	0.4±0.01
5	86.6	8.3±0.6*	3.9±0.1*	3.3±0.6	8.3±0.6	0.04±0.006	0.36±0.05
6	81.2	8.3±1*	3.7±0.15*	3.3±0.6	8.7±0.6	0.04±0.003	0.37±0.02*
10	50	13±1*	4.8±0.27*	3.3±0.6	9.7±0.6*	0.05±0.007*	1.6±0.08*

Table 1: In vitro shoot multiplication and growth parameters of stevia during ten successive sub-cultures, three weeks/each, on MS medium with 0.25 mg/L BAP.
Values are means of three replicates ± standard deviation (SD)
Statistical significance of differences compared to control (initial culture): *significant at P<0.05.

When the shoots were multiplied on an MS medium containing 0.25 mg/l BAP for three weeks, 10% of the in vitro obtained shoots showed vitrification signs. The minority of these signs (3%) was represented in the appearance of enlarged and deformed leaves, and it was associated with enlarged stems (Figure 1A). Plant shoots that expressed vitrification in this way could not be used to produce artificial seeds. Also, the majority of vitrified shoots represented an increase in leaf thickness and the formation of a tissue mass at the base of shoots (Figure 1B). Rarely, necrosis of apical shoot tips and loss of apical dominance was detected on shoot multiplication medium

Figure 1. Shoot multiplication at the third for three weeks. A: Vitrification sign is represented by enlargement and deformation of the plant leaves. B: The vitrification sign is represented by a tissue mass on the base of the explant.

During the fourth, fifth, and sixth subcultures, *in vitro* cultured shoots showed a high percentage of shoot multiplication, ranging from 81% to 86% (Table 1). During those three subcultures, the shoots showed the best value in terms of shoot number/explant, and the determined growth parameters. Therefore, shoots subjected to these numbers of subcultures were exposed to silver nitrate in order to avoid vitrification completely. Shoots were sub-cultured on MS medium containing 1.7 mg/l $AgNO_3$ in addition to 0.25 mg/l BAP. The addition of 1.7 mg/l $AgNO_3$ resulted in an improvement in the multiplication percentages and the number of shoots/explant compared to their counterparts that were sub-cultured on a medium containing 0.25 mg/l BAP only. On the contrary, the weight of the shoots/shoot cluster was lower in the presence of silver nitrate media than in its absence (Table 2).

Table 2. In vitro shoot multiplication and growth of stevia during three successive subcultures, three weeks/each, on MS medium with 0.25 mg/l BAP and 1.7 mg/l AgNO3.

No.of sub-culture	Shoot formation frequency (%)	No. shoots /Explant	Length/ shoot (cm)	No. nodes /shoot	No. leaves /shoot	F.W./shoot (g)	F.W./shoot cluster (g)
4	84.6	12±1*	3.7±0.2*	3.7±0.6	7.7±0.6	0.052±0.004*	0.34±0.005*
5	92.6	9.7±0.58	3.3.±0.2	3.6±0.57	9.3±1.2	0.05±0.006*	0.31±0.045*
6	85	9±1*	3.1±0.1	3±0	7.7±0.6	0.04±0.007	0.3±0.03*

Values are means of three replicates ± standard deviation (SD)
Statistical significance of differences compared to control (0.25 BAP at first subculture): *significant at P<0.05.

The presence of 1.7 mg/l AgNO3 in the shoot multiplication medium resulted in the disappearance of vitrification (Figure 2A). Therefore, tissue masses and enlargement of shoot leaves were not detected. In addition, the yielded shoots were healthy and could be used entirely to produce artificial seeds or induction of root formation. The fresh weight of the shoot cluster that resulted from a single explant on MS media containing 1.7 mg/l AgNO3 was higher than that of the shoot cluster formed on MS media free of it. This increase resulted from the increase in the number of shoots that were produced under the influence of AgNO3.

Since the production of artificial seeds was fulfilled using a nodal segment, the production of the largest number of shoots/explant helped to produce synseeds in mass quantity. Therefore, a valuable increase in the number of shoots/explant was obtained by increasing the culture period for more than three weeks, with the absence (Figure 2B) or presence of 1.7 mg/l AgNO3 (Figure 2C), but its presence was qualitatively better.

Figure 3. Shoot multiplication at the third subculture. A: Shoot multiplication on MS medium supplemented with 0.25 mg/l BAP and 1.7 mg/l $AgNO_3$ for 3 weeks representing healthy shoot formation. B: *In vitro*, **s**hoot multiplication appeared during subculture for the fourth time on MS medium supplemented with 0.25 mg/l BAP for 5 weeks. C: Shoot multiplication as it appeared during subculture for the fourth time on MS medium supplemented with 0.25 mg/l BAP and 1.7 mg/l $AgNO_3$ for 5 weeks.

It is clear from the results (Tables 1 and 2) that each *in vitro* obtained explant had between 3 to 4 nodes. This means that each shoot could be used to produce 3 to 4 artificial seeds. Then, each single *in vitro* obtained explant formed a shoot cluster with about 8 shoots or more that

could be used to produce about 32 artificial seeds or more. Also, it was clear that the use of silver nitrate led to an increase in the frequency of shoot formation that resulted from each *in vitro* obtained explant as well as the number of shoots/explant. In addition, the presence of silver nitrate masked the appearance of the vitrification, which made all the formed shoots suitable for the production of artificial seeds capable of conversion.

For the production of synseeds, small nodal cuttings with one node were encapsulated using MS medium containing 4% (w/v) of sodium alginate as a gel matrix and 75 mM CaCl2 as a complexing agent. The formed synseeds were uniform granules with a diameter of 2-3 mm (Figure 4A). The seeds showed the ability to convert when placed directly on MS media containing 0.25 mg/l BAP or after keeping them for a month in a refrigerator at 4 °C in a closed glass jar containing a thin layer of liquid MS media supplemented with 0.25 mg/l BAP. However, the seed's conversion was also affected by the absence (Figure 4B) or presence (Figure 4C) of 1.7 mg/L AgNO3. The absence of silver nitrate resulted in the appearance of vitrification through enlarged leaves and stems. On the contrary, the presence of silver nitrate in the conversion medium resulted in healthy and sufficient conversion for a further subculture or root formation for the purpose of acclimatization and translocation to *ex vitro* conditions

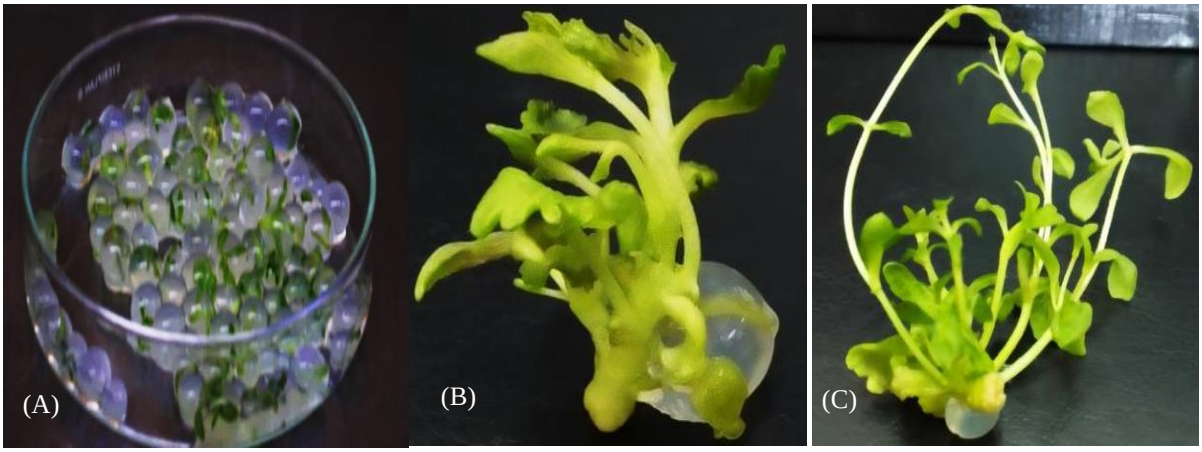

Figure 4. Synseeds formation (A) and conversion (B and C). The converted seeds showed enlargement vitrification (B) on MS medium containing 0.25 mg/L BAP or healthy shoots (C) on MS medium containing 0.25 mg/L BAP and 1.7 mg/L $AgNO_3$ for 4 weeks.

Data recorded for synseeds formation and conversion showed that storing the synseeds for a month at a low temperature negatively affected, to a very limited extent, the ability of the seeds to convert, where the conversion was 90%. The results also indicated that storing the synseeds for four weeks resulted in an increase in the number of shoots/synseeds and growth parameters of the obtained shoots compared to synseeds that were not stored at all (Table 3).

Table 3. Synseeds were stored for different periods at 4 ^{o}C and converted for three weeks at 4 ^{o}C.

Storage period (day)	Synseed conversion (%)	No.of shoots/ synseed	Length/ shoot (cm)	No. nodes /shoot	No. leaves /shoot	F.W./ shoot (g)	F.W./shoot cluster (g)
0	100 %	5.7±0.58	1.3±0.15	2.3±0.58	4.3±0.58	0.038±0.002	0.54±0.044
30	90 %	11.3±1.5	3±0.15	4.7±0.58	8.3±0.58	0.054±0.005	0.89±0.09

In vitro formed roots were investigated using a full-strength MS medium containing 15 g/l sucrose and different concentrations of IBA (Table 4). Increase the concentration of IBA to more than 0.5 mg/l fasted root formation. The best frequency of root formation, the highest number of

roots/shoot cutting, and the best growth/ root were obtained when 1 mg/l IBA was used (Figure 5). Also, the application of 1 mg/l IBA resulted in the best shoot growth parameters (shoot length, number of nodes, and fresh weight).

Table 4. Stevia root formation is influenced by the concentration of IBA.

IBA Conc. (mg/l)	Root initiation period (days)	Rooting frequency (%)	No. of roots/ explant	Length of the root system (cm)	Shoot length (cm)	No. of nodes/ shoot	Shoot fresh weight (g)
0.5	10	80.1%	3.7±0.6	2.4±0.1	4.27±0.2	4.3±0.6	0.05±0.005
1	7	100%	8.0±1*	3.3±0.1*	6.4±0.15*	5.7±0.6	0.09±0.007*
1.5	8	70.1%	6.0±1*	1.6±0.1*	6.3±0.15*	5.3±0.6	0.07±0.006*

Values are means of three replicates ± standard deviation (SD)

Statistical significance of differences compared to control (0.5 IBA): *significant at P<0.05.

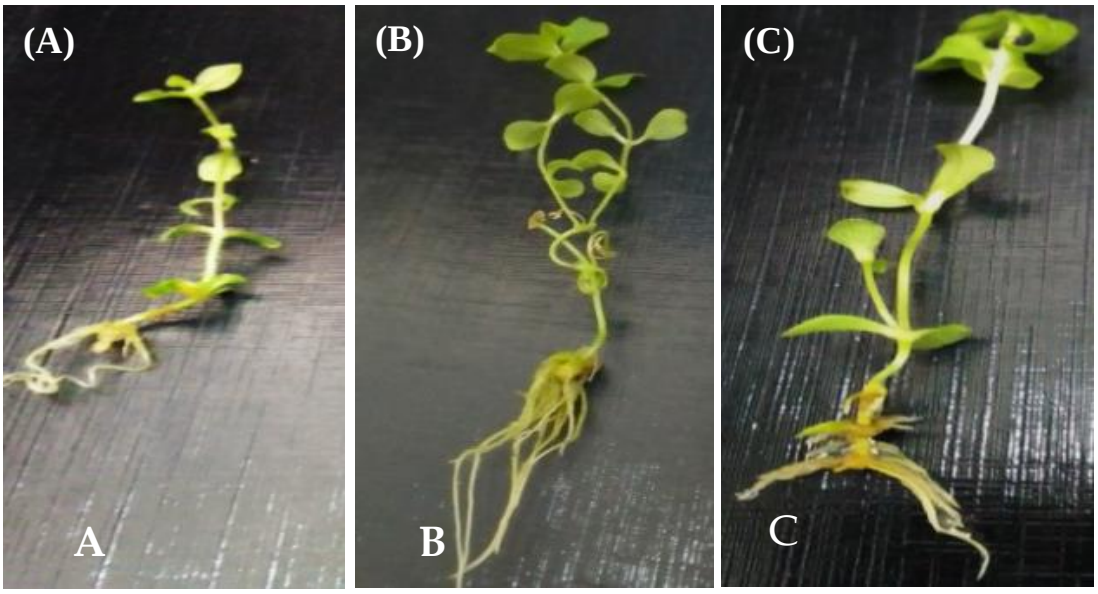

Figure 5. Roots formation on half-strength MS medium containing 0.5 (A), 1 (B), and 1.5 (C) mg/l IBA for four weeks.

In vitro formed shoots free from any appearance of vitrification were subcultured on MS medium containing 1 mg/l IBA. Within five days, the base of the shoots began to show enlargement, and then the roots began to appear. The roots were clearly seen within 7 days. The roots formed on the base of the shoot cuttings continued to grow as well as the shoot system. Each shoot cutting formed 6.3 roots with a length of 1.8 cm within two weeks. Necrosis of apical shoot tips as an appearance of vitrification was detected when shoot cuttings were cultured on MS medium containing 1 mg/l IBA leading to loss of apical dominance. Therefore, the branched shoot tip of the rooted plantlets was detected (Figure 6A).

Successful acclimation of the in vitro obtained plantlets was fulfilled when shoots with extensive root systems were transferred to plastic pots containing a mixture of sand and peat moss (1:2 v/v) under the conditions of the tissue culture room (Figure 6B). Placing plantlets under transparent polyethylene bags saved relatively high humidity where it measured 87%. By pouring the transparent plastic bag and increasing its diameter with time, the gradual reduction in relative humidity around the acclimatized plantlets was established in three weeks.

Figure 6. Acclimatization of *in vitro* grown stevia plant. A: Roots formation on MS medium containing 1 mg/l IBA for two weeks. The plantlet shows an extensive root system and branched shoot tip. B: Acclimatized stevia plant grew for two months under *ex vitro* conditions.

4. Discussion

An efficient sterilization procedure was established when stevia shoots segments of field-grown plants as well as other plant species were sterilized using sodium hypochlorite and mercuric chloride. Sodium hypochlorite is a common choice for surface sterilization of field or greenhouse-grown plants where it is effective and available at a low price. While mercuric chloride is a very strong disinfectant against contaminants but it is very dangerous and toxic to plants and humans. To avoid the phytotoxicity of these substances and obtain the right balance between the concentration of the disinfectant substance and subjection time, commercial bleach (5 % v/v) was used for 5 min and HgCl2 (0.2% w/v) was used for 2 min. Since explants must be viable after sterilization time, disinfectant substances were removed by washing the explants in sterilized deionized water (Hassanein, 2019). The applied sterilization protocol minimized explants injury and achieve better explant survival (85%).

The production of artificial seeds in stevia required several basic factors. The first factor that influenced the production of synseeds in stevia was the production of mass quantities of nodal cuttings that could be encapsulated in calcium alginate beads (Hassanein *et al.*, 2005). It was established by the subculture of stevia nodal or shoot tip cuttings on MS medium containing 0.25 mg/l BAP for three weeks as was described by Salem (2020). The used protocol resulted in 100% shoot formation during the first three subcultures but an increase in the number of subcultures resulted in a decrease in shoot multiplication to 50% by the tenth subculture. On the contrary, by increasing the number of subcultures, the number of shoots/explant increased significantly. This increase in shoot number compensated for the decrease in shoot frequency by increasing the number of subcultures. Upon the first subculture, shoot length increased and continued during the successive subculture; it also, saved valuable plant materials for the encapsulation process to produce synseeds (Hassanein *et al.*, 2005; Salem 2020).

The second factor that influenced artificial seed production in stevia was the production of healthy plant materials. The applied protocol for stevia shoot multiplication should produce

healthy shoots and be suitable for the encapsulation process. This means that the multiplied plant materials should be free of any appearance that would retard synseeds formation and their conversion. One of these aspects that must be avoided is the phenomenon of tissue vitrification during shoot multiplication before the production of calcium alginate beads. Stevia shoot multiplication on MS medium containing 0.25 mg/l BAP for three weeks resulted in low vitrification frequency (10%); it was avoided by incorporation of 1.7 mg/l AgNO3 in multiplication medium. In our work, stevia nodal segments were subcultured on a MS medium containing a low concentration of BAP (0.25 mg/l) to obtain sufficient plant materials for the mass production of synseeds. In other work, nodal or shoot tip explants of stevia cultured on MS medium containing a relatively high concentration of BAP (2 mg/l) resulted in the highest number of regenerated shoots without any sign of vitrification (Jahan et al., 2014). The incorporation of 1.7 mg/l AgNO3 in MS medium improved multiplication and growth parameters in stevia and other plant species (Drisya Ravi et al., 2019). The third factor that influenced artificial seed production in stevia was the production of healthy-unvitrified plant materials. Manifestations of vitrification in moringa appeared through the formation of tissue mass at the base of cultured plant material, enlargement of stem and leaves of the in vitro formed shoots, or necrosis of shoot tips (Hassanein et al., 2018). Appearance of vitrification was disappeared when 1.7 mg/l AgNO3 was used and it associated with the increase in shoot multiplication (Salem 2020) and growth. Under in vitro culture conditions, cells show cell division without sufficient protection where their cell walls lack sufficient lignin and cellulose (Kevers & Gaspar, 1986). Cells lack these components under a high relative humidity atmosphere in a closed vessel diffused large amounts of water leading to the appearance of highly vacuolated cells (Safrazbekyan *et al.*, 1990). Application of cytokinin such as BAP may lead to the induction of rapid cell divisions in meristem regions leading to necrosis of shoot tips and other vitrification symptoms such as stem and leaf enlargement (Abdalla et al., 2022).

The fourth factor that influenced the production of synseeds in stevia was the suitability of the used chemicals and their concentration, they should be in appropriate concentrations to avoid their negative impact on the vitality of encapsulated tissues or the ability of synseeds to convert (Hassanein *et al.*, 2011). These conditions were attained when small nodal cuttings were encapsulated within artificial endosperm containing MS medium supplemented with 3% (w/v) of sodium alginate as a gel matrix and 75 mM CaCl2 as a complexing agent. The resulting synseeds were uniform granules with a diameter of 2-3 mm. The synseeds showed the ability to convert when placed directly on MS media containing 0.25 mg/l BAP or after keeping them for a month in a refrigerator at 4 ºC in a closed glass jar containing a thin layer of liquid MS with 0.25 mg/l BAP. To avoid vitrification during synseeds conversion, 1.7 mg/l AgNO3 was added to the conversion medium. For the production of synseeds from stevia, an ion exchange took place during the nodal segment encapsulation leading to the replacement of sodium ions by calcium ions forming calcium alginate beds. In 30 min ion exchange, the obtained beads had suitable hardening (Bapat and Mhatre, 2005; Hassanein et al., 2005; Hassanein *et al.*, 2011). The used protocol led to the formation of synseeds with a nutritive complex (artificial endosperm) that supplemented the conversion process of the encapsulated plant materials like the endosperm of the natural seed (Bapat and Mhatre, 2005). Production of calcium alginate capsules using potassium nitrate resulted in synseeds with suitable softening and allowed the easy emergence of shoots from alginate beads (Onishi *et al.*, 1994). In our work, the right component of the artificial endosperm

consisted of basal MS medium supplemented with 3% sucrose and 0.25 mg/l BAP. The created synseeds were able to convert even after conservation under low temperatures for one month.

In stevia root formation frequency, root number, and root growth was influenced by the concentration of IBA, 1 mg/l was the best. IBA has various aspects in root development, including regulation of the size of root apical meristem, lateral root development, formation and elongation of root hair, and *ex vitro* and *in vitro* adventitious root formation. It is well known that IBA is a 'synthetic auxin' that elicited auxin-like effects and it can convert into IAA. For example, in arabidopsis, IBA promotes adventitious root formation through its conversion to IAA (Veloccia *et al.*, 2016). In successful micropropagation, better shoots with extensive root systems can produce healthy plants under greenhouse conditions (Lawson et al., 2023). In stevia application of 1 mg/l, IBA resulted in the best growth parameters of *in vitro* formed roots and shoots. In addition, shoot cuttings derived from *in vitro* cultured shoots or synseeds conversion, and free from any appearance of vitrification formed extensive root systems on MS medium containing 1 mg/l IBA. In stevia and other plant species, shoot tip necrosis led to loss of apical dominance but it did not retard root formation or plantlets acclimatization (Kataeva, 1986).

Plantlets with extensive root systems were transferred to small plastic pots containing a mixture of sand and peat moss (1:2 v/v) and covered by transparent polyethylene bags. Pored the plastic cover and increase its diameter with the time-saving gradual reduction in the humidity of the atmosphere around the acclimatized plantlets. In three weeks, acclimatized plants (60%) were able to grow under ex-vitro conditions. The poor survival rate of the acclimatized plantlets was attributed to the non-functionality of the in vitro-developed rooting system (Aygun and Dumanoglu 2015). During the acclimatization, the in vitro plantlets overcome the abnormalities of the in vitro growth period through the gradual reduction of the relative humidity under transparent plastic bags (Ubalua and Nsofor, 2017). In addition, during the acclimatization procedure, the structure of plant cuticle and stomata was improved to regulate plant water loss (Seelye *et al.*, 2003). The gradual reduction in humidity during stevia acclimatization was attained by poring the plastic bags starting from the fifth day, but it started from the third day by others (Ubalua and Nsofor 2017). Acclimatization of stevia plantlets was fulfilled under tissue culture room conditions where the temperature was 25 ^{o}C. The relative humidity under transparent plastic pages was 87%. Successful in vitro acclimatization of Phalaenopsis orchid plantlets was carried out under low temperature (15-25 ^{o}C) and low relative humidity (60±5%RH). The acclimatized plants showed high levels of photosynthetic pigments and net photosynthetic rate as well as stomatal conductance and low transpiration rate, leading to enhancement of the determined growth parameters. In contrast, orchid plantlets acclimatized under low relative humidity (50%) and extreme temperature (35 ^{o}C) showed wilting symptoms and chlorophyll degradation leading to growth retardation (Jeon *et al.*2006).

5. Conclusions

To avoid vitrification during shoot multiplication and synseeds conversion, MS medium supplemented with 0.25 mg/l BAP and 1.7 mg/l $AgNO_3$ was used. Uniform synseeds with a diameter of 2-3 were obtained when small stevia nodal cuttings were encapsulated within artificial endosperm containing MS medium supplemented with 4% (w/v) of sodium alginate as a gel matrix and 75 mM $CaCl_2$ as a complexing agent. Within 30 min, ion replacement of sodium ions by calcium ions formed calcium alginate beds (artificial endosperm) as a nutritive complex. This complex supplemented the conversion process of the encapsulated plant materials and allowed the easy emergence of shoots from alginate beads. The synseeds showed the ability to convert

in vitro even after their preservation for a month in a refrigerator at 4 ^{o}C in a closed glass jar containing a thin layer of liquid MS.

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مقال

الملخص العربي

الاستنساخ المعملي لنبات الإستيفيا لتجنب الظاهرة الزجاجية وإنتاج البذور الاصطناعية

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نبات الإستيفيا نبات طبي هام، تستخدم أوراق نبات الإستيفيا كمصدر للسكر الغير معطي للسعرات الحرارية، تقدر حلاوة سكر الإستيفيا أكثر 300 مرة من حلاوة قصب السكر. معدل إنبات بذور نبات الإستيفيا منخفض جدا، ولذلك فإن الإكثار المعملي الدقيق لنبات الإستيفيا متطلب أساسي لإنتاج كميات من الإستيفيا وتعديلها. ظروف زراعة الأنسجة المحددة تم تطبيقها لاستنساخ الأفرع الخضرية وتكوين البذور الاصطناعية. تضاعف الأفرع كان على الوسط المغذي موراشيجي وسكوج المضاف إليها 0.25 مجم/لتر من البنزيل أمينو بيورين. أفضل تكرار لتكوين الأفرع الخضرية تم الحصول علية عند زراعة المستقطعات العقدية كان في الجيل الخامس وبإضافة نترات الفضة بتركيز 1.7 مجم/لتر لوحظ زيادة في تكرار تكوين الأفرع الخضرية وتقليل الظاهرة الزجاجية. لإنتاج البذور الاصطناعية تم تغليف المستقطعات العقدية الصغيرة (تقريبا بطول 3 مم) بمحلول ألجينات الصوديوم بتركيز 4% وكلوريد الكالسيوم بتركيز 75 مل مول كمنبت هلامي وعامل تركيبي على التوالي. مستقطعات الأفرع الخضرية الناتجة من تحول البذور الاصطناعية وزراعة الأفرع الخضرية تم استخدامها لتأسيس تكوين الجذور على الوسط المغذي موراشيجي وسكوج المضاف إلية 1 مجم/لتر من حمض الإندول بيوتيريك. النبتات ذات المجموع الجذري الممتد تم تثبيتها في التربة.

الكلمات الرئيسية: البذور الاصطناعية، الإكثار المعملي الدقيق، الإستيفيا، الظاهرة الزجاجية، نترات الفضة..