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The Optimized Protocols for Production, Adaptation and Keeping of the Produced Artificial Seeds from Encapsulated Lateral Buds in *Stevia Rebaudiana* (Bertoni)

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ABSTRACT

Stevia, as an anti-diabetic medicinal plant, is estimated to be about 300 times sweeter than sugar cane. Low seed germination is a major problem in stevia propagation. Artificial seeds (ASs) technology is a valuable method for it's rapid and massive propagation. In this study, the effective factors on production, the produced plantlet adaptation and keeping of the ASs produced from encapsulated lateral buds were efficiently optimized for the first time. The results showed that 2.5% sodium alginate with 1% calcium chloride was the best composition for coating the ASs. The bigger lateral buds (2-3 mm) with MS medium resulted in the highest percent and rate of germination. Germination and growth of the ASs were increased using 1 mg l^{-1} BAP with 1.5 mg l^{-1} Kn in the pre-culture environment. The findings indicated that transmission of the ASs-plantlets to cocopeat seedbed increased the growth characters. Keeping the produced ASs in the MS medium had a significant ($p<0.05$) and positive effect on germination characters. Among the treatments for keeping artificial seeds in 90 days, the treatment of liquid paraffin at 4°C showed the best response for regrowth. In this research, the possibility of routine and massive production of ASs by encapsulating the lateral buds has been reported in stevia. Therefore, using this new protocol, the rapid and fast micro-propagation of this plant could be achieved through lateral buds for inexpensive and commercial purposes in the future.

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1. Introduction

Iran is a part of the arid and semi-arid regions of the world; therefore, water is the most important element for the sustainable production of agricultural products. The plant's water requirement depend on several factors, such as cultivars, climatic and agronomic*Stevia rebaudiana* (Bertoni) is a perennial, herbaceous and very important anti-diabetic medicinal plant (Ghaheri *et al*., 2019; Jalili *et al*., 2019; Ansari *et al*., 2018). It's natural and non-caloric sweetener substance, namely stevioside, is about 300 times sweeter than sugar. Stevioside is not absorbed in the digestive system, therefore; it can be used as a good alternative to other synthetic sweeteners, such as Aspartame and Sodium Saccharin (Esmaeili *et al*., 2018; Ghaheri *et al*., 2018; Akbari *et al*., 2018). Stevia, as a self-incompatible and cross-pollinated plant, due to very small and empty grains with low germinating percent, cannot routinely produce true seeds (Ghaheri *et al*., 2018; Kahrizi *et al*., 2018; Hashempoor *et al*., 2018). Therefore, artificial seeds (ASs) production can be an effective technology for massive and uniform propagation and maintenance of this medicinal plant what a low cost (Keshvari *et al*., 2018). This technology is an efficient method for keeping germplasm and massive propagation of virus-free plantlets (Sharma *et al*., 2013). The seeds produced by this method, having the appropriate size and lack of quarantine due to the structure of the seed coating and reducing the costs of production and transfer, can be a good alternative to the actual seed in many plants (Sharma *et al*., 2013). The ASs also has the long-term storage capability, along with maintaining seedling power. in order to adapt seedlings to the greenhouse or field conditions (Rai, 2009; Hung and Truman, 2012).

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In order to produce ASs in different plants, some encapsulated generative-units such as somatic embryos, lateral buds, nodules segments, end buds, or meristematic parts can be used (Sharma and Shahzad, 2012; Standardi and Michelle, 2013; Teixeira DA Silva, 2015). Synthetic seeds are often produced due to the easier and simultaneous development of roots and stem using somatic embryos (Gantit *et al*., 2015). However, somatic embryos are hardly produced in stevia, meanwhile; the created plantlets of the embryos show high genetic diversity. Therefore, application of the vegetative generative-units such as lateral buds, nodules segments, end-buds, or meristematic parts can be proposed as an economic and an alternative strategy to produce uniform and massive ASs (Hayat *et al*., 2010; Ravi *et al*., 2012).

So far, in addition to stevia, the protocols of production of ASs in different medicinal plants, such as cannabis (Latta *et al*., 2009), ginger (Sandraj, 2010), eucalyptus (Hung and Truman, 2012), albizia (Parveen and Anis, 2014), vitex (Ahmed *et al*., 2015) and basil (Saha *et al*., 2015) have been previously reported. Indeed, although Sivaram and Mukandan (2003), Hung (2006), Hetmant et al. (2010), Ahmed et al. (2013), and Nower (2014) have previously reported the production of ASs in the stevia through end-buds and nodules-segments, in our study, the possibility of routine and massive production of ASs by encapsulating the lateral buds, has been established in stevia for the first time.

One of the important factors in the production of ASs is detecting the proper concentration of sodium alginate and calcium chloride, which significantly affects the encapsulation of explants. Among the jelling agent, sodium alginate due to its negligible toxicity, the cost low, the ability to jell in the shortest time and the properties of biotic compatibility are the best material for the formation of artificial seed shells (Chung *et al*., 2014). Nower (2014) encapsulated the shoot tips of stevia in 4 % calcium alginate.

Selection of suitable culture medium, size and type of explants for regeneration of ASs are effective factors on increasing the germination efficiency, so that the tissue of the explants is younger, more active and more meristematic have more ability to germinate and grow (Bustam *et al*., 2013). Laribi et al. (2012) reported the maximum micro propagation on MS medium containing 1 mg l^{-1} BAP and 0.5 mg l^{-1} IBA in stevia. The yield of seedlings in different concentrations and the use of different hormones vary in different plants. Using growth regulators and their appropriate combination in the endosperm environment of explants can increase germination efficiency, viability and vegetative growth of shootings in somatic embryos and capsulated propagules (Sharma and Shahrzad, 2012). The induction of shoots from nodal segments was the maximum in MS medium containing 1 mg l^{-1} BAP (Nower, 2014). In a research (Parveen and Shahzad, 2014), the ideal ASs was obtained by a composition of 3 % sodium alginate and 100 mM calcium chloride. Meanwhile, the ideal results of ASs were obtained in MS medium supplemented with 2.5 µM BAP and 0.4 µM NAA. Artificial seeds in different substrates such as coco peat, compost, vermiculite, perlite and sand to increase the power of converting capsulated explants to seedlings and also increasing their adaptation with a greenhouse and field conditions are planted (Rihan *et al*., 2011; Ravi *et al*., 2012). Artificial seeds can be kept at low temperatures, especially at 4ºC for a long time. In addition, the storage of ASs in liquid paraffin can be effective in prolonging the storage period of seedlings (Zhang *et al*., 2011). Nower (2014) suggested that the growth of the shoot was increased in capsules stored for 5 weeks on MS than other MS strengths.

Therefore, the present research carried out (i) - to optimize the best protocol for producing the ASs from encapsulated lateral buds in stevia for the first time, (ii)- to evaluate the effect of different type and the different size of the explants, various concentrations of sodium alginate and calcium chloride, different type of medium, and different concentrations of BAP, NAA, and Kn hormones in two medium of pre-culture and endosperm on quality of the obtained ASs , (iii)- to establish the possibility of long-term keeping the ASs at 4ºC and adaptation, and also (iv)- to study the best adaptation conditions for the produced plantlets.

2. Materials and methods

2.1. The site and plant materials of the experiment

The experiment was carried out at the Plant Breeding Laboratory, Department of Agronomy and Plant Breeding, Faculty of agriculture, Bu-Ali Sina University, Hamedan, Iran, in cooperation with the Agricultural and Natural Resources Research and Education Center of Kermanshah, Kermanshah, Iran. The mother seedlings of Stevia rebaudiana (Bertoni), prepared from" Golsaran company", Rasht, Gilan, Iran.

They were transported from the culture- trays to the pots and then, the mother seedlings were kept in an adaptive room for 45 days in order to provide sufficient lateral buds. After that, in order to facilitate the sterilization of the plant material parts, we cut the seedlings to pieces of about 3-10 cm (Fig. 1 a). After that, they were washed for 10 minutes with sterilized-water. The pieces were finally put in plastic bags and then transferred under the laminar hood. To sterilize the explants, seedlings were placed on ethanol 70% for 1 minute, and then washed with sterilized-water. The seedling pieces were placed in sodium hypochlorite 3% for 15 minutes and then rinsed in sterilized distilled water 3 times (each time for 5 minutes). After that, the sterilized seedling pieces were fixed using forceps under the laminar hood, and the lateral or apical buds were separated using a scalpel under the binocular. Before preparing the artificial seeds, the buds were placed in a solid MS medium (as the pre-culture medium) at a pH of 5.7 for 2 days, using 30 g l-1 sucrose and 8 g l-1 of agar.

2.2. Encapsulation of the buds

After the mentioned steps, to produce the synthetic endosperm for artificial seeds, different concentrations of autoclaved sodium alginate (pH=7.5) were mixed with MS liquid medium. Then, the buds were transferred from pre-culture medium and were immersed in a 100 ml solution of prepared artificial endosperm for 5 minutes. While in the first and second experiments, a hormone-free liquid MS was used, in other experiments, a hormone- containing liquid MS was applied. After that, encapsulation was completed by mixing the buds into the alginate and dropping these buds containing synthetic endosperm into the shaking Ca Cl2 solution by 1000 µl sampler. The droplets containing the explants were finally kept for at least 30 min to achieve polymerization of the sodium alginate and the obtained droplets were collected and rinsed 3 times with sterile distilled water to wash away CaCl2 residues. To remove the excess water, the prepared artificial seeds were put on filter paper. The obtained artificial seeds imposed to cold treatment in Petri dishes at 4ºC for 48 hours. After that, the artificial seeds were then placed in a growth chamber at 24ºC and 2000 lux light intensity with a light period of 16 hours and 8 hours of darkness on a regrowth medium consisting of solid MS medium (pH 5.7) with 30 g l^{-1} sucrose and 8 g l^{-1} of agar without growth regulators. Then, the rate and speed of germination were recorded until 21 days (Fig. 1 c and Fig. 1 d). In each experiment, the regrowth rate was defined as the percentage of the capsulated buds that elongated and emerged out of the capsule wall to produce in vitro plantlets. Regrowth speed was calculated according to Maguire (1962), which is $M = [N_1 / T_1 + N_2 / T_2 +... N_n / TN]$, where ($N_1$, N_2, etc.) are the number of germinated artificial seeds at times T_1, T_2 and etcetera.

2.3. Effect of various concentrations of sodium alginate and calcium chloride

This experiment was conducted to determine the most suitable concentration of sodium alginate and calcium chloride to encapsulate the buds. Sodium alginate with three concentrations (2, 2.5, and 3% w/v) and calcium chloride with three concentrations (0.75, 1, and 1.25% w/v) were used and their effect on forming seed crust, percent and germination rate were investigated (Fig. 1 b).

2.4. Effect of size of explants, type of explants and culture medium

This experiment was performed to study the effect of the size of explants, type of explants and the type of medium on germination traits of artificial seeds. Two types of explants (lateral and apical buds), two sizes of explants (1-1.99 and 2-3 mm) with two types of MS medium, (Ms:Murashig and Skoog 1962) and B5 (Gombur 1968) were used to study the effect of these factors on germination rate and speed.

2.5. Effect of composition and different concentrations of growth regulators

In this experiment, the bigger lateral buds (2-3 mm) with MS medium were used to study the effect of using different concentrations of growth regulators with two methods in the pre-culture medium of buds for 2 days and the matrix culture medium of the encapsulated buds in three sections. The first section consists of four concentration levels (0, 0.5, 1 and 1.5 mg l^{-1}) of BAP and four concentration levels (0, 0.5, 1 and 1.5 mg l^{-1}) of Kn, the second section consists of four levels (0, 0.5, 1 and 1.5 mg l^{-1}) of NAA and four levels (0, 0.5, 1 and 1.5 mg l^{-1}) of BAP, and the third part contains four concentration levels (0, 0.5, 1 and 1.5 mg l^{-1}) of Kn and

four concentration levels (0, 0.5, 1 and 1.5 mg l^{-1}) of NAA.

2.6. Effect of seed application method and the seedbed type

In this experiment, two different type of seed application (including direct use of encapsulated lateral buds and germinated seeds on MS medium) with three types of seedbed (including cocopeat, perlite and potting soil) (Fig. 1 e) were used. Direct seeds were immediately transferred into previously sterilized media by autoclave after encapsulating the lateral buds under the laminar hood. Also germinated seeds after 26 days of exposure to non-hormone solid MS were transferred to sterilize media under the laminar hood. In this experiment, polyethylene coatings with controlled moisture kept artificial seeds and adaptation conditions until root and stem emerged. After 2 weeks of placement in the media, full seedlings percent, mean shoot length, the mean number of leaflets and mean stem diameter traits were recorded.

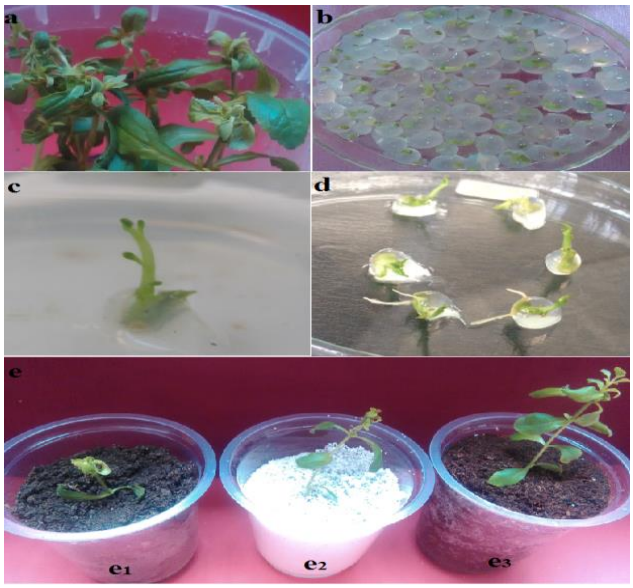

Figure 1. Steps of production of artificial seeds with lateral buds of *Stevia rebaudiana* (Bertoni). a: The branches of original plant including lateral buds. b: Encapsulated lateral buds with 2.5% sodium alginate and 1% calcium chloride. c: Emerging and elongation of the shoot of the artificial seed. d: Shoot and root emergence from produced artificial seeds. e: Three different types of the used seedbed of artificial seed for adaptation of produced planets including; potting soil (e1), perlite (e2) and cocopeat (e3).

2.7. Effect of method and time of artificial seed storage on regrowth

In this experiment, the effect of storage time on germination ability and regrowth of encapsulated lateral buds over time (0, 30, 60 and 90 days) was investigated. The encapsulated lateral buds were transferred into blue cap glass water containers free of the medium, containing MS medium and containing liquid paraffin. In each container, 144 artificial seeds were kept at 4ºC at the indicated intervals, 48 seeds were selected from each of the glasses and placed in 4ºC Petri dishes containing a non-hormone-solid MS medium, so that (each Petri dish was considered as one replicate and each replication included 10 seeds), after 19 days, germination rate and percent were measured.

2.8. Data analysis

The experiments were carried out based on a completely randomized design (CRD) with four replications (Petri dishes) and each Petri dish containing 10 artificial seeds. Data analysis was performed using SAS 9.2 and Minitab 16 software. The data of each experiment were tested for "treatment equal of variance" and "residual normality test" before analysis of variance. Comparison of means was done by Duncan's multiple-range test (Duncan, 1955) at the level of 0.05.

3. Results and discussion

3.1. Effect of various concentrations of sodium alginate and calcium chloride

Determination of the best concentrations of sodium alginate and calcium chloride is the first important step in encapsulating the different types of explants. This affects the placement of explants in the center of the capsules to absorb more nutrients. While the use of 2% sodium alginate produced fine and often non-uniform capsules, the use of 3% sodium alginate resulted in the formation of uniform and circular but hard capsules that delayed germination. Therefore, the results showed that the use of sodium alginate 2.5% with calcium chloride 1% resulted in the production of transparent, homogeneous synthetic seeds with displacement and storage capacity. It resulted in the maximum percent of ASs germination (46.25%) and the highest placement of the explants in the center of the capsules (83.75%) (Table 1). Nower (2014) reported that the use of 4% sodium alginate with calcium chloride 1% is the best treatment for encapsulating apical buds in stevia. While Nower ′s results (2014) did not completely agree with

our results, Mishra et al. (2011) and Parveen et al. (2013) stated that the use of sodium alginate more than 3% (due to hardening of the capsule shell) and the application of sodium alginate at concentrations of less than 2.5% (due to the formation of fine capsules) would reduce germination and growth traits.

Table 1. Effect of three concentrations of sodium alginate and three concentrations of $CaCl_2$ on the germination percentage and placement of the explants in the center of the capsules after 3 weeks of culture.

Sodium alginate (%)	CaCl2 (%)	Germination (%)	Placement of the explants in the capsule (%)
2.00	0.75	25.00^{c}	28.75^{d}
	1.00	25.00^{c}	25.00^{c}
	1.25	25.00^{c}	28.75^{d}
2.50	0.75	25.00^{c}	42.50^{c}
	1.00	46.25^{a}	83.75^{a}
	1.25	28.75^{b}	77.50^{b}
3.00	0.75	21.25^{d}	17.50^{g}
	1.00	21.25^{d}	21.25^{f}
	1.25	17.50^{e}	17.50^{g}

Different letters indicate significant differences according to Duncan's multiple test range with $p \leq 0.05$.

3.2. Effect of size of explants, type of explants and type of medium

Apical and lateral buds have uniform cells with remarkable genetic stability (Sharma et al. 2014). Using the appropriate size of explants and selecting the appropriate medium can be two effective factors in increasing the efficiency of capsuled buds.

The use of 2-3 millimeter buds in MS medium showed the highest percentage (68%) and speed (0.24) of germination compared to the B5 medium (Table 2). Also in another experiment, the use of lateral buds with a size of 2-3 millimeters in MS medium, produced the highest speed of germination (0.23) in comparison with the apical buds (Table 3). Apical buds should have a better performance due to the absence of end-domination, but in this experiment, the lateral buds performance significantly increased in comparison with the apical buds. According to laboratory observations, the reason can due to the greater ability of lateral buds in pre-cultivation and endosperm conditions, which are more vigorous in the stevia. Bustam et al. (2013) reported that the growth and germination of encapsulated lateral buds with larger explants increased significantly in orchids. The highest seedling regeneration was observed in the nodule segment of *Corymbia citriodora* and *Decaepis hamilttoui* in MS medium (Hung and Truman, 2012; Sharma *et al*., 2012). MS medium was better than WP medium for shoot numbers and shoot length of *Dalbergia sissoo* Roxb (Thirunavoukkarasu *et al*., 2010). Eighty-seven percent of the encapsulated nodal segment of *Orchid hybrid* in the B5 medium became seedlings after two months (Mahendran *et al*., 2014). The highest percentage and rate of germination of potato-capsulated buds were reported for 2-3 mm buds in MS medium (Ghanbarali *et al*., 2016).

Table 2. Effect of explants size and culture medium on regrowth in encapsulated buds after 3 weeks of culture.

Explants size (mm)	Culture medium type	Regrowth	
		Rate (%)	Speed
1.00-1.99	MS	31.00^{c}	0.09^{bc}
	B5	31.00^{c}	0.07^{c}
2.00-3.00	MS	68.00^{a}	0.24^{a}
	B5	50.00^{b}	0.13^{a}

Different letters indicate significant differences according to Duncan's multiple test range with $p \leq 0.05$.

Table 3. Effect of explants type and explants size on regrowth speed of capsulated buds after 3 weeks of culture.

Explants type	Bud size (mm)	Regrowth
Lateral bud	1.00-1.99	0.10^{bc}
Lateral bud	2.00-3.00	0.23^{a}
Apical bud	1.00-1.99	0.06^{c}
Apical bud	2.00-3.00	0.14^{b}

Different letters indicate significant differences according to Duncan's multiple range test range with $p \leq 0.05$.

3.4. Effect of growth regulators

The composition of the gel matrix is an important factor that significantly affects the performance and utility of artificial seeds (Sharma *et al*., 2014). The use of 1 mg l^{-1} of BAP with 1.5 mg l^{-1} Kn showed the highest germination percent (87.50), germination rate (0.44) and shoot length (2.25 cm) (Table 4). The use of 1 mg l^{-1} BAP in endosperm medium (matrix) had the highest shoot length (2.04 cm) (Fig. 2). The maximum length and number of shoots in the encapsulated nodules of stevia were reported at 1 mg / L BAP. Also, the maximum number of leaflets was reported with 1.5 mg l^{-1} Kn in MS medium (Ahmed *et al.*, 2013). Using 1.3 mg l^{-1} BAP in MS medium showed the highest yield of artificial seeds in sugar beet (Roba *et al*., 2014). The highest growth and germination characteristics of capsuled apical buds were reported in *Ficus carical* on MS media with 5 µM BAP with 5 µM Kn (Sharma and Shahrzad, 2014).

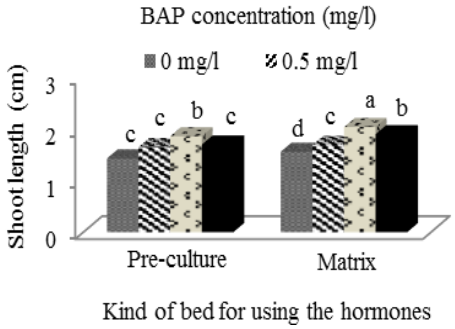

Figure 2. Effect of different concentrations of BAP in two kind of bed on shoot length of encapsulated lateral buds during 3 weeks after the artificial seeds culture. Different letters indicate significant differences according to Duncan's multiple range test.

Applying 1.5 mg l^{-1} BAP with 0.5 mg l^{-1} NAA showed the highest percentage (83.75) and speed (0.43) of germination and shoot length (1.96 cm) (Table 5). In addition, the use of 0.5 mg l^{-1} NAA in the matrix medium of capsuled lateral buds showed the highest regrowth (Fig. 3). The use of 0.3 µM NAA with 2.2 µM BAP showed the highest increase in germination characteristics in the encapsulated nodules segments in the *Corymbia citriodora* (Hung and Truman, 2012). Using 0.2 mg l^{-1} NAA showed the highest shoot length in the encapsulated nodule segments of the stevia (Ahmed *et al*., 2013). Adhikari et al. (2014) proposed using 4.4 µM BAP with 0.6 µM NAA as the best treatment for encapsulating the apical buds in *Cucumis sativus* L. Ahmed et al. (2015) reported the use of 5 µM BAP and 0.5 µM of NAA in encapsulated nodules segments of *Vitex trifolia*.

Table 5. Effect of the different concentrations hormones BAP and NAA on regrowth rate, speed and shoot length encapsulated lateral buds after 3 weeks of culture. Different letters indicate significant differences according to Duncan's multiple range test.

BAP concentration (mg l^{-1})	NAA concentration (mg l^{-1})	Regrowth		Shoot length (cm)
		Rate	Speed	
	0.0	71.2^{b}	0.3^{e}	1.2^{k}
0.0	0.5	71.2^{b}	0.3^{e}	1.3^{jk}
	1.0	73.1^{b}	0.3^{de}	1.4^{ij}
	1.5	75.0^{b}	0.3^{bcde}	1.4^{hi}
	0.0	73.1^{b}	0.3^{cde}	1.5^{h}
0.5	0.5	73.1^{b}	0.3^{bcde}	1.5^{gh}
	1.0	75.0^{b}	0.3^{bcde}	$1.6f^{g}$
	1.5	75.0^{b}	0.3^{bcde}	1.6^{ef}
	0.0	73.1^{b}	0.3^{bcde}	1.7^{de}
1.0	0.5	75.0^{b}	0.3^{bcde}	1.7^{cd}
	1.0	75.0^{b}	0.3^{bcde}	1.8^{bcd}
	1.5	75.0^{b}	0.3^{bcd}	1.8^{bc}
	0.0	75.0^{b}	0.3^{ab}	1.8^{ab}
1.5	0.5	83.7^{a}	0.4^{a}	1.9^{a}
	1.0	75.0^{b}	0.3^{abc}	1.8^{ab}
	1.5	75.0^{b}	0.3^{bcd}	1.8^{abc}

Different letters indicate significant differences according to Duncan's multiple range test range with $p \leq 0.05$.

Using 1 mg l^{-1} Kn and 0.5 mg l^{-1} NAA showed the highest shoot length (1.80 cm) (Fig. 4).

The use of 1 mgl^{-1} Kn in the endosperm medium of encapsulated lateral buds showed the highest shoot length (1.76 cm) (Fig. 5). Rihan et al. (2011) reported the highest levels of germination and growth of artificial seeds of *Brassica olerace* using 2 mg l^{-1} Kn and 1 mg l^{-1} IBA in MS medium. The use of 0.5 µM NAA in hydrogel medium of apical encapsulated in *Cassia angustifolia* vahl showed the highest survival (95%) and growth of germinated seeds (Parveen and Shahrzad, 2014).

Using 1 mg l^{-1} of BAP and 1.5 mg l^{-1} Kn led to the highest efficiency in germination percent and rate (19 and 0.2 respectively), as well as the length of the shoots in the germinated seeds was reported in comparison with to the combination of BAP and NAA, as well as Kn and NAA (2.40 cm). In reality, many studies found

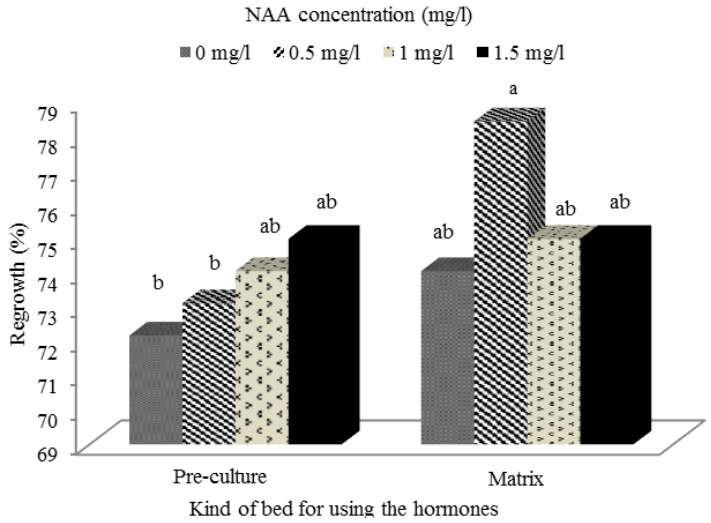

Figure 3. Effect of different concentrations of NAA in two kind of bed on regrowth of encapsulated lateral buds during 3 weeks after the artificial seeds culture. Different letters indicate significant differences according to Duncan's multiple range test.

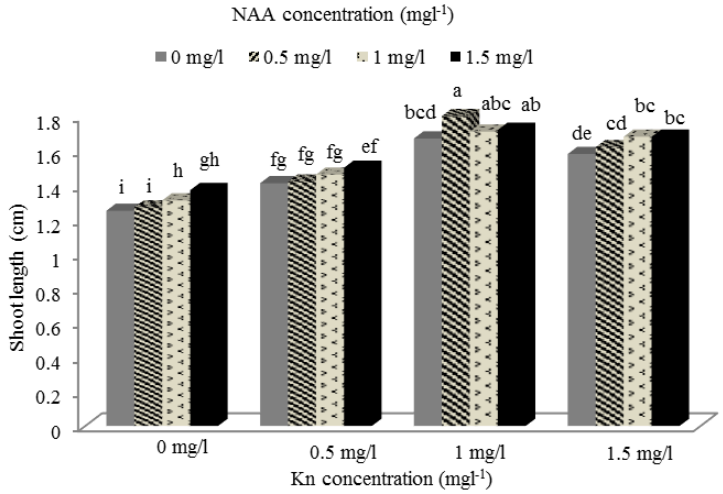

Figure 4. Effect of different mixed concentrations of NAA and Kn hormones on length shoot of encapsulated lateral buds during 3 weeks after the artificial seeds culture. Different letters indicate significant differences according to Duncan's multiple range test.

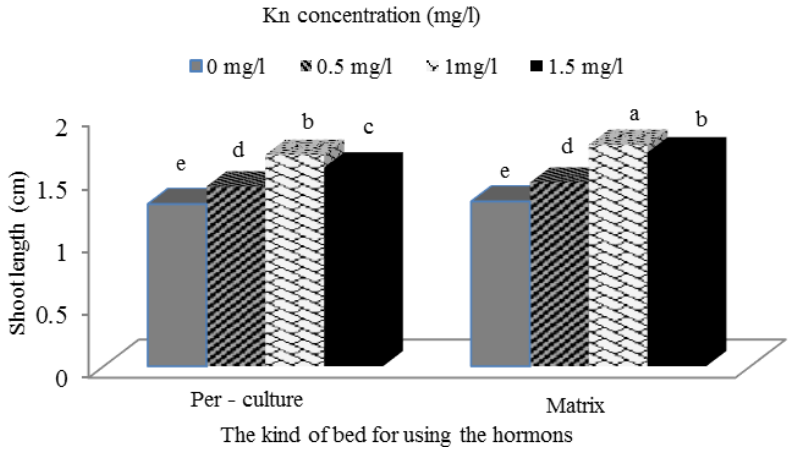

Figure 5. Effect of different concentrations of Kn used in two kind of bed on shoot length of encapsulated axillary buds during 3 weeks after the artificial seeds culture.

that using the Kn hormone in combination with other growth regulators can be desirable. So that, in a study (Gopinath *et al*., 2014) the use of this hormone with BAP had a key role in increasing germination.

3.5. Effect of seed application method and the seedbed type

The results showed a significant effect of the seed application method and the type of medium the number of leaflets and stem diameter. So that the treatment of seed germinated on the MS medium with cocopeat seedbed showed the highest number of leaflets (10.20) and stem diameter of (1.88 mm). In fact, the better ability of this seedbed to maintain moisture compared to perlite and garden soil may be due to this result (Table 6).

Table 6. Effect of seed application method and seedbed type on the number of leaflets and stem diameter of encapsulated lateral buds after 3 weeks of culture.

Seed application method	Substrate	Number of leaflets	Stem diameter (mm)
Direct seed application	cocopeat	5.75^{c}	1.27^{bc}
	perlite	4.75^{cd}	1.22^{c}
	garden soil	4.25^{d}	1.12^{c}
Seed germinated on MS	cocopeat	10.20^{a}	1.88^{a}
	perlite	9.33^{ab}	1.50^{b}
	garden soil	7.25^{b}	1.30^{bc}

Different letters indicate significant differences according to Duncan's multiple range test with $p \leq 0.05$.

Another reason for this observation needs to create a second shell in the artificial seeds in order to maintain the germination power of the encapsulated seeds as well as increasing the compatibility with in vitro conditions. Using a commercial medium may vary depending on the type and species of the plant, for example, using a mixture of garden soil and sand with equal proportions in the *Spilantes acmelia* L. (*Singh et al*., 2010), peat moss in *Dalbergia sissoo* Roxb (Chand and Singh, 2010), perlite in potato cultivar M26 (Michel *et al*., 2010), the mixture of perlite and compost in *Brassica oleracea* (Rihan *et al*., 2011) and *Corymbia citriodora* (Hung and Truman, 2012), perlite and compost in the encapsulated nodules of *Decaepis hamilttoui* (Sharma and Shahrzad, 2012), peat moss and Sand in Sugar beet (Roba *et al*., 2014) and cocopeat in potato (GhambarAli *et al*., 2016) was reported.

3.6. Effect of method and time of artificial seed storage on regrowth

Maintaining encapsulated buds inside the MS medium and liquid paraffin at 4ºC showed the highest amount of germination characteristics (Table 7).

Considering the significant reduction of germination traits in the lateral buds after 90 days, the highest percentage and rate of germination of artificial seeds were seen at 67.50 and 0.18 in liquid paraffin, respectively. Indeed, Among the treatments for keeping artificial seeds in 90 days, the treatment of liquid paraffin at 4ºC showed the best response for regrowth (Table 7). It shows the impact of liquid paraffin on increasing the possibility of maintaining artificial seeds over longer periods. Reducing germination characteristics of stored seeds at 4ºC after 90 days could be due to the possibility of endosperm tissue respiration and loss of moisture in the tissue during maintenance periods (Sharma *et al*., 2014). Also, due to the proper function of stored artificial seeds in liquid paraffin after 90 days, it can be concluded that the use of liquid paraffin to keep artificial seeds in longer periods at low temperatures can be a very effective method .This can be explained by the ability of liquid paraffin to keep intact the plant cells at low temperatures (Zhang *et al*., 2011). Ghambarali et al. (2016) also reported a significant decrease in the yield of encapsulated buds of potato in Sante and Agria cultivars after 90 days of storage. The storage of artificial seeds in most plant species at 4ºC in many studies such as maintaining the tip of the *Helianthus annus* (Majd *et al*., 2011), parts of the encapsulated proteasomes in the *Orchid hybrid* Aranda (Gantit *et al*., 2012), the maintenance of somatic encapsulated embryos of *Brassica napus cv*. (Zeynali *et al*., 2013) and the maintenance of encapsulated nodal parts in *Vitex trifolia* (Ahmed *et al*., 2015) have been successful, which are consistent with the results of this study. In this study, the production of artificial seeds in stevia was studied by investigating different treatments. After increasing the germination efficiency of encapsulated lateral buds, compatibility and its methods along with the methods of encapsulated buds storage were investigated. Results showed that about 70% of seedlings had favorable growth in proper conditions.

Table 7. Effect of three different maintenance methods and four times of storage at 4ºC on regrowth rate and speed of encapsulated lateral buds after 3 weeks of culture.

Maintenance methods	Time of storage (day)	Regrowth	
		Rate	Speed
Without MS medium at 4ºC	0.0	71.2^{a}	0.3^{a}
	30.0	71.2^{a}	0.3^{a}
	60.0	53.7^{b}	0.2^{abc}
	90.0	50.0^{b}	0.1^{d}
Inside MS medium at 4ºC	0.0	71.2^{a}	0.3^{a}
	30.0	71.2^{a}	0.3^{a}
	60.0	71.2^{a}	0.3^{ab}
	90.0	53.7^{b}	0.1^{cd}
Inside liquid paraffin at 4ºC	0.0	71.2^{a}	0.3^{a}
	30.0	71.2^{a}	0.3^{a}
	60.0	71.2^{a}	0.2^{bc}
	90.0	67.5^{ab}	0.1^{cd}

Different letters indicate significant differences according to Duncan's multiple range test range with $p \leq 0.05$.

4. Conclusion

The obtained results indicated that sodium alginate 2.5% plus $CaCl_2$ 1% was the best composition for the coating of artificial seeds (ASs). Meanwhile, the lateral buds with a bigger size (2-3 mm) in MS medium with 1 mg l^{-1} BAP plus 1.5 mg l^{-1} Kn, as the pre-culture environment of lateral buds, represent better explants than apical buds for regrowth speed and germination rate in the created-ASs. Transmission of germinated ASs onto MS medium to cocopeat seedbed showed the highest seedling yield and increased growth characteristics. Besides, keeping the capsulated buds in the MS medium had a significant effect on keeping germinating traits. Among the treatments for keeping artificial seeds in 90 days, the treatment of liquid paraffin at 4ºC showed the best response for regrowth. Therefore, in the present study, in addition to producing the ASs created from lateral buds in stevia for the first time, the possibility of long-term adaptation and maintenance of ASs was also investigated. Since the encapsulated lateral bud is an efficient method in germplasm storage and it can lead to minimizing the cost of production in stevia, as an anti-diabetic medicinal plant.

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Author contributions

BS carried out all the experiments and she helped in preparing the manuscript, SSM designed the project, analyzed the data and wrote the manuscript, MRA helped in the analysis of the data , and HS contributed to the supply of laboratory equipment.

Conflict of Interest

The authors declare no conflict of interest.

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