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Plant regeneration and cold preservation of Eureka lemon (*Citrus limon* [L.] Burm. f. ['Eureka']) by using sodium alginate-encapsulated shoot tips

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ABSTRACT

In this study, in vitro-derived shoot tips of Eureka lemon (Citrus limon [L.] Burm. f. ['Eureka']) were encapsulated in various concentrations of sodium alginate (Na-alginate) (3%, 4%, and 5%) dissolved in liquid MS medium supplemented with 50 g l^{-1} sucrose + 10 mg l^{-1} BAP + 1 mg l^{-1} NAA (SA1); liquid MS medium supplemented with 50 g l^{-1} sucrose (SA2) and distilled water with 50 g l^{-1} sucrose (SA3). Encapsulated and non-encapsulated shoot tips were stored at 4°C for different storage periods (1-8 weeks). Alginate matrix of 4% Na-alginate and 100 mM $CaCl_2 \cdot 2H_2O$ was found to be suitable for the production of alginate beads. Shoots obtained by root induction were transferred to MS medium containing 5 mg l^{-1} IBA. Encapsulated shoot tips on SA1 treatment were viable (50%) after 3 weeks of cold storage while non-encapsulated shoot tips lost their viability completely after 3 weeks. Addition of MS nutrient and hormones to the alginate matrix significantly affected the regeneration of shoot tips. The survival rate of shoot tips encapsulated in SA1 after 4 weeks was 30% while that of encapsulated in SA2 was 20%. However, shoot tips encapsulated in SA3 lost their viability. Conversion frequencies of shoot tips encapsulated with SA1 in MS medium with hormones and without hormones were 60% and 30%, respectively, after 1 week. Thus, the current synthetic seed technology could be useful in large-scale propagation and short-term conservation of citrus.

Keywords: Alginate matrix, Cold storage, Encapsulation, Synthetic seed, Eureka lemon.
Abbreviations: *BA, N^6-benzyladenine; NAA, α-naphthaleneacetic acid; MS, Murashige and Skoog; IBA, indole-3-butyric acid*

INTRODUCTION

Synthetic seed production technology is one of the most important applications of plant tissue culture. The alginate encapsulation technique combines the advantages of clonal propagation and the characteristics of seed propagation and storage (i.e., storage, easy handling and transport, use of sowing equipment, and protection against pathogens) [2, 18, 30]. Synthetic seed technology via alginate encapsulation is an efficient method for both propagation and short-term storage of various important plants [10, 26]. In crop plants with long juvenile periods, e.g., citrus and grapes, the sowing efficiency can be significantly improved by using synthetic seeds instead of cuttings [21]. Alginate encapsulation of shoot tips together with cold preservation presents a possibility of converting plant materials between laboratories and an alternative for saving space while storing plants at temperatures below 0°C [6, 24, 28, 29]. Cold storage can reduce the cost of preserving germplasm cultures by reducing manual labor and can decrease genetic variation because of less frequent sub-culturing and adventitious regeneration. Shoot tips encapsulated in alginate are suitable for preserving vegetative propagated woody plants because meristems and shoot tips are appropriate explants for synthetic seeds to preserve water. Moreover, maintenance of genetic fidelity

of donor plant is assured [7]. In addition, shoot tips can regenerate directly without callus formation, which reduces the risk of somaclonal variation [33]. Furthermore, plant regeneration from shoot tips is easy and quick, thus shortening the duration for regeneration [33] and making the plant available at any time of the year [25]. In such cases, synthetic seed production from shoot tips can be used in place of germplasm storage and tissue cultured plants [28]. Ballester et al. [3] reported that among several non-embryogenic propagules, shoot tips are more responsive than other explants because of the higher mitotic activity in the meristems.

In vitro germplasm preservation is often achieved using conditions that minimize the rate of tissue growth. Encapsulation of tissues in alginate bead reduces their respiration, decreases their growth, and allows their maintenance and storage [4]. Viability and germination of encapsulated shoot tips can be improved by manipulating the composition of synthetic endosperm and the media, changing the media type, and optimizing growth regulators.
Because the citrus plant is one of the most economically important fruit tree in the world, its propagation has been studied worldwide to highlight the importance of commercial propagation of healthy citrus plants at low per unit cost. An efficient protocol was developed for short-term storage and conservation of commercial woody and citrus plants by using encapsulated shoot tips. In citrus, various materials such as ovules [14], somatic embryos [14], embryonic axes [5], embryonic cells [22], and shoot tips [13, 33, 34] have been successfully cryopreserved. In this study, we reported cold preservation of synthetic seeds of Eureka lemon by employing encapsulation of shoot tips and successful regeneration of these encapsulated shoot tips into plantlets to improve their preservation. We also reported the effect of different periods of cold storage, different concentrations of sodium alginate, and different compositions of alginate matrix on the viability of encapsulated shoot tips.

MATERIALS AND METHODS

Plant material

The plant material included shoot tips sampled into *in vitro* plantlets of Eureka lemon (*Citrus limon* [L.] Burm. f. ['Eureka']), which were obtained from seeds germinated *in vitro*. The seeds were obtained from citrus orchards in the east of Mazandaran province, Iran. The seeds were sterilized by submerging them in 70% (v/v) ethanol for 5 min, followed by immersion in 4% sodium hypochlorite (w/v) for 10 min. After rinsing with sterile distilled water for 5 times, the seeds were placed on MS [20] medium supplemented with 30 g l^{-1} sucrose and 500 mg l^{-1} malt extract. Shoot tips from *in vitro* grown seedlings were excised and cultured on MS basal medium supplemented with 10 mg l^{-1} BAP and 1 mg l^{-1} NAA for proliferation. The pH of the medium was adjusted to 5.8 ± 0.1 before adding 0.8% (w/v) agar-agar. The media were sterilized by autoclaving at 121°C for 15 min. All cultures were incubated in a culture room at 25°C ± 1°C under 16-h day length, with an illumination of 100 µmol m^{-2} s^{-1} provided by Osram cool white 18 W fluorescent lamps. Every month, all cultures were transferred to fresh MS medium supplemented with 500 mg l^{-1} malt extract and 30 g l^{-1} sucrose. Approximately 60-day-old plantlets were used in the experiments.

Encapsulation procedure

To study the role of different concentrations of sodium alginate (Na-alginate), different concentrations of NA-alginate (3%, 4% and 5%) were prepared using MS liquid medium supplemented with 50 g l^{-1} sucrose, 10 mg l^{-1} BAP, and 1 mg l^{-1} NAA. To study the effect of alginate matrix composition, alginate matrices were prepared as follows: SA1 was prepared with Na-alginate 4% (w/v) and liquid MS medium supplemented with 50 g l^{-1} sucrose, 10 mg l^{-1} BAP, and 1 mg l^{-1} NAA; calcium chloride ($CaCl_2 \cdot 2H_2O$; 100 mM) and 50 g l^{-1} sucrose were added to the liquid MS medium. SA2 was prepared with Na-alginate 4% (w/v) and liquid MS medium supplemented with 50 g l^{-1} sucrose; $CaCl_2 \cdot 2H_2O$ (100 mM) and 50 g l^{-1} sucrose added to the liquid MS medium. SA3 was prepared with Na-alginate 4% (w/v) and distilled water containing 50 g l^{-1} sucrose; $CaCl_2 \cdot 2H_2O$ (100 mM) and 50 g l^{-1} sucrose added to the liquid MS medium. The gel matrix and complexing agent were autoclaved separately at 121°C for 15 min after adjusting the pH to 5.8. *In vitro* shoot tips were excised from proliferated Eureka lemon shoots and used for encapsulation. The shoot tips were individually encapsulated in Na-alginate beads by transferring them drop-by-drop with a pipette from Na-alginate solution into calcium chloride solution. Droplets containing shoot tips were maintained in calcium chloride solution for 20 min to achieve polymerization of Na-alginate. After the beads were hardened, they were rinsed 3 times with sterilized distilled water for 10 min to remove excess calcium chloride.

Culture media for plant regeneration

After cold storage for 1-8 weeks, Na-alginate beads of each alginate matrix composition were cultured on MS basal medium supplemented with 50 g l^{-1} sucrose, 10 mg l^{-1} BAP, and 1 mg l^{-1} NAA. To determine the effect of culture media type on synthetic seed conversion, some Na-alginate beads encapsulated with SA1 were cultured on 2 different media, MS basal medium supplemented with 50 g l^{-1} sucrose, 10 mg l^{-1} BAP, and 1 mg l^{-1} NAA and hormone-free MS basal medium, after cold storage for 1-8 weeks. All cultures were maintained as previously described. After the regenerated shoots reached a height of 1-2 cm, they were transferred to MS basal medium

supplemented with 5 mg l^{-1} IBA for root development. Complete plantlets were obtained after 2 months of incubation.

Cold storage

The encapsulated shoot tips of each alginate matrix composition and non-encapsulated shoot tips were transferred in empty Petri dishes covered with aluminum foil and stored in a refrigerator at 4°C for 1-8 weeks. After each storage period, the encapsulated and non-encapsulated shoot tips were cultured on MS medium with or without growth regulators to evaluate conversion.

Statistical analysis

The percentage of stored and non-stored encapsulated shoot tips that developed plantlets was recorded weekly for 3 consecutive weeks. The conversion of encapsulated shoot tips was determined by evaluating developed shoots with apparent 2 leaves after 4 weeks and roots after 3 weeks. In every experiment, 20 synthetic seeds in 4 culture plates were treated. Each culture plate containing 5 synthetic seeds was considered as 1 replicate. Descriptive statistics such as mean, standard deviation, and bar chart and inferential statistics, including analysis of variance in factorial design (2 factors) by 10 repetitions were performed. Significant differences between means were assessed using Duncan's multiple range tests, with $p<0.05$ being statistically significant (statistical package for social science [SPSS] ver. 16).

RESULTS

Alginate matrix containing 4% Na-alginate and 100 mM calcium chloride was found to be suitable for the formation of firm and isometric alginate beads and for the conversion of shoot tips into complete plantlets. However, lower concentration (3%) of Na-alginate was unsuitable because the alginate beads were diaphanous and asymmetrical and could not be easily manipulated. Higher concentration of Na-alginate (5%) was found to be unsuitable because the alginate beads were hard, making it impossible for the shoot tips to emerge. Alginate beads encapsulated in 4% Na-alginate and 100 mM calcium chloride were used for encapsulating shoot tips in all further experiments. Regardless of the composition of the alginate matrix, shoots grew from alginate beads after 4 weeks of culturing. Roots emerged after approximately 3 weeks of culturing. Complete plantlets were obtained 2 months after culturing the synthetic seeds.

As shown in Table 1, the conversion percentage of encapsulated and non-encapsulated shoot tips that were immediately cultured without storage was 70% and 60%, respectively, which was statistically significant ($p<0.05$). However, with an increase in storage time at 4°C, the regeneration frequency gradually decreased such that after 2 weeks, the conversion percentage of non-encapsulated and encapsulated shoot tips was 10% and 50%, respectively (Table 1).

Table 1. Effects of different storage durations on the conversion of encapsulated and non-encapsulated shoot tips of Eureka lemon into plantlets after 8 weeks of culture on MS medium supplemented with 10 mg l^{-1} BAP and 1 mg l^{-1} NAA

	Frequency of plantlet conversion (%)	
Storage duration (weeks)	Encapsulated shoot tip with SA1	Non-encapsulated shoot tips
0	70 ± 8.944a	60 ± 6.325b
1	60 ± 6.325b	40 ± 6.325d
2	50 ± 6.325c	10 ± 8.944g
3	50 ± 8.944c	00 ± 0.00h
4	30 ± 8.944e	00 ± 0.00h
5	20 ± 6.325f	00 ± 0.00h
6	10 ± 10.954g	00 ± 0.00h
7	10 ± 8.944g	00 ± 0.00h
8	00 ± 0.00h	00 ± 0.00h

Values are expressed as mean ± standard error (SE). SA1: 4% (w/v) sodium alginate + liquid MS medium + 50 g l^{-1} sucrose + 10 mg l^{-1} BAP + 1 mg l^{-1} NAA + 100 mM calcium chloride

During storage, encapsulated shoot tips showed a higher survival rate than non-encapsulated shoot tips. After 3 weeks of storage at 4°C, encapsulated shoot tips showed approximately 50% viability while non-encapsulated shoot tips completely lost their viability. Encapsulated shoot tips lost their viability after 8 weeks of storage at 4°C (Table 1).

The conversion percentage of shoot tips encapsulated with SA2 (50 g l^{-1} sucrose and liquid MS) was not significantly different from that of shoot tips encapsulated with SA1 (control) after the first and second week of storage. However, after 3 weeks of storage, their conversion percentage significantly decreased because of which they lost their viability in the sixth week (Table 2). Conversion percentage of shoot tips encapsulated with SA3 (50 g

l^{-1} sucrose and distilled water) was 60% even after immediate encapsulation, which was significant compared to that of shoot tips encapsulated with SA1 (control) (Table 2).

Table 2. Effects of alginate matrix composition on the conversion percentage of encapsulated shoot tips of Eureka lemon after storage

	Frequency of plantlets conversion (%)		
Storage Duration (weeks)	Alginate matrix SA1	Alginate matrix SA2	Alginate matrix SA3
0	70 ± 8.944a	70 ± 6.325a	60 ± 10.954b
1	60 ± 6.325b	60 ± 6.325b	30 ± 8.944e
2	50 ± 6.325c	40 ± 10.954d	20 ± 6.325f
3	50 ± 8.944c	30 ± 8.944e	10 ± 6.325g
4	30 ± 8.944e	20 ± 6.325f	00 ± 0.00h
5	20 ± 6.325f	10 ± 6.325g	00 ± 0.00h
6	10 ± 10.954g	00 ± 0.00h	00 ± 0.00h
7	10 ± 8.944g	00 ± 0.00h	00 ± 0.00h
8	00 ± 0.00h	00 ± 0.00h	00 ± 0.00h

Values are expressed as mean ±SE. Evaluation was made after 3 weeks of culture

Table 3. Effects of different media on the conversion of encapsulated shoot tips (with SA1) of Eureka lemon into plantlets

	Percentage of plantlet conversion (%)	
Storage duration (weeks)	Encapsulated with SA1 on MS with hormones	Encapsulated with SA1 on MS hormone free
0	70 ± 8.944a	60 ± 8.944b
1	60 ± 6.325b	30 ± 6.325d
2	50 ± 6.325c	20 ± 10.954e
3	50 ± 8.944c	10 ± 8.944f
4	30 ± 8.944d	10 ± 6.325f
5	20 ± 6.325e	00 ± 0.00g
6	10 ± 10.954f	00 ± 0.00g
7	10 ± 8.944f	00 ± 0.00g
8	00 ± 0.00g	00 ± 0.00g

Values are expressed as mean ±SE. MS with hormones: solid MS medium + 50 g l^{-1} sucrose + 10 mg l^{-1} BAP + 1 mg l^{-1} NAA

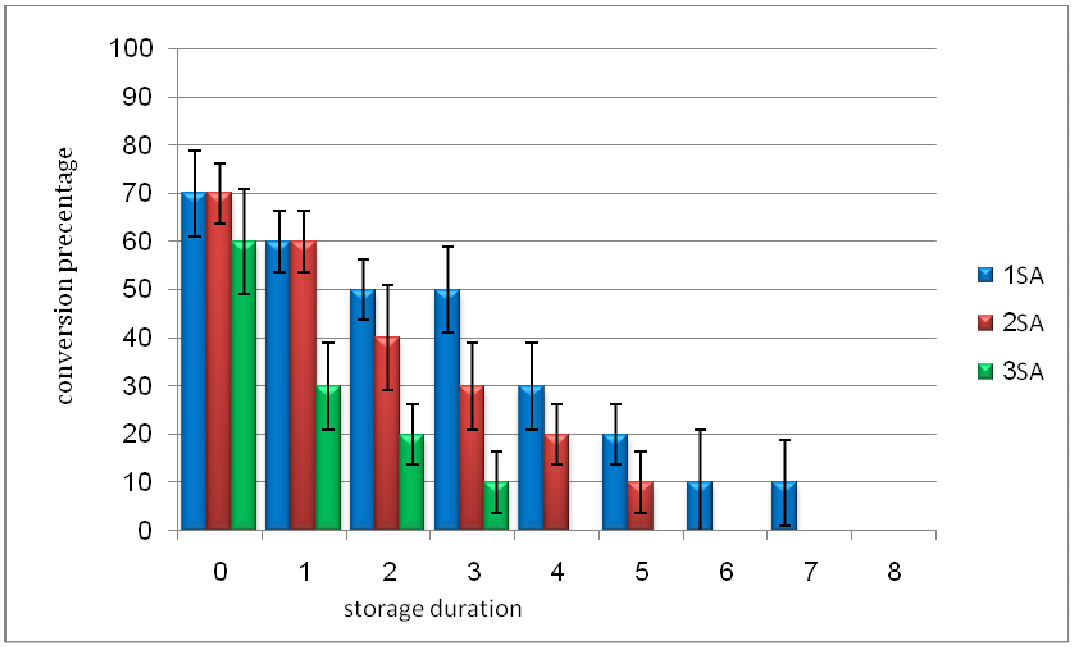

Fig. 1. Effect of alginate matrix composition and duration of storage on plant recovery in encapsulated shoot tips of Eureka lemon. The bars represent mean ± SE. Bars followed by the same latter indicate no significant difference (p = 0.05), according to Duncan's multiple range test.

During cold dark storage, conversion percentages of shoot tips encapsulated with SA3 in the first, second, and third week were 30%, 20%, and 10%, respectively; however, in the fourth week, these shoot tips lost their viability (Fig. 1).

Our results showed that synthetic seed culture on hormone-free MS medium immediately after encapsulation significantly decreased ($p<0.05$) plantlet conversion percentage; in addition, the generated plantlets had feeble rooting. As shown is Table 3, the conversion frequency of beads encapsulated in liquid MS medium supplemented

with 10 mg l^{-1} BAP and 1 mg l^{-1} NAA (SA1) and cultured on hormone-free MS medium after first, second, third, and fourth week of cold storage decreased to 30%, 20%, 10%, and 10%, respectively; in the fifth week, these shoot tips lost their viability completely (Fig. 2). The conversion frequency of beads encapsulated with SA1 and cultured on solid MS medium containing BA and NAA after 7 weeks was 10%; in the eighth week, these shoot tips lost their viability completely (Table 3).

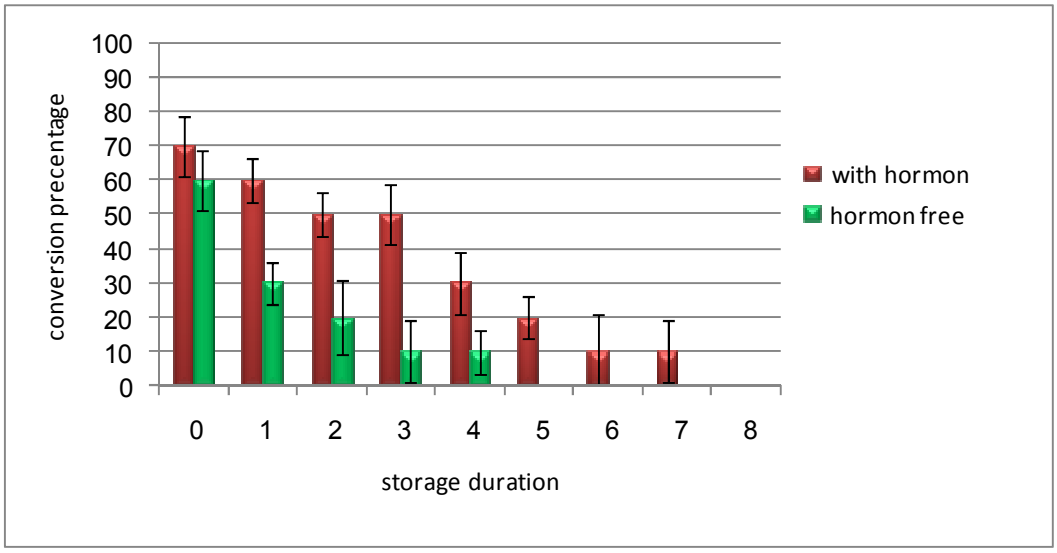

Fig. 2. Effect of media type (MS medium with or without hormones) on the conversion of encapsulated shoot tips of Eureka lemon after cold storage. The bars represent mean ± SE. Bars denoted by the same latter indicate no significant difference (p = 0.05), according to Duncan's multiple range test. Data were recorded after 3 weeks of culture.

DISCUSSION

The key factor in successful production of synthetic seeds and capsule quality is to use the correct composition of Na-alginate and calcium chloride. The shape and diameter of alginate beads depend on Na-alginate and calcium chloride composition. Our results correspond to the findings by Rady and Hanafy [23], Kavyashree et al. [17], and Sundararaj et al. [31] who reported that 4% Na-alginate was the optimum concentration for alginate bead formation in *Gypsophila paniculata*, *Morus alba* and *Zingiber officinale*, respectively. However, most reports on 3% Na-alginate and 100 mM calcium chloride have shown that this is the optimum combination for alginate bead formation [1, 15, 16, 27]. Similar to the findings by Germana et al. [12] in citrange, our results showed that the addition of 10 mg l^{-1} BAP and 1 mg l^{-1} NAA (for shoot production) and 5 mg l^{-1} IBA (for root production) to the MS medium significantly improved the regeneration percentage of Eureka lemon plantlets.

The conversion percentage of non-encapsulated shoot tips gradually decreased after prolonged storage at 4°C such that after 3 weeks, they lost their viability completely. In contrast, encapsulated shoot tips showed higher resistance to cold. Therefore, an artificial coat is essential to protect shoot tips during cold storage. Similar to the findings by Germana et al. [12] in *Carrizo citrange* and Katouzi et al. [15] in *Helianthus annuus* L., our results showed that encapsulated shoot tips had a higher regeneration capacity than non-encapsulated shoot tips. Ahmad and Anis [1] reported that encapsulated nodal segments of *Vitex negundo* showed 50% viability after 8 weeks of storage. Our data indicated that only 30% of Eureka lemon synthetic seeds converted into plantlets after 4 weeks. In contrast to the findings by West et al. [36], which showed that encapsulated nodal segment of *Hibiscus moscheut*os had 80% viability even after 7-8 weeks of cold storage at 5°C, our data indicated that encapsulated shoot tips of Eureka lemon lost their viability after 8 weeks of storage at 4°C. These differences may be related to plantlet type or genotype. Although the survival rate of encapsulated shoot tips of Eureka lemon in cold storage was better than that of non-encapsulated shoot tips, their conversion percentage to plantlets decreased gradually after prolonged storage, indicating that conservation frequency was affected by storage time. Our results correspond to the findings of Katouzi et al. [15] in *Helianthus annuus* L, which showed that regeneration capacity of encapsulated shoot tips decreased after prolonged storage. The decline in the conversion of encapsulated shoot tips after a prolonged storage time may be attributed to the inhibitory respiration tissues resulting from the alginate coat or loss of moisture due to partial desiccation during storage, as reported earlier [6, 8].

Our data indicated a significant difference between encapsulated shoot tips cultured on liquid MS supplemented with BA and NAA (SA1) and those cultured on hormone-free liquid MS (SA2). These findings are comparable to

those by Danso and Ford-Lloyd [6], which showed that addition of BAP and NAA during *Cassava* nodal cuttings and shoot tips encapsulation process significantly enhanced the plant re-growth. Similar to the findings by Faisal et al. [8], Faisal and Anis [9], and Saiprasad and Polisetty [26], our results showed that addition of BA and NAA to artificial endosperm and provision of conditions similar to natural seeds increased plantlet regeneration. However, a study by Zych et al. [37] on *Rhodiola Kirilowii* showed that supplementation of Na-alginate with MS medium containing growth regulators had no effect on the viability of encapsulated axillary buds, callus, or shoots. Shoot tips encapsulated with SA3 showed a significant decline compared with those encapsulated with SA1 (control) and SA2. This difference was particularly obvious after storage. Similar to the results by Faisal et al. [8] and Germana et al. [11], our results showed that a completely artificial endosperm, including nutrient, sucrose, and growth regulators, is necessary for supporting the survival and further growth of synthetic seeds of Eureka lemon stored at cold temperatures. Our results corresponded to those by Tsvetkov et al. [32] in hybrid aspen (*Populus tremula* L. × *P. tremuloides* Mincx.) and indicated that alginate matrix composition was an important factor that significantly affected the conversion of encapsulated hybrid aspen apical segment. Moreover, addition of assistant components to the alginate solution improved the re-growth parameters.

Because 50 g l^{-1} sucrose was used in all alginate matrices, the synthetic seeds of Eureka lemon required plant growth regulators other than sucrose for their conversion to plants. However, a study by Makowczynska and Andrzejewska-Golec [19] on *Plantago asiatica* showed that presence of sucrose in capsules had more distinct effect than IBA on the germination and conversion of synthetic seeds into plants.

Our data indicated that presence of hormones (BAP and NAA) both inside the artificial coat and in the medium possibly had a positive effect on increasing the conversion of Eureka lemon synthetic seeds into plants. In comparison to the findings by Saiprasad and Polisetty [26] in *Dendrobium*, our findings showed that encapsulation matrix prepared with MS medium supplemented with 0.44 µM BA and 0.54 µM NAA and culturing on MS media with different concentrations of BA and NAA showed 100% conversion rate to plants after storage periods.

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