Plant regeneration of *Citrus sinensis.*var. Thamson navel using sodium alginate-encapsulated shoot tips

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Abstract

Citrus genus is known as one of the world's major economic agricultural products. In the current research, a method is reported for artificial seed production technology by shoot tips in *Citrus sinensis.*var. Thamson navel. Alginate is one of the most efficient gelling agents in the production of capsule coating in artificial seeding technique. In the current research, impact of different constituents of alginate matrix was investigated on artificial seed germination. In order to produce artificial seeds, shoot tips of Thamson navel were coated by three alginate coating including SA_1 containing liquid MS and BA and NAA hormones, SA_2 with liquid MS without hormone, and SA_3 with distilled water. Alginate coating with BA and NAA hormones was used as control environment. The artificial seeds were then stored in the refrigerator (4 °C). Following passing storage period, seeds were placed in MS solid medium with BAP (10 mgl^{-1}) and NAA (1 mgl^{-1}) for conversion to seedlings. Using distilled water instead of MS medium for the preparation of alginate coating significantly reduced the percentage of conversion into seedlings.

***Keywords*:** alginate matrix; cold storage; encapsulation; synthetic seed; Thamson navel

Abbreviations
BA: N^6-benzyladenine; IBA: indole-3-butyric acid; NAA: α-naphthalene acetic acid; MS: Murashige and Skoog

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Introduction

Citrus is one of the most important horticultural products in the world. Variety in products and the high performance make this crop play special role in the agriculture and international economy. Using tissue culture techniques and artificial seeds, seedlings can be produced to replace costly conventional methods of vegetative propagation of plants.

Artificial seed is a plant propagule coated on a platform (matrix), which has the ability to grow into a plant. Plant propagule may include somatic embryos, shoot tips, lateral buds, plantlet, protoplasm, etc. which grows in sterile conditions in cultures. Propagules are easily grown in culture and can be converted into seedlings. Capsuling is the best method for providing protection and conversion of cuttings *in vitro* into the artificial seeds (Ara et al., 2000).

Since the plant propagules like shoot tips do not have important attached tissues including endosperm and the protective cover, when the

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shoot tips are exposed to the natural environment, they are susceptible to drought and pathogens and generally they lack sleep stage and are not able to pass through stress. Thus, coating (encapsulation) is considered as a first step to produce artificial seeds. It is expected that masking is the best way to protect the shoot tips and convert them to provide artificial seeds. In addition, coated artificial seeds should have growth materials, microorganisms that proceed the growth (like mycorrhiza) and other compounds which are essential for embryo development and its conversion into the seedlings (Ara et al., 2000; Sharma, 2010).

Shoot tips encapsulated in alginate are suitable for preserving vegetatively propagated woody plants because meristem and shoot tips are appropriate explants for synthetic seeds to preserve water. Moreover, maintenance of genetic fidelity of donor plant is assured (De carlo and Lambardi, 2005). In addition, shoot tips can regenerate directly without callus formation, which reduces the risk of somaclonal variation (Wang and Deng, 2001). Furthermore, plant regeneration from shoot tips is easy and quick, thus shortening the duration for regeneration (Wang and Deng, 2001) and making the plant available at any time of the year (Reed, 2002). In such cases, synthetic seed production from shoot tips can be used in place of germplasm storage and tissue cultured plants (Singh et al., 2006). Ballester et al. (1997) reported that among several non-embryogenic propagules, shoot tips are more responsive than other explants because of the higher mitotic activity in the meristems.

Sodium alginate solution in the presence of calcium salts such as calcium chloride comes in solid form. The ion substitution reaction occurs, and sodium ions are replaced by calcium ions and alginate is solid. Difficulty level of produced encapsulates depends on the alginate concentration, concentration of sodium chloride, and sodium alginate contact with calcium chloride solution (Sharma, 2010).

Since the alginate matrix is similar to the endosperm in the natural seed, compounds of this artificial endosperm considerably affects germination and growth of encapsulated shoot tips. The aim of this study was to investigate the endosperm synthetic compounds suitable for encapsulation of Novell Thomson shoot tips and the effect of these compounds on the growth of artificial seed.

Materials and Methods

Plant material

The plant material included shoot tips sampled into *in vitro* plantlets of Citrus sinensis.var. Thamson navel, which were obtained from seeds germinated *in vitro*. Seeds were obtained from citrus orchards in the east of Mazandaran province, Iran. Seeds were sterilized by submerging 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 (Murashige and Skoog, 1962) medium supplemented with 30 gL−1 sucrose and 500 mgl^{-1} malt extract. Shoot tips from *in vitro* grown seedlings were excised and cultured on MS basal medium supplemented with 10 mgl^{-1} BAP and 1 mgl^{-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. All cultures were transferred to fresh MS medium supplemented with 500 mgl^{-1} malt extract and 30 gl^{-1} sucrose every month. Approximately 60-day-old plantlets were used in the following experiments.

Encapsulation matrix

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 gl^{-1} sucrose, 10 mgl^{-1} BAP, and 1 mgl^{-1} NAA; calcium chloride ($CaCl_2 \bullet 2H_2O$; 100 mM) and 50 gl^{-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 gl^{-1} sucrose; $CaCl_2 \bullet 2H_2O$ (100 mM) and 50 gl^{-1} sucrose were added to the liquid MS medium. SA3 was prepared with Na-alginate 4% (w/v) and distilled water

containing 50 gl^{-1} sucrose; $CaCl_2 \bullet 2H_2O$ (100 mM) and 50 gl^{-1} sucrose were 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.

Encapsulation

In vitro shoot tips were excised from proliferated Thamson navel 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 gl^{-1} sucrose, 10 mgl^{-1} BAP, and 1 mgl^{-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 gl^{-1} sucrose, 10 mgl^{-1} BAP, and 1 mgl^{-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 mgl^{-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 growth regulators to evaluate conversion.

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 $P \leq 0.05$ being statistically significant (SPSS ver. 16).

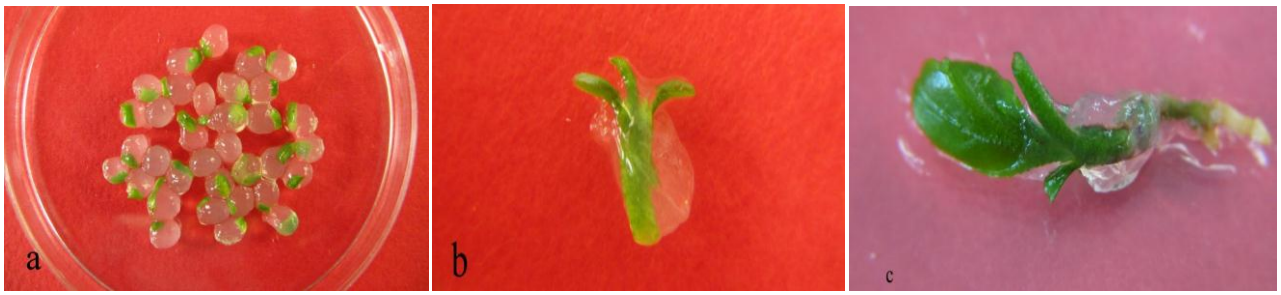

Fig. I. Different stages of plantlet regeneration from encapsulated shoot tips of Thamson navel: (a) Shoot tips encapsulated in 4% sodium alginate and 100 mM calcium chloride; Synthetic seeds are firm and isometric. (b) Shoots emerging from encapsulated shoot tips; (c) Root induction in MS medium supplemented with 5 mgl^{-1} IBA

Results

To obtain synthetic seeds, shoot tips of Thamson navel were excised from *in vitro*

proliferated shoots and encapsulated with alginate matrix (Fig. I, a). Encapsulated and non-encapsulated shoot tips were stored at 4 °C in darkness for 1–8 weeks. The stored encapsulated and non-encapsulated shoot tips were cultured after storage on MS basal medium supplemented with 10 mgl^{-1} BAP and 1 mgl^{-1} NAA to induce shooting. Regardless of the composition of the alginate matrix, shoots grew from alginate beads after 4 weeks of culturing (Fig. I, b). When the shoots grew to a height of 1–2 cm, they were placed on MS medium supplemented with 5 mgl^{-1} IBA to induce rooting. Roots emerged after approximately 3 weeks of culturing (Fig. I, c). 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 ($P \leq 0.05$).

However, with an increase in storage time at 4 °C, the regeneration frequency gradually decreased so that after 2 weeks, the conversion percentages of non-encapsulated and encapsulated shoot tips were 10% and 50%, respectively (Table 1).

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).

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

	Frequency of plantlet conversion (%)	
Storage duration (weeks)	Encapsulated shoot tip with (SA1)	Non-encapsulated shoot tips
0	70±8/944 a	60±6/325 b
1	60±6/325 b	40±6/325 d
2	50±6/325 c	10±8/944 g
3	50±8/944 c	00±000 h
4	30 ±8/944 e	00±000 h
5	20±6/325 f	00±000 h
6	10±10/954 g	00±000 h
7	10±8/944 g	00±000 h
8	00±000 h	00±000 h

Values are expressed as mean ± standard error (SE). Four replicates, each containing 5 synthetic seeds were used for each treatment. Evaluation was made after 3 weeks of culture. SA1: sodium alginate 4% (w/v) + liquid MS medium + 50 gl^{-1} sucrose + 10 mgl^{-1} BAP + 1 mgl^{-1} NAA + 100 mM calcium chloride

Table 2
Effects of alginate matrix composition on the conversion percentage of encapsulated shoot tips of Thamson navel after storage

	Percentage of Plantlet Conversion Frequency		
Storage Duration (weeks)	Alginate Matrix SA1 (control)	Alginate Matrix SA2	Alginate Matrix SA3
0	70±8/944 a	70±6/325 a	60±10/954 b
1	60±6/325 b	60±6/325 b	30±8/944 e
2	50±6/325 c	40±10/954 d	20±6/325 f
3	50±8/944 c	30±8/944 e	10±6/325 g
4	30 ±8/944 e	20±6/325 f	00±000 h
5	20±6/325 f	10±6/325 g	00±000 h
6	10±10/954 g	00±000 h	00±000 h
7	10±8/944 g	00±000 h	00±000 h
8	00±000 h	00±000 h	00±000 h

Values are expressed as mean ± SE. Four replicates, each containing 5 synthetic seeds were used for each treatment. Evaluation was made after 3 weeks of culture.

The conversion percentage of shoot tips encapsulated with SA2 (50 gl^{-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 cold storage, their conversion percentage significantly decreased because of which they lost their viability in the sixth week (Table 2).

Alginate coating of the third solution, which was mixed only with distilled water, was used along with sucrose as a coating for shoot tips. The results showed that when distilled water was used instead of liquid MS culture medium for preparation of gel foundation, conversion into seedling was reduced (Fig. I). In fact, conversion percentage in shoot tips coated with SA3 was 60% even after immediate encapsulation, which was significantly differfent compared to that of shoot tips encapsulated with SA1 (control) (Table 2).

During storage period, conversion percentage in shoot tips coated with SA3 reduced to 30 percent in the first week, 20 percent in the second and 10 percent in the third week and they lost their viability totally in the fourth week, and such reductions are significant compared to control group (SA1) (Fig. II).

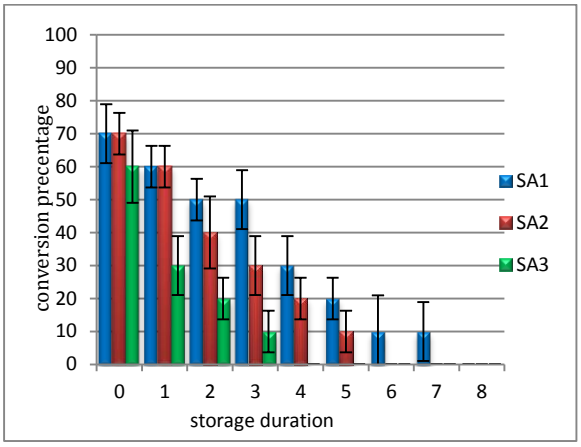

Fig. II. Effect of alginate matrix composition and duration of storage on plant recovery in encapsulated shoot tips of Thamson navel; the bars represent mean ± SE. Bars followed by the same latter indicate no significant difference ($p \leq 0.05$) according to Duncan's multiple range test.

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 (Singh, 2006). The shape and diameter of alginate beads depend on Na-alginate and calcium chloride composition. In this study, Na-alginate 4% and calcium chloride 100 mM were used to produce hardy and identical beads.

Our results correspond to the findings by Rady and Hanafy (2004), Kavyashree et al. (2006), and Sundararaj et al. (2010) who reported that 4% Na-alginate as 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 (Ahmad and Anis, 2010; Sharma and Shahzad, 2012; Katouzi et al., 2011).

Similar to the findings by Germana et al. (2011) in citrange, our results showed that addition of 10 mgl^{-1}BAP and 1 mgl^{-1} NAA (for shoot production) and 5 mgl^{-1} IBA (for root production) to the MS medium significantly improved the regeneration percentage of Thamson navel plantlets.

In this study, the conversion percentage of encapsulated shoot tips was significantly higher than that of non-encapsulated shoot tips, indicating that alginate beads not only prevented the growth and regeneration of plantlets but also positively affected their regeneration.

The conversion percentage of non-encapsulated shoot tips gradually decreased after prolonged storage at 4 °C so 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. (2011) in Carrizo citrange and Katouzi et al. (2011) in Helianthus annuus L., our results showed that encapsulated shoot tips had a higher regeneration capacity than non-encapsulated shoot tips.

Anis and Ahmad (2010) reported that encapsulated nodal segments of Vitex negundo showed 50% viability after 8 week of storage. Our data indicate that only 30% of Thamson navel synthetic seeds converted into plantlets after 4 weeks.

In contrast to the findings by West et al. (2006), which showed that encapsulated nodal segment of Hibiscus moscheutos had 80% viability even after 7–8 weeks of cold storage at 5 °C, our data indicated that encapsulated shoot tips of Thamson navel 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 Thamson navel in cold storage was better than that of non-encapsulated shoot tips, their conversion percentage into 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. (2011) on 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 (Danso and Ford- Lloyd, 2003; Faisal et al., 2006).

Our data indicate 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 (2003), who showed that addition of BAP and NAA (plant growth regulators) during Cassava nodal cuttings and shoot tips encapsulation process significantly enhanced the plant regrowth.

Similar to the findings by Faisal et al. (2006), Faisal and Anis (2007), and Saiprasad and Polisetty (2003), 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. (2005) 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. (2006) and Germana et al. (1998), 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 citrus stored at cold temperatures.

Our results correspond to those by Tsvetkov et al. (2006) in hybrid aspen (Populus tremula L. × P. tremuloides Mincx.) and indicate that alginate matrix composition is an important factor that significantly affects the conversion of encapsulated hybrid aspen apical segment. Moreover, addition of assistant components to the alginate solution improves the regrowth parameters.

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