Encapsulated somatic embryos and shoot tips of rapeseed (*Brassica napus* L.): An efficient way for stor­age, transport and multiplication of plant material

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**Abstract**

The present paper reports the encapsulation of Somatic embryos and in vitro-derived shoot tips of rapeseed (*Brassica napus* L.) for evaluate somatic embryos response to different storage periods and germplasm exchange purposes. In this experiment, the effects of several concentrations will be studied for: a) sodium alginate as gelling agent; b) calcium chloride as complexing agent; c) preparation of sodium alginate and d) IAA concentration for enhance root formation in re-growth shoot tips after recovery. At other experiment, encapsulated somatic embryos (ESEs) were encapsulated in 2% sodium alginate, stored for several periods (10, 20, 30 and 40 days) at 4°C. Results showed that, a gelling matrix of 4% sodium alginate and 1.4% calcium chloride were found most suitable for formation of ideal beads in shoot tips. Encapsulated shoot tips exhibited the best shoot development on Murashige and Skoog (MS) medium supplemented with 2 mg l-1BAP and 2mg l-1IAA (70.2 %) followed by hormone-free MS basal medium (63.19%) and distilled water (62.16%).Furthermore, it illustrated that2 mg l-1 IAA produced a highest root in plantlets derived from encapsulated shoot tips. The second experiment results indicated that Maximum conversion frequency of 55.5% was obtained from encapsulated embryos cultured on MS medium without plant growth regulators stored for 10 days. The longer duration of cold storage, caused the less in plantlet recovery.  This work indicates that encapsulated somatic embryos of canola could be regenerated for a limited period time.

**Key word**: cold storage; Encapsulated somatic embryos (ESEs); shoot tips encapsulation; sodium alginate

**Introduction**

Numerous studies were undertaken in this practice-oriented field of plant biotechnology (Tsvetkov and Hausman 2005)since the concept of ‘‘artificial seeds’’ was formulated by Murashige (1997).Encapsulation technology has been applied to crop species to produce artificial seeds from somatic embryos and buds (Bajaj 1995). Synthetic seed technologies are useful tools for the field delivery of in vitro derived plantlets. On the other hands, the artificial cover protects the embryoid or meristematic tissues against mechanical damage, drying or dehydrating during storage and transportation. Capsules with sterile material can be exchanged easily between laboratories (Zych et al. 2005). Encapsulation technology has been widely used for biopharmaceutical purposes (Blandino et al. 2000). In addition, the synthetic seed technology could be useful in germplasm conservation of elite (superior species), endangered and commercially important plants by using appropriate storage technique as well as exchange of axenic plant material between laboratories (Hasan and Takagi 1995, Maruyama et al. 1997, Nadeem et al. 2000, Ara et al. 2000, Danso and Ford-Lloyd 2003, Naik and Chand 2006).Encapsulation of somatic embryos or vegetative propagules and regeneration of complete plantlets from them has been reported for cereals, vegetables, fruits, ornamentals, conifers and woody crop plants (Ara et al. 2000, Ipekci and Gozukirmizi 2003, Tsvetkov and Hausman 2005). However, the encapsulation of somatic embryos and shoot tips could be an attractive alternative for producing plantlets in some species recalcitrant to somatic embryogenesis (Tsvetkov and Hausman 2005).

In first experiment, parameters for the encapsulation of axenic rapeseed shoot tips derived from plantlets resulting from seed cultures in sodium alginate gel and subsequent conversion of encapsulated shoot tips into plantlets was optimized. Then, encapsulation of somatic embryos of *Brassica napus* in sodium alginate beads was encapsulated and conversion of them into plantlets was investigated. Effect of different storage durations on the conversion of encapsulated somatic embryos was discussed.

**Materials and Methods**

Plant material and culture conditions

*Brassica napus* cv. Tallayeh was grown under aseptic conditions in the Biotechnology and Molecular Genetics Lab., Zanjan University. Sterilized seeds were cultured on Murashige and Skoog (1962) (MS) basal medium with 10 g l-1 agar and 20 g l-1 sucrose without any plant growth regulator (PGR). The Shoot tips developed from the rapeseed cultured in vitro condition were used for encapsulation. The plants were placed under standard conditions at 25±1°C temperature, 16/8 h photoperiod under white fluorescent tubes for recovery of plantlets. Shoot tips (meristems with one or two leaf primordial) 2–4 mm in length, were excised from 2weekold in vitro grown plants using two hypodermic needles under a stereo microscope in sterile conditions. For somatic embryo encapsulation, Embryogenic callus formation from hypocotyls and somatic embryos obtain described by author previously (Zeynali et al. 2010).

*Encapsulation of somatic embryos and shoot tips*

For encapsulation of somatic embryos, sodium alginate (Sigma ChemicalsTM, USA) at the concentration of 2% (w v-1) was prepared in MS medium containing 2 mg l-1  BAP and 2 mg l-1  IAA ( Indole acetic acid), and 0.75 (g 50 ml-1 water) of calcium chloride (MerckTM, Germany), that it was prepared in double distilled water. For shoot tip encapsulation, three concentrations of (2, 4 and 6%) were used for sodium alginate solution. One of these concentrations was prepared by three various mediums/media of distilled water, basal liquid free MS medium with 2.5% sucrose and basal liquid MS medium supplemented with 2.5% sucrose, 2 mg l-1 IAA and 2 mg l -1 BAP. For calcium chloride solution, three concentrations were prepared: 1, 1.4 and 2%. . Both the gel matrix and complexing agent were autoclaved at 121°C for 15 min. Encapsulation was accomplished by mixing the somatic embryos at globular stage and individual shoot tips into the sodium alginate solution and dropping these into the calcium chloride solution. Each drop contained one and SE. The beads containing one somatic embryos and shoot tip were held for 20 to 30 min in the calcium chloride solution and after hardening of the beads, encapsulated somatic embryos were washed with sterilized distilled water 2 and 3 times to remove traces of calcium chloride. Encapsulated somatic embryos were stored in refrigerator at 4 °C for different periods (10, 20, 30 and 40 days). At the end of each storage period, stored encapsulated somatic embryos were immediately transferred to fresh germination medium (growth regulator free solid MS medium supplemented with 2.5% sucrose and 0.7% agar) and placed under standard conditions at 25±1°C temperature, 16/8 h photoperiod under white fluorescent tubes for recovery of plantlets. The frequency of plantlet conversion was calculated as the percentage of encapsulated somatic embryos showing well\_developed shoot and root out of total number of somatic embryos encapsulated. For shoot tips encapsulation, the survival percentage and the numbers of shoot per encapsulated beads were recorded after this time. In addition the shoots with fully expanded leaves emerging from encapsulated shoot tips to induce root formation, were placed on MS medium supplemented with a range of (1-3 mg l-1) IAA. The percentage of root formation was recorded after one week.

*Experimental design and data analysis*

The percentage of encapsulated shoot tips that developed shoots or roots was recorded weekly for one month. Each experiment was replicated five times with five explants per replicate. Encapsulation experiment was set up on factorial experiment in completely Randomized Design (CRD) and rooting experiment was carried out using CRD from five replications. For somatic embryos encapsulation experiment, 12 replicates were used for this treatment and each experiment was repeated three times. The mean standard error and one-way ANOVA were calculated using the MSTATC software. The mean separations were carried out using Duncan’s multiple range tests (Duncan, 1955) and significance was determined at (p<0.05).

**Results and discussion**

*Development of plants by encapsulated shoot tips*

Encapsulated shoot tips resumed growth within 7 days after planting and developing of normal shoots without callus formation within 4 weeks. The regrowth events could be observed in whole illustrate (Figure 1).Shoots and plantlets developed from synthetic seeds are used for further micro propagation of the plants.

*Effects of different concentrations of sodium alginate and calcium chloride*

The results indicated that there was significant difference (Table 1) among several concentrations of sodium alginate, in a manner the increasing of sodium alginate from 2 to 6 %,decreased the percentage conversion of encapsulated shoot tips. The formation of fir, uniform sodium alginate capsules was achieved with 4% sodium alginate complexed with 1.4% CaCl2( Figure 2).In this experiment, 4 % sodium alginate has the highest recovery of encapsulated shoot tips, but, 2% and 6% has not a significant difference. On the other hand, although in high levels of sodium alginate (6%) beads were isodiametric but were enough hard to cause considerable delay in sprouting.

Different concentrations of sodium alginate ranging from 1.5 % to 6 % have been used for different plants (Vij et al. 2001). Hassan (2003) reported that 6% sodium alginate was most suitable for encapsulation of shoot tips in *Simmondsiachinensis* L. Zych et al.(2005), encapsulated the micro shoots of *Rhodiola Kirilowii* via 5% sodium alginate. Rady and Hanafy (2004) confirm that high percent vitrification of *Gypsophila paniculata*was observed in shoots derived from shoot tips coated by 2 % sodium alginate. But, Sandoval-Yugar et al. (2008) encapsulated the micro shoots of *Musa sp*. cv. ‘Grand Naine’ through 1% sodium alginate. Singh (2008) introduced 3% sodium alginate for shoot tips encapsulation in Sikkim Himalayan Rhododendron (*R. maddeni* Hook. f.).

A range of calcium chloride concentrations has been experimented for shoot tips encapsulation. Results indicated that, increasing in CaCl2 had not significant effects on plantlet recovery (Table 2). But, by concentration increase, the capsules were so hard that prevented the proliferation of shoot tips. Although there was no significant difference between calcium chloride concentration, but 1.4% concentration provided more suitable hardness and shapes of capsules. Lower concentrations of sodium alginate or CaCl2 prolonged the time of ion exchange of sodium and calcium ions and resulted in fragile capsules.

Rai *et al*. (2008) reported that a gelling matrix of 3% sodium alginate and 100 mM calcium chloride was found most suitable for formation of firm, clear and isodiametric beads. Similar observations were also registered in *Punica granatum*L. (Naik and Chand 2006).Halmagyi and Deliu (2007) used 3% sodium alginate solution and 1.0 M calcium chloride for encapsulation of carnation shoot tips. Singh (2008) utilized 60 mM calcium chloride solutions for encapsulation of *Rhododendron maddeni*. Sandoval-Yugar et al.(2008) dropped sodium alginate beads in 100mM CaCl2 solution for encapsulation of micro shoots in *Musa* sp. cv. ‘Grand Naine.

Conversion of encapsulated shoot tips into plantlets was significantly affected by the concentration of sodium alginate, calcium chloride and planting media. A key factor for synthetic seed technology is the effects of various concentrations of sodium alginate and calcium chloride on the texture, shape and size of the bead. It seems that the source of the alginate affects was the quality of capsules (Hassan 2003).Sodium alginate concentration, degree of viscosity of the alginate used, CaCl2 concentration are important parameters for determining the permeability, resistance and hardness of the resulting beads and the subsequent success of the encapsulation method (Block 2003).

*Effects of different material used to prepare the sodium alginate*

In this research, we tested distilled water, liquid free MS medium and MS medium supplemented with 2.5% sucrose, 2 mgl-1 BAP and 2mgl-1IAAfor sodium alginate’s preparation. The analysis of variance revealed that sprouting was significantly affected by planting medium. Out of the three planting media evaluated, the best percentage of sprouting was observed in liquid free MS medium supplemented with 2.5% sucrose, 2 mg l-1 BAP and 2 mg l-1 IAA show highest recovery (70.2%) followed by liquid free MS medium (63.19%) and distilled water (62.16%)(Table3). Our result was in agreement with that of Hassan’s (2003) results that the appearance of encapsulated buds exhibit the best shoot development on Murashige and Skoog (MS) medium supplemented with 1.0 mg l-1 BAP, 40 mg l-1 adenine sulfate and 3.0 mg l-1 IAA and gelled with 0.8% bacteriological agar (73% +5.17conversion). Halmagyi and Deliu (2007) used liquid MS medium for encapsulation of *Dianthus caryophyllus* L. shoot tips. Ganapathi et al. (2001) reported that among the different media for somatic embryos encapsulation, the MS medium with sucrose gave the maximum conversion (66%) into plantlets. Sandoval-Yugar et al. (2008) stated that encapsulated and non-encapsulated micro shoots exhibited 100% germination in response to MS culture medium. The complex composition of endosperm makes difficult its reconstitution and the use of standard culture medium in the capsule may increase the conversion of the propagule to plantlets (Sandoval-Yugar et al. 2008). This is in agreement with our results, where the addition of MS increased the micro shoots conversion in the alginate matrix together with 2 mg l -1 BAP and 2 mg l -1 IAA treatment. Anand and Bansal (2002) believe that the presence of nutrients and plant growth regulators (PGRs) in the encapsulation matrix is more important than in the inoculation medium.

The alginate matrix supplemented with PGRs serves as an artificial endosperm; thereby the nutrients are supplied to the encapsulated explants for plant regrowth (Bapat and Rao 1992; Nieves et al. 1998).

*Effects of different rooting medium*

After 4 weeks the encapsulated shoot tips produced low root, then for increased root induction, the proliferated rapeseed shoots were placed in liquid MS medium supplemented with three different concentration of IAA. The root formation was increased following two 2 weeks. The analysis illustrated that, there was significant difference among several concentrations of IAA and the maximum root formation was obtained from3% calcium chloride (90%)(Table 4).In many species an appropriate root induction treatment should be integrated with the encapsulation protocols (Hasan and Takagi, 1995; Maruyama *et al*. 1997). Gangopadhyay et al. (2005), used liquid MS medium supplemented with 0.01 mM IBA (indole-3-butyric acid) and 0.002 mM kinetin for root induction in encapsulated pineapple shoot tips.

To best of our knowledge, this is first report for encapsulation of *Brassica napus* L shoot tips into an alginate matrix followed by successful in vitro regeneration. The results obtained could be considered as a prerequisite for exploring the encapsulation technique for development of a procedure for production of synthetic seeds from non-embryonic explants in Rapeseed.

*Somatic embryos encapsulation*:

The effect of the length of storage periods at 4 oC on subsequent development of ESEs was evaluated. Immediately after storage the ESEs were cultured in gelled MS medium. Encapsulation and subsequent plant recovery are illustrated in Figure 3. During the germination process, the encapsulated, ivory-colored embryos turned green. After 7-9 d, roots emerged through the alginate coat. Formation of secondary embryos was observed at the root pole. The effect of storage period (temperature 4º C, RH 70%) on formation of root and shoot of encapsulated somatic embryos was significant (Figure 4). Analysis of variance of data showed that 40 days of cold storage of encapsulated somatic embryos caused significant reduction in plantlet recovery (P = 0.05). The highest percentage of germination of the ESEs was obtained after storage for 10 days (55.5%) whereas the lowest (11.1%) was observed after 40 days of cold storage (Table 5). The growth rate (shoot length mm) of encapsulated somatic embryos varied over time. It was very slow during the first week, then, it considerably increased, and then it decreased 12 days after culture initiation (Figure 5). Root formation varied from 72.4% to 52.8%, the highest percentage of root formation was obtained after 10 days, and the lowest was recorded for 30 and 40 days of storage. The 30 and 40 days cold storage also resulted in drastic reduction of the root development and conversion levels (Table 6).The regeneration data from the experiment with encapsulated somatic embryos in rapeseed were similar to the results obtained with *Citrus reticulata* by Antonietta et al. (2007). Similarly, the conversion frequency of encapsulated nodal segments of *Punica granatum* also declined markedly following storage at low temperature (Naik and Chand 2006). It seems that 10 days cold storage kept the ESEs quiescent and contributed to a better maturation and accumulation of reserve compounds like storage proteins and carbohydrates in the growing points resulting in improvement of the conversion frequency. An interesting feature of the ESEs was their ability to retain their sprouting potential even after storage of 40 days at 4°C.  Bekheet (2006) noted that storage of encapsulated bulblets of garlic at 15 ºC was more effective than storage at 25 ºC. Rise in survival and conversion rates of encapsulated embryos after cold storage was also demonstrated for other plant species, such as *Paulownia elongata* and *Quercusrobur* L. (Ipekci and Gouzukirmizi 2003). Machii and Yamanouchi (1993) observed root development in the synthetic seeds of mulberry only when these were stored at 4 °C for 30 days before sowing. The alginate matrix supplemented with MS medium and 2 mg L-1 BAP + 2 mg L-1 IAA serves as an artificial endosperm and is necessary for cold storage after dehydration and encapsulation; it, also increases the stability of membranes under severe dehydration.  The decline in plant recovery from stored encapsulated vegetative propagules may be due to oxygen deficiencies in the calcium alginate bead (Redenbaugh et al. 1991). The technique of sodium alginate, due to the stimulatory effect, in encapsulation matrix is  reinforces  the  tolerance of  the tissues to the cold storage.

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Table 1 Comparison of various sources and concentration of sodium alginate used for producing capsules of suitable hardness

|  |  |  |
| --- | --- | --- |
| Sodium alginate concentration (%) | | Frequency of shoot recovery (%) |
| 2 | 55.59 b | |
| 4 | 86.06 a | |
| 6 | 53.92 b | |

Data was recorded after one month of transfer on germination medium. Mean values sharing the same letter do not differ significantly (α =0.01) according to Duncan’s multiple range test.

Table 2 Comparison of various sources and concentration of calcium chloride used for producing capsules of suitable hardness

Data was recorded after one month of transfer on germination medium. Mean values sharing the same letter do not differ significantly (α =0.01) according to Duncan’s multiple range test.

Table 3 Effects of different media on conversion of encapsulated shoot tips into plantlets in Brassica napus L.

|  |  |  |
| --- | --- | --- |
| Sodium alginate gel preparation | Frequency of shoot recovery (%) | |
| MS liquid medium with hormones | | 70.2 a |
| MS liquid medium | | 63.19 b |
| Distilled water | | 62.16 b |

Data was recorded after one month of transfer on germination medium. Mean values sharing the same letter do not differ significantly (α =0.05) according to Duncan’s multiple range test.

Table 4 Effects of different rooting medium on root formation after plantlet transfer to several rooting medium (after 2 wks)

|  |  |  |
| --- | --- | --- |
| IAA ( mg l-1) | | Frequency of plantlet rooting (%) |
| 1 | 43.11 b | |
| 2 | 90 a | |
| 3 | 75 ab | |

Data was recorded after 2wks of transfer plantlet medium. Mean values sharing the same letter do not differ significantly (α =0.05) according to Duncan’s multiple range test.

Table 5 Plantlet conversion from encapsulated somatic embryos of *Brassica napus* cv. Tallayeh after storage at low temperature (4 °C) for different duration

|  |  |  |
| --- | --- | --- |
| Frequency of plantlet conversion (%) | Storage period (days) | |
| 55.5±2 a | 10 |
| 27.7±2 ab | 20 |
| 16.6 ±2 b | 30 |
| 11.1±2 b | 40 | |

Values are means ± S.E. of conversion experiment .Data was recorded after three week of transfer on germination medium. Mean values sharing the same letter do not differ significantly (P =0.05) according to Duncan’s multiple range test.

Table 6 Plantlet rooting from encapsulated somatic embryos of *Brassica napus* cv. Tallayeh after storage at low temperature (4 °C) for different duration

|  |  |
| --- | --- |
| Frequency of plantlet rooting (%) | Storage period (days) |
| 72.4 a | 10 |
| 56.6 a | 20 |
| 52.8 a | 30 |
| 52.8 a | 40 |

Data was recorded after three weeks of transfer on germination medium. Mean values sharing the same letter do not differ significantly (P =0.05) according to Duncan’s multiple range test.