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A PROTOCOL FOR SYNTHETIC SEEDS FROM *SALVIA OFFICINALIS* L. SHOOT TIPS

IZABELA GRZEGORCZYK* AND HALINA WYSOKIŃSKA

Department of Biology and Pharmaceutical Botany, Medical University of Lodz, Muszyńskiego 1, 90–151 Lodz, Poland

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Shoot tips excised from shoot culture of *Salvia officinalis* were encapsulated in 2% or 3% (w/v) sodium alginate and exposed to 50 mM calcium chloride for complexation. Immediately or after 6, 12 or 24 weeks of storage at 4°C, the synthetic seeds were cultured for 6 weeks on half-strength MS medium supplemented with indole-3-acetic acid (IAA) (0.1 mg/l) and solidified with 0.7% agar. The frequency of shoot and root emergence from encapsulated shoot tips was affected by the concentrations of sodium alginate and additives in the gel matrix (sucrose, gibberellic acid, MS nutrient medium) as well as duration of storage. The frequency of shoot and root induction of non-stored synthetic seeds was highest with shoot tips encapsulated with 2% sodium alginate containing 1.5% sucrose and 0.5 mg/l gibberellic acid (GA_3). Shoot tips maintained their viability and ability to develop shoots even after 24 weeks of storage when they were encapsulated in 3% alginate with 1/3 MS medium, sucrose (1.5%) and GA_3 (0.25 mg/l). Root formation tended to decrease with storage time. Overall, 90% of the plantlets derived from stored and non-stored synthetic seeds survived in the greenhouse and grew to phenotypically normal plants. This procedure can enable the use of synthetic seed technology for germplasm conservation of *S. officinalis*, a plant species of high medical and commercial value.

Key words: Calcium alginate, cold storage, *Salvia officinalis* L., shoot tip encapsulation, synthetic seeds.

INTRODUCTION

Sage (*Salvia officinalis* L.), a perennial plant native to the Mediterranean area, is widely used in medicine as well as in the food and cosmetic industries. Leaves of the species have been introduced into the pharmacopoeias of many countries. They possess antibacterial (Farag et al., 1989), antioxidant (Cuvelier et al., 1994), antiviral (Tada et al., 1994) and anti-inflammatory (Baricevic et al., 2001) properties. These activities are due mainly to the presence of essential oils, phenolic acids, di- and triterpenoids, flavonoids and tannins.

In a previous study we found that tissue culture using apical buds can be an effective alternative to the conventional method of propagating *S. officinalis* (Grzegorczyk and Wysokińska, 2004). Here for the first time we report encapsulation of shoot tips for development of *S. officinalis* synthetic seeds. A synthetic seed is a somatic embryo or any non-embryogenic vegetative propagule (shoot tip, shoot primordial, axillary bud, nodal segment) inside an alginate hydrogel coating, possessing the ability to convert to a plant in vitro or ex vitro, an ability it can retain after storage. The use of unipolar propagules for development of synthetic seeds has been reported in numerous medical plant species, such as *Valeriana wallichii* (Mathur et al., 1989), *Rauvolfia serpentina* (Ray and Bhattacharya, 2008), *Cineraria maritima* (Srivastava et al., 2009), *Cannabis sativa* (Lata et al., 2009), *Fragaria ananasa* and *Rubus idaeus* (Lisek and Orlikowska, 2004). This kind of explant is very useful in such plant species, where somatic embryogenesis is not well established or else good quality somatic embryos are not produced.

To the best of our knowledge there are no published data on regeneration of plants from somatic embryos of sage. Synthetic seed technology offers an efficient means for mass propagation of a commercially important plant species all year round, irrespective of season and climatic conditions. The advantages of synthetic seeds for propagation include easy handling and potential long-term storage. The seeds can also be used for conservation of germplasm and exchange of axenic plant material between laboratories.

Many factors have been shown to be significant for shoot regrowth and rooting of those shoots after

*e-mail: izabela.grzegorczyk@umed.lodz.pl

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TABLE 1. Effect of gel matrix composition on shoot and root emergence from non-stored synthetic seeds of *S. officinalis*. Data were recorded after 6 weeks of culture on regrowth medium (1/2 MS medium with 0.1 mg/l IAA)

Alginate matrix composition			Formation	
Alginate concentration [%]	Sucrose [%]	GA_3 [mg/l]	shoots * [%]	shoots with roots ** [%]
2			40 ± 4.89	9.1 ± 3.76
2	1.5		55 ± 9.73	23.3 ± 5.44
2		0.5	61 ± 4.76	9.1 ± 2.93
2	1.5	0.5	69 ± 5.21	32.0 ± 4.67
2		1	59 ± 3.18	19.1 ± 3.28
2	1.5	1	61 ± 4.41	23.5 ± 6.87
3			43 ± 5.60	27.77 ± 5.87
3	1.5		29 ± 4.51	15.0 ± 8.65
3		0.5	47 ± 5.73	24.3 ± 3.87
3	1.5	0.5	64 ± 4.73	14.5 ± 4.40
3		1	55 ± 6.57	17.64 ± 4.25
3	1.5	1	56 ± 5.29	25.0 ± 5.83

Values are means ± SE.
* percentage of encapsulated shoot tips that formed shoots with normal morphology.
** percentage of obtained shoots that formed roots.

encapsulation. We undertook this study to evaluate the effect of alginate matrix components (sodium alginate concentration, the presence of sucrose, GA_3 and MS nutrient salts) and storage at low temperature (4°C) for different periods (6, 12 and 24 weeks) on the morphogenetic response of encapsulated shoot tips of *S. officinalis*, that is, their ability to develop shoots and plantlets.

MATERIAL AND METHODS

Shoot tips of *S. officinalis* (0.5–1 cm long) were excised from shoots proliferated in vitro on agar (0.7%) with MS (Murashige and Skoog, 1962) medium supplemented with IAA (0.1 mg/l) and BAP (0.45 mg/l) (Grzegorczyk and Wysokińska, 2004).

For encapsulation the explant was immersed in 2% or 3% (w/v) sodium alginate solution and dropped into the complexing solution of 50 mM calcium chloride using a sterile pipette of appropriate diameter. Each drop contained one shoot tip. The beads were kept in this solution for 30 min in a 600 ml beaker on a magnetic stirrer for hardening. Both the gel matrix and the complexing agent were autoclaved at 121°C for 17 min and then the beads were washed 3 times with sterilized distilled water to remove traces of calcium chloride. After encapsulation the shoot tips were placed individually in culture tubes containing 25 ml regrowth medium (MS agar medium supplemented with 0.1 mg/l IAA). The culture was maintained in a growth chamber at 26±2°C under cool-white fluorescent light (40 µmol $m^{-2}s^{-1}$) under a 16 h photoperiod. The data on the percentage of shoot development from encapsulated explants (i.e., when the elongating shoot tips burst through the alginate capsule walls) and rooting of the shoots were recorded after 6 weeks of culturing on the regrowth medium.

To study the effect of alginate bead composition on shoot and root emergence, shoot tips were immersed in sterilized mixtures of the following composition:

- 2 or 3% (w/v) sodium alginate + distilled water
- 2 or 3% (w/v) sodium alginate + sucrose (1.5%)
- 2 or 3% (w/v) sodium alginate + GA_3 (0.5 or 1 mg/l)
- 2 or 3% (w/v) sodium alginate + sucrose (1.5%) + GA_3 (0.5 or 1 mg/l)
- 3% (w/v) sodium alginate + sucrose (1.5%) + GA_3 (0.5 mg/l) + full-strength MS medium (MS)
- 3% (w/v) sodium alginate + sucrose (1.5%) + GA_3 (0.5 mg/l) + half strength MS medium (1/2 MS)
- 3% (w/v) sodium alginate + sucrose (1.5%) + GA_3 (0.25 or 0.5 mg/l) + one-third strength MS medium (1/3 MS)

Tables 1 and 2 specify the composition of the alginate matrix used for encapsulating *S. officinalis* shoot tips.

To examine the effect of storage duration on shoot and root development, some of the capsules obtained as described above were placed in Petri dishes sealed with Parafilm (Fig. 1) and refrigerated at 4°C in darkness. After 6 (short-term storage), 12 (medium-term storage) or 24 (long-term storage) weeks, the beads were transferred to regrowth medium (½ MS medium with 0.1 mg/l IAA) for inducing

TABLE 2. Effect of gel matrix composition and storage period on shoot and root emergence from synthetic seeds of *S. officinalis*. Data were recorded after 6 weeks of culture on regrowth medium (1/2 MS with 0.1 mg/l IAA)

Duration of storage [weeks]	Composition of alginate matrix				Formation	
	Alginate concentration [%]	Sucrose [%]	GA_3 [mg/l]	MS nutrient medium	shoots * [%]	shoots with roots** [%]
6	2				0	0
6	2	1.5			61 ± 4.48	50.0 ± 8.22
6	2		0.5		0	0
6	2	1.5	0.5		87 ± 7.41 (6.7)	69.5 ± 10.50
6	2		1		0	0
6	2	1.5	1		88 ± 5.78 (24.0)	72.0 ± 20.45
6	3				0	0
6	3	1.5			16 ± 4.96	50.0 ± 5.6
6	3		0.5		0	0
6	3	1.5	0.5		83 ± 6.61	64.3 ± 7.68
6	3		1		0	0
6	3	1.5	1		80 ± 5.21	40.5 ± 8.5
12	3	1.5	0.5		56 ± 4.60 (3.4)	52.6 ± 12.6
12	3	1.5	0.5	MS	52 ± 2.5 (6.9)	33.3 ± 7.22
12	3	1.5	0.5	½ MS	48 ± 5.1 (4.0)	50 ± 12.5
12	3	1.5	0.5	⅓ MS	72 ± 8.48 (9.4)	87.0 ± 20.55
24	3	1.5	0.25	⅓ MS	63 ± 6.89	33.3 ± 3.05

Values are means ± SE. The percentage of synthetic seeds that developed shoots during storage in the refrigerator is given in parentheses.
* percentage of encapsulated shoot tips that formed shoots with normal morphology
** percentage of obtained shoots that formed roots

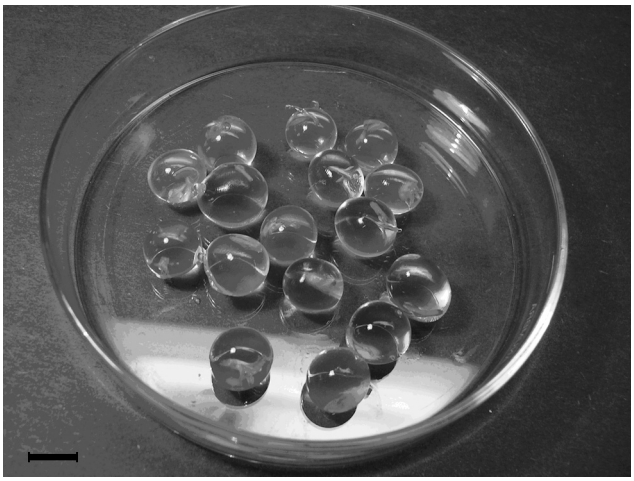

Fig. 1. *Salvia officianalis* shoot tips encapsulated in calcium alginate. Bar = 1 cm.

shoots and roots. The incubation conditions were the same as used for non-stored encapsulated shoot tips.

In all experiments, 7–14 seeds were used for each treatment (respective composition of alginate matrix and length of storage period) and each experiment was done in three replicates. Well-rooted shoots regenerated from encapsulated shoot tips (Fig. 2) were transferred to 10 cm diameter pots containing a mixture of sand, peat and soil (3:3:4, v/v/v). Survival rates were determined after 10 weeks in the greenhouse.

RESULTS AND DISCUSSION

In this work, shoot tips from *S. officinalis* shoots proliferated in vitro were encapsulated in 2% or 3% sodium alginate and hardened with 50 mM calcium chloride to prepare synthetic seeds (Fig. 1). Both concentrations of sodium alginate proved suitable for forming uniform beads. The synthetic seeds were transferred to the regrowth medium (1/2 MS agar medium supplemented with 0.1 mg/l IAA) directly after encapsulation or after refrigerated storage at 4°C for 6, 12 or 24 weeks (42, 84 or 168 days). After 6 weeks of culture we recorded the percentage of synthetic seeds that developed shoots and the percentage of those shoots that formed roots.

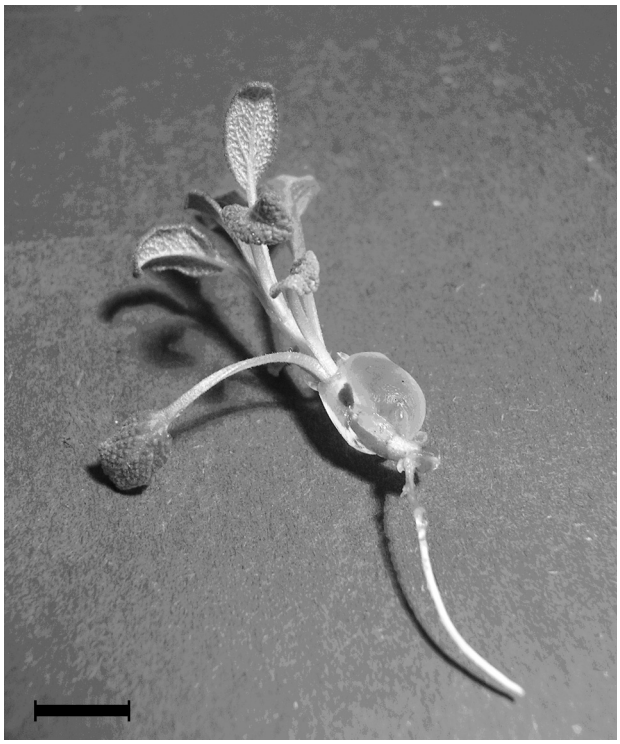

Fig. 2. *S. officinalis* plantlet obtained from the encapsulated shoot tip after 6 weeks on MS medium containing IAA. Bar = 1 cm.

We found that the regrowth of encapsulated explants to shoots and the rooting of emerged shoots depended on the bead composition and the duration of storage. Shoots emerged from non-stored encapsulated shoot tips, breaking the capsule wall, at the end of the first week, and did so most intensively in the second week of incubation on regrowth agar medium. Later (at 4–6 weeks), roots formed at the base of shoots on the same medium (Fig. 2). Among the various types of tested gel matrices (2% or 3% sodium alginate solution with or without addition of sucrose and GA_3 alone or in combination) (Tab. 1), the results for shoot and root emergence were best with shoot tips encapsulated in 2% sodium alginate with sucrose (1.5%) and GA_3 (0.5 mg/l). Almost 70% of these beads regenerated shoots with normal morphology (i.e., a shoot with well-developed leaves, without signs of hyperhydricity); 32% of the obtained shoots had roots (Tab. 1). The shoot development response was similar (64%) for beads with a 3% alginate concentration, but decreased rooting of the shoots that emerged resulted in lower conversion to plantlets (14.5%). We should stress that the presence of both concentrations of GA_3 (0.5, 1 mg/l) in the encapsulation matrices promoted the regrowth of *S. officinalis* shoot tips to shoots and improved the morphology of emerged shoots. Pattnaik et al. (1995) reported that supplementing the alginate matrix with 0.3 mg/l GA_3 increased shoot formation from encapsulated axillary buds of different *Morus* species, and attributed the effect of GA_3 to improvement of shoot internode elongation and/or stimulation of vegetative bud germination.

We also studied the effect of storage duration at low temperature (4°C) on emergence of shoots and roots from encapsulated *S. officinalis* shoot tips. Synthetic seeds can be used for preservation of germplasm and can be exchanged between laboratories only if they remain viable after storage for a reasonable period. After each storage period (6, 12 or 24 weeks), encapsulated shoot tips were cultured on regrowth medium for 6 weeks. The experiments with encapsulated propagules of *S. officinalis* stored for 6 weeks at low temperature (short-term storage) showed that shoots and roots developed only in the presence of sucrose in the gel matrix (Tab. 2). In contrast, at least 40% of non-stored shoot tips encapsulated in alginate beads without sucrose developed shoots, although their rooting ability was poor (Tab. 1). Sucrose as a carbon source for encapsulated explants enables their survival during storage. The presence of sucrose in the alginate gel matrix also improved emergence of shoots from encapsulated shoot tips of *Camelia japonica* (Ballester et al., 1997) and axillary buds of *Betula pendula* (Piccioni and Standardi, 1995). Rai et al. (2008) demonstrated complete inhibition of shoot development from encapsulated shoot tips of *Psidium guajava* in medium without sucrose. Our combination of 2% sodium alginate, sucrose (1.5%) and GA_3 (0.5 or 1 mg/l) in the alginate matrix proved most effective for shoot and root regeneration from encapsulated shoot tips after 6 weeks of storage, giving a result similar to that for non-stored synthetic seeds, Almost 90% of such beads developed shoots and ~70% of them demonstrated simultaneous formation of roots (Tab. 2). However, some of the stored encapsulated shoot tips (from 7 to 24%, depending on the GA_3 concentration) broke through the alginate matrix and developed shoots in the refrigerator at the end of the fifth week of storage. In practice this would complicate transport of synthetic seeds and exchange of plant material between laboratories. Murayama et al. (1997a) showed that synthetic seeds of *Jacaranda mimosaefolia* could not be stored because of shoot development resumption during storage at low temperature. In the case of *S. officinalis* synthetic seeds, the problem could be overcome by increasing the concentration of sodium alginate in the encapsulation matrix from 2% to 3% (w/v). None of the shoot tips encapsulated in 3% alginate and stored for 6 weeks at low temperature were able to "germinate" in the refrigerator. After these seeds were transferred to regrowth medium the

shoot and root development response (83% and 64%, respectively) was only slightly lower than that of shoot tips encapsulated in 2% alginate with the same adjuvants (1.5% sucrose and 0.5 mg/l GA_3). At the higher sodium concentration the alginate beads were harder, presumably suppressing shoot emergence during the storage period, so we chose the 3% alginate concentration for subsequent experiments with *S. officinalis* shoot tips after medium-term (12 weeks) and long-term (24 weeks) storage. After storage for 12 weeks, 55% of the shoot tips encapsulated in 3% sodium alginate with sucrose (1.5%) and GA_3 (0.5 mg/l) formed shoots, and ~50% of them had well developed roots after 6 weeks of culture on regrowth medium. To increase shoot and root development, the alginate matrix was additionally enriched with MS nutrient medium. Among the different strengths of MS medium used in our experiments (full, half, and one-third), only the presence of 1/3 MS medium in beads gave a higher percentage of shoot and root formation than the control (shoot tips stored for 12 weeks and encapsulated in 3% sodium alginate prepared in distilled water without MS medium) (Tab. 2). In other work, alginate supplemented with MS nutrient medium has been used to improve shoot and root emergence from encapsulated explants of *Carica papaya* (Castillo et al., 1998) and *Chonemorpha grandiflora* (Nishitha et al., 2006). Adding MS medium to the alginate matrix reduced gel viscosity (Piccioni and Standardi, 1995). This was probably why shoots formed in the refrigerator from ~10% of the stored shoot tips encapsulated in beads containing 1/3 MS medium. That percentage is almost three times higher than in the control (gel matrix without MS medium). Our results with short-term storage of *S. officinalis* synthetic seeds indicated that GA_3 also increased shoot development during the storage period (Tab. 2). Based on that outcome, we prepared beads for long-term storage with 3% sodium alginate dissolved in 1/3 MS medium supplemented with 1.5% sucrose and with the GA_3 concentration reduced to 0.25 mg/l to limit initiation of shoot regrowth in the refrigerator. The result was that 63% of the synthetic seeds developed shoots, but only 33% of the shoots formed roots within 6 weeks of culture on regrowth medium. Reduction of the frequency of plantlet recovery from stored encapsulated explants has been observed in work with synthetic seeds of many other plant species. For example, after 45 days of storage at 4°C, the rate of germination of *Ananas comosus* synthetic seeds was down by ~20%, and after 90-day storage the synthetic seeds lost viability completely (Soneji et al., 2002). Danso and Ford-Lloyd (2003) reported a nearly complete decline in rooting of encapsulated *Manihot esculenta* shoots after one month of storage. Redenbaugh et al. (1987) suggested that the decline could be due to desiccation of alginate seeds during storage, oxygen deficiency, inhibition of plant tissue respiration, lack of nutrients, or accumulation of metabolic waste within the alginate capsule.

Here we demonstrated that encapsulated *S. officinalis* shoot tips could be stored for 6 months (24 weeks) at 4°C without a marked loss of viability or of the ability to develop shoots (Tab. 2). However, the propagules' low ability to form roots after long-term storage is a problem. Probably it can be overcome with a two-step procedure: transferring the sprouted shoots of encapsulated explants to rooting medium. In such experiments done with *S. officinalis* shoots proliferated in vitro, 74% of them developed roots after 5 weeks of culture on 1/2 MS medium (Grzegorczyk and Wysokińska, 2004). The two-step procedure has been used for conversion of synthetic seeds of other plant species to plantlets (Hasan and Takagi, 1995; Maruyama et al., 1997b). For example, inhibition of rooting was overcome in encapsulated *Psidium guajava* shoot tips stored short-term (6 weeks) by transferring sprouted shoots to agar-solidified MS medium. Rooted shoots obtained from stored and non-stored synthetic seeds of *S. officinalis* were transferred to pots containing a mixture of sand, peat and soil (3:3:4 v/v/v) and grown in the greenhouse. After 10 weeks the estimated survival frequency was 90%. The plants were phenotypically similar to normal plants.

Here we reported a procedure for encapsulation of shoot tips of *S. officinalis*. Manipulation of the sodium alginate concentration (2 or 3%) and the presence of such components as sucrose, GA_3 and nutrient medium (1/3 MS) in the gel matrix ensured viability and a high frequency of shoot emergence from encapsulated explants after even 6 months of low-temperature storage. Further work should improve the frequency of root formation from the shoots that emerge from encapsulated shoot tips, especially from those stored long-term. At present that is the main factor limiting the use of *S. officinalis* synthetic seeds for germplasm propagation and conversion of this important medicinal plant.

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