

ENCAPSULATION OF *CENTAURIUM ERYTHRAEA* RAFN – AN EFFICIENT METHOD FOR REGENERATION OF TRANSGENIC PLANTS

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An effective procedure for producing transformed *Centaurium erythraea* plants from synthetic seeds is described. Explants were encapsulated in 3% sodium alginate with 3% sucrose. Encapsulated hairy roots were cultured on half-strength Murashige and Skoog (1/2 MS) or Woody Plant (WPM) agar-solidified regeneration media supplemented with 6-benzylaminopurine (BAP) or without the cytokinin. The use of WPM with 0.5 mg/L of BAP gave the best shoot formation frequency (86%) and mean number of shoots (15) per root segment. Shoots rooted with 97% frequency on 1/2 MS without growth regulators. Encapsulated shoot buds were cultured on one-third-strength MS agar medium (1/3 MS) supplemented with indole-3-butyric acid (IBA) (0.05 mg/L). The plantlet conversion frequency was 32%. The encapsulated hairy roots and shoot buds were stored for 4, 6 or 14 weeks at 4°C. Synthetic seeds encapsulated with 3% sodium alginate with 3% sucrose stored at 4°C remained viable for 6 weeks but their developmental parameters significantly decreased. Adding nutrient medium and growth regulator to the alginate matrix increased plantlet recovery from both non-stored and stored synthetic seeds: synthetic seeds retained their viability and ability to form plantlets even after 14 weeks of storage. Regenerated transformed plantlets of *C. erythraea* were acclimatized in the greenhouse.

Key words: *Centaurium erythraea*, hairy roots, transgenic plants, synthetic seeds.

INTRODUCTION

Infection of sensitive dicotyledonous plants by *Agrobacterium rhizogenes* causes hairy roots to form at infection sites, due to the transfer, integration and expression in the plant cell genome of T-DNA from the Ri (root-inducing) plasmid. These genetically transformed roots are capable of growing intensively *in vitro* in liquid media without growth regulators. Hairy root culture has applications in many areas of research. It has been used for production of useful secondary metabolites, regeneration of transgenic plants, as an experimental system to study interactions with other pathogenic organisms, and for synthetic seed production (Guillon et al., 2006).

We have established a hairy root culture of *Centaurium erythraea* by *Agrobacterium rhizogenes*-mediated transformation (strain LBA 9402) and obtained transgenic plants from roots (Piątczak et al., 2006). *C. erythraea* is a valuable medicinal plant listed in the pharmacopoeias of many European and American countries. The pharmaco-

logical activity of *C. erythraea* is due mainly to the presence of bitter secoiridoid glycosides such as gentiopicroside, sweroside and swertiamarin. They show fungistatic, antibacterial, choleric, pancreatic and hepatoprotective activities (Kumarasamy et al., 2003). Due to their bitterness, these secoiridoids are also used in the manufacture of some commercial beverages or soft drinks (Vágnerová, 1992). We found that Ri-transformed regenerants of *C. erythraea* represent a valuable source of secoiridoids, particularly gentiopicroside and swertiamarin. The yield of these bioactive compounds in transformed plants grown in the greenhouse for 10 weeks was 8 times higher than in commercially available *Centaurii herba* and double that of non-transformed plants regenerated *in vitro* (Piątczak et al., 2006).

The major problem associated with using hairy root culture for transgenic plant production is that the cultures require continuous transfer to fresh medium, which is expensive and time-consuming. Synthetic seed production using hairy root fragments has been reported (Uozumi and Kobayashi,

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1997; Pietrosiuk et al., 2007; Gongopadhyay et al., 2011), but whole transgenic plants from encapsulated hairy root fragments have been obtained only for *Armoracia rusticana* so far (Nakashimada et al., 1995; Repunte et al., 1995; Nakashimada et al., 1996). Synthetic seeds are structures obtained by sodium alginate encapsulation of somatic embryos or vegetative propagules that can produce whole plants. The synthetic seeds can be stored at low temperature and can be used for clonal propagation, conservation and exchange of in vitro plant material between laboratories (Kikowska and Thiem, 2011). Encapsulation of in-vitro-derived vegetative propagules for production of synthetic seeds has been widely studied in different plant species such as *Momordica dioica* (Thiruvengadam et al., 2012), *Salvia officinalis* (Grzegorzczak and Wysokińska, 2011), *Psidium guajava* (Rai et al., 2008), *Withania somnifera* (Singh et al., 2006), *Rhodiola kirilowii* (Zych et al., 2005), *Hibiscus moscheutos* (West et al., 2006) and *Ocimum* sp. (Mandal et al., 2000).

In this study we produced synthetic seeds from hairy root segments and transformed shoot buds of *C. erythraea* and assessed their ability to regenerate transgenic plants. We also examined the effects of storage duration (at 4°C) and alginate matrix composition on plantlet formation from encapsulated hairy roots and shoot buds.

MATERIAL AND METHODS

PLANT MATERIAL

Hairy root culture (line L-1) of *Centaureum erythraea* was used in this study. The roots were induced by infecting sterile shoots with *Agrobacterium rhizogenes* (LBA 9402). The transformation event was confirmed at the molecular level by PCR (Piątczak et al., 2004). The hairy roots were subcultured every 4 weeks in 300 ml Erlenmeyer flasks containing 80 ml growth regulator-free liquid woody plant medium (WPM) (Lloyd and McCown, 1980) with 3% sucrose. The flasks were maintained at 26°C in darkness on a rotary shaker at 100 rpm. Under these conditions the hairy roots of *C. erythraea* readily produced shoot buds. Shoot buds (0.3 cm long) excised from 4-week-old hairy root culture were transferred to Murashige and Skoog (MS) (1962) agar medium supplemented with 0.1 mg/L indole-3-acetic acid (IAA) and 1.0 mg/L BAP and cultured under cool white fluorescent lamps (40 $\mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ flux) at 26 \pm 2°C. They were subcultured every 4 weeks. The media used for all experiments were adjusted to pH 5.7–5.9 with 0.1 M NaOH. The growth regulators were added before autoclaving (0.1 MPa, 121°C for 17 min).

PREPARATION OF SYNTHETIC SEEDS

Hairy root segments (HRs) (0.3–0.5 cm long) and transformed adventitious shoot buds (SBs) (0.3 cm long) of *C. erythraea* were used for synthetic seed production by encapsulation with 3% (w/v) sodium alginate (Sigma) and complexation in 50 mM CaCl₂ solution. The alginate gel and complexing agent were sterilized by autoclaving at 121°C under 0.1 MPa for 17 min. Encapsulation was done by mixing the explants into the sodium alginate solution and dropping them into the calcium chloride solution. Droplets each containing one shoot bud or hairy root fragment were stirred for 30 min in a flask on a magnetic stirrer, then rinsed three times in sterile water to remove traces of calcium chloride. This process produced alginate beads 0.5–0.6 cm in diameter (Fig. 1a,b). All operations were performed in sterile conditions.

EXPERIMENTS WITH ENCAPSULATED HAIRY ROOT SEGMENTS

Two series of experiments were performed. In the first, HRs were encapsulated in 3% sodium alginate dissolved in water with 3% sucrose and transferred to test tubes containing 25 ml of one of two regeneration media solidified with 0.7% (w/v) agar: half-strength MS medium (1/2 MS) or full-strength WPM. Both media were supplemented with 3% sucrose. The choice of media was based on our earlier experiments which showed them to be effective for growth of *C. erythraea* hairy roots and rooting of transformed adventitious shoots (Piątczak et al., 2006). To study the effect of BAP on shoot proliferation, the regeneration media (1/2 MS, WPM) were supplemented with BAP (0.05, 0.1 or 0.5 mg/L) or contained no growth regulators.

The second series was conducted to examine the effect of storage duration and alginate matrix composition on shoot regeneration and plantlet development from HR synthetic seeds. HRs encapsulated either in 3% sodium alginate dissolved in water with 3% sucrose or WPM with 3% sucrose and BAP (0.5 mg/L) were transferred to Petri dishes (6–7 synthetic seeds per dish) and refrigerated at 4°C in darkness for 4, 6 or 14 weeks. After each storage period, encapsulated HRs were placed on agar-solidified WPM supplemented with 0.5 mg/L BAP for shoot regeneration. Non-stored synthetic seeds were transferred to regeneration medium of the same composition directly after encapsulation.

For all experiments with encapsulated HRs, the percentage of synthetic seeds forming adventitious shoots and the mean number of shoots per root segment were recorded after 5 weeks of culture. For rooting, shoots regenerated from stored and non-

stored synthetic seeds were transferred individually to agar solidified 1/2 MS medium without growth regulators, supplemented with 3% sucrose. After 4 weeks the percentage of rooted shoots, mean number of roots per shoot, and mean root length (mm) were recorded.

EXPERIMENTS WITH ENCAPSULATED SHOOT BUDS

In these experiments, shoot buds (SBs) encapsulated with 3% sodium alginate dissolved in water with sucrose (3%) were cultured on MS medium with 1/3 MS macro- and micronutrients (1/3 MS) supplemented with sucrose (3%) and 0.05 mg/L IBA. Some SBs were placed individually in test tubes containing 25 ml agar-solidified 1/3 MS medium with IBA directly after encapsulation, without prior storage. In other treatments, synthetic seeds with SBs were transferred to Petri dishes and sealed with Parafilm to study the effect of storage on plantlet development. The Petri dishes, each containing 8–10 synthetic seeds, were refrigerated at 4°C in darkness for 4, 6 or 14 weeks. After each storage period, encapsulated SBs were individually transferred to tubes containing agar-solidified 1/3 MS medium with IBA. The medium was chosen based on our earlier studies on plantlet conversion by nontransformed shoot buds of *C. erythraea* encapsulated in 3% sodium alginate (data not published). For non-stored and stored synthetic seeds we determined the rate of conversion to plantlets (%), mean number of roots per shoot and root length (mm) after 6 weeks of culture. Conversion of encapsulated shoot buds to plantlets is expressed as the percentage of encapsulated shoot buds developing both shoots and roots.

STATISTICAL ANALYSIS

All the experiments were conducted in three replicates of 18–43 synthetic seeds per treatment. The presented data are means \pm SE. The significance of differences was checked with the Mann-Whitney U-Test at $P \leq 0.05$ using STATISTICA ver. 10 (STATSoft).

TRANSFER OF PLANTLETS TO SOIL

Plantlets (4–6 weeks old) with well developed roots (derived from encapsulated SBs and HRs) were washed in water to remove agar from the roots and transferred to pots (\varnothing 10cm) containing a sterile mixture of soil, sand and peat (4:3:3, v/v/v). The plantlets were covered with glasses to ensure high humidity. The covers were gradually opened during the acclimatization period and removed completely after 14 days. The potted plantlets were maintained inside a growth chamber at $26 \pm 2^\circ\text{C}$ under cool white fluorescent lamps ($40 \mu\text{M}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ flux) with a

16 h photoperiod for 4 weeks. After this time the pots with acclimatized plants were placed in a greenhouse and the survival rate was recorded 4 weeks later.

RESULTS

In this work we made synthetic seeds from transformed tissues of *C. erythraea* and evaluated their ability to form plantlets. The transformed tissues were hairy root segments (HRs) and also adventitious shoot buds excised from shoots regenerated from hairy roots.

The explants were encapsulated in 3% sodium alginate with 3% sucrose (Fig. 1a,b). Our preliminary experiments had shown that no encapsulated transformed tissues of *C. erythraea* survived when sucrose was omitted from the encapsulation procedure (data not published).

PLANTLET REGENERATION FROM ENCAPSULATED HAIRY ROOT SEGMENTS

HRs after encapsulation were transferred to agar-solidified 1/2 MS or WPM regeneration media supplemented with BAP (0.05, 0.1, 0.5 mg/L) or without the cytokinin. On media without growth regulators, explants protruded from the gel matrix but did not show any shoot formation. Regeneration of adventitious shoots was observed on media supplemented with BAP. Small calluses appeared on the root surface within two weeks of culture. Shoots developed on the callus surface in week 3 of culture and the process continued up to 5 weeks, but root development from these shoots was not observed (Fig. 1c). As shown in Figure 2, the BAP concentration affected both the shoot formation frequency and the number of shoots formed per encapsulated HR. The response after 5 weeks was highest on WPM medium supplemented with 0.5 mg/L BAP: 86% of synthetic seeds developing shoots, with a multiplication rate of 15 shoots per root segment. In light of this result we used that medium for subsequent experiments with stored encapsulated *C. erythraea* HRs. After encapsulation in the gel matrix composed of either distilled water with 3% sucrose or regenerating medium additives (WPM nutrients, 3% sucrose and 0.5 mg/L BAP), the HRs were stored at 4°C for 4, 6 or 14 weeks. After the storage period, synthetic seeds were transferred to agar-solidified WPM regeneration medium with 3% sucrose and 0.5 mg/L BAP and cultured for 5 weeks. We found that shoot regeneration from encapsulated root segments was lower after storage. This was evident after 4 weeks of storage in HRs encapsulated with water and sucrose in the alginate matrix (Tab. 1). After 6 weeks of storage 77% of the synthetic seeds developed

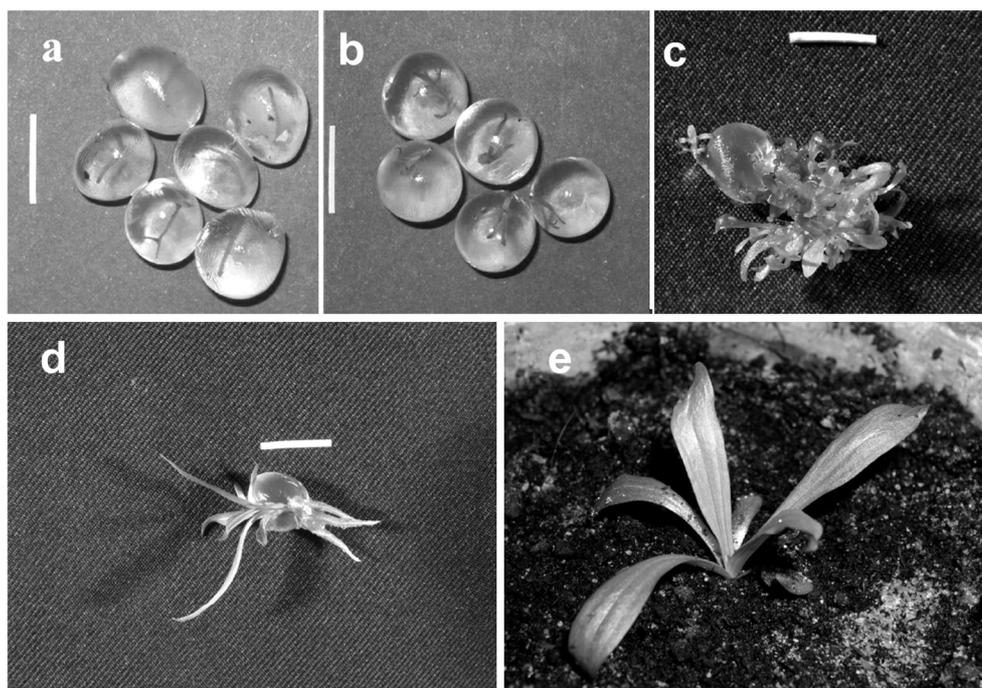


Fig. 1. Encapsulation of *C. erythraea* transformed tissues. (a) Synthetic seeds produced by alginate encapsulation of hairy root segments, (b) Shoot buds, (c) Multiple shoots regenerated from an encapsulated hairy root fragment after 5 weeks of culture on agar WPM medium supplemented with BAP (0.5 mg/L), (d) Conversion of an encapsulated transformed shoot bud into a plantlet after 6 weeks of growth on agar-solidified 1/3 MS medium with IBA (0.05 mg/L), (e) Acclimatized transformed plant 8 weeks after transfer to soil. Bars = 1 cm.

shoots, with a mean proliferation rate of 10 shoots per root segment, lower than for non-stored encapsulated HRs (86% regeneration frequency, mean 15 shoots per root fragment). All synthetic seeds lost viability after 14 weeks of storage; the explants browned and died. In the synthetic seeds encapsulated with WPM and 0.5 mg/L BAP in the alginate matrix, shoot regeneration after storage was significantly higher: after 6 weeks of storage all synthetic seeds responded, with 10.5 shoots per root segment on average (Tab. 1), and even after 14 weeks of storage, 68% of the seeds regenerated shoots (Tab. 1). Irrespective of the alginate bead composition, however, the mean number of shoots produced by non-stored encapsulated HRs was higher than from stored encapsulated HRs (Tab. 1).

The shoots regenerated from non-stored and stored hairy roots were rooted on 1/2 MS agar medium without growth regulators (Tab. 2). After 4 weeks almost all (97%) of the shoots regenerated from non-stored synthetic seeds formed 4 roots per shoot on average (Tab. 2). Mean root length was 13 mm. For stored synthetic seeds the shoot rooting frequency depended on the duration of storage. All shoots regenerated from encapsulated HRs stored for 4 weeks formed roots after 4 weeks of culture on 1/2 MS medium. Rooting was lower for HRs stored longer: 75% for those stored 6 weeks and 80% for

those stored 14 weeks. Also their roots were shorter than those from non-stored seeds. The number of roots per shoot was not affected significantly by storage for up to 6 weeks and declined slightly after 14 weeks of storage (Tab. 2).

PLANTLET DEVELOPMENT FROM ENCAPSULATED TRANSFORMED SHOOT BUDS (SBs)

In subsequent experiments, encapsulated transformed SBs of *C. erythraea* were used for plantlet formation. Explants encapsulated in 3% sodium alginate dissolved in water with 3% sucrose or in 1/3 MS salts, 3% sucrose and 0.05 mg/L IBA were incubated on 1/3 MS agar medium containing 0.05 mg/L IBA directly after encapsulation or after storage for 4, 6 or 14 weeks at 4°C. At week 6th of culture, shoot growth and root formation were observed (Fig. 1d). Thus, in vitro root induction was not needed prior to acclimatization. As shown in Table 3, 32% of the non-stored encapsulated SBs developed into plantlets. Similarly, 35% of encapsulated SBs stored for 4 weeks developed plantlets. Longer storage clearly affected the regeneration frequency (Tab. 3), which declined to 25% after 6 weeks of storage. The mean number of roots per shoot also decreased with storage time. After 14 weeks of storage all the encapsulated shoot buds browned and could not

TABLE 1. Effect of alginate matrix composition and storage period on shoot development from encapsulated *C. erythraea* hairy roots cultivated on WPM agar medium with BAP (0.5 mg/L) and 3% sucrose for 5 weeks

Composition of alginate matrix	Time of cold-storage (weeks)	% of roots forming shoots	Number of shoots per encapsulated root fragment
3% SA + %S*	0	86.4 ^a	15.2 ± 1.83 ^a
3% SA + M**		100.0 ^b	18.4 ± 3.29 ^b
3% SA + 3% S	4	85.3 ^a	11.1 ± 1.85 ^c
3% SA + M		96.7 ^b	8.9 ± 1.69 ^d
3% SA + 3% S	6	77.1 ^c	9.8 ± 1.03 ^c
3% SA + M		100.0 ^b	10.5 ± 1.42 ^c
3% SA + 3% S	14	0.0 ^d	0.0
3% SA + M		67.7 ^e	8.5 ± 1.92 ^d

*3% SA + 3% S – 3% sodium alginate + 3% sucrose

**3% SA + M – 3% sodium alginate + 3% sucrose + WPM medium with BAP (0.5 mg/L)

Values bearing the same letter within column do not differ significantly at P≤0.05 by the Mann-Whitney U-Test. Data are means ± SE of 3 replicated experiments.

TABLE 2. Rooting of *C. erythraea* shoots regenerated from non-stored and stored encapsulated hairy root fragments. Shoots were rooted on solid 1/2 MS medium without growth regulators for 4 weeks

Storage period (weeks)*	% of shoots forming roots	Average number of roots/shoot	Average root length (mm)
0	97.1 ^a	3.6 ± 0.30 ^a	13.1 ± 1.12 ^a
4	100.0 ^a	2.9 ± 0.33 ^a	6.4 ± 0.73 ^b
6	75.0 ^b	3.2 ± 0.39 ^a	9.0 ± 0.95 ^c
14	80.0 ^b	2.0 ± 0.24 ^b	6.5 ± 1.10 ^b

Data are the means ± SE of 3 replicated experiments

*time of cold storage of encapsulated hairy root segments. Alginate matrix contained WPM medium with 3% sucrose and 0.05 mg/L BAP
 Values bearing the same letters within column do not differ significantly at P≤0.05 by the Mann-Whitney U-Test.

form plantlets. As with encapsulated HRs, the rate of conversion of encapsulated SBs to plantlets increased when the nutrients and growth regulators of regeneration medium (1/3 MS with 3% sucrose and 0.05 mg/L IBA) were incorporated into the alginate matrix: the frequency of plantlet formation from 6-week stored synthetic seeds more than doubled from 25% (seeds encapsulated in alginate dissolved in water with 3% sucrose) to 56%, and the number of roots per shoot and root length also increased (Tab. 3).

ACCLIMATIZATION OF PLANTS IN SOIL

Well-rooted plantlets (4–6 weeks old) raised from encapsulated transformed root segments and shoot buds were transferred from aseptic culture to a sterilized mixture of soil, sand and peat (4:3:3 v/v/v) and were grown in the greenhouse (Fig. 1e). Humidity

around the plantlets was controlled at the initial stage for ~2 weeks. Ninety percent of the plants survived 8 weeks of growth in that substrate. The transformed plants did not show any evident morphological differences from the respective donor plants but differed from non-transformed *C. erythraea* plants as previously described by Piątczak et al. (2006).

DISCUSSION

In this study we used hairy root segments and adventitious shoot buds from hairy roots to obtain transformed *C. erythraea* plants from synthetic seeds. We found hairy root segments to be the most suitable material for this purpose: a high percentage of the explants encapsulated in 3% sodium alginate responded, yielding a high number of shoots per root segment, and root length of ~0.5 cm. High-density culture of *C. erythraea* hairy roots is feasible (Piątczak et al., 2006), producing a large number of explants from culture. The procedure for obtaining complete plantlets from encapsulated *C. erythraea* HRs goes in two stages: shoot proliferation on agar WPM in the presence of BAP as cytokinin (optimum BAP concentration is 0.5 mg/L); and rooting of shoots on 1/2 MS medium without growth regulators.

It takes 9 weeks from fragmentation and encapsulation of hairy roots to transplantation of plantlets to soil. In stage 1, lasting 5 weeks, ~260 shoots can be produced from a hairy root segment 10 cm long, calculated as follows: (20 synthetic seeds each containing a root segment 0.5 cm long) × (86% shoot regeneration) × (15 shoots per root segment). In stage 2, lasting 4 weeks, 250 plants can be obtained: (260 shoots) × (97% rooting). Optimizing the gel matrix composition (WPM with 3% sucrose and 0.5 mg/L BAP) can improve the outcome further.

TABLE 3. Effect of alginate matrix composition and storage period on plantlet development from encapsulated transformed *C. erythraea* shoot buds cultivated on 1/3 MS agar medium with IBA (0.05 mg/L) for 6 weeks after storage

Composition of alginate matrix	Time of cold-storage (weeks)	Conversion to plantlets (%)	Number of roots per shoot	Length of roots (mm)
3% SA + 3% S*	0	32.26 ^a	5.45 ± 0.9 ^{ac}	9.51 ± 1.0 ^a
3% SA + M**		56.67 ^b	6.08 ± 1.2 ^c	12.40 ± 2.6 ^b
3% SA + 3% S	4	34.88 ^a	4.94 ± 0.6 ^a	7.39 ± 1.1 ^c
3% SA + M		44.44 ^d	5.91 ± 1.4 ^c	8.73 ± 1.8 ^a
3% SA + 3% S	6	25.00 ^c	3.15 ± 1.0 ^b	5.66 ± 0.9 ^d
3% SA + M		55.55 ^b	4.4 ± 0.8 ^a	13.58 ± 1.9 ^b
3% SA + 3% S	14	0.00 ^e	0.00 ^d	0.00 ^e
3% SA + M		40.00 ^d	3.7 ± 0.5 ^b	10.13 ± 2.1 ^a

*3% SA + 3% S – 3% sodium alginate + 3% sucrose

**3% SA + M – 3% sodium alginate + 3% sucrose + 1/3 MS medium with IBA (0.05 mg/L)

Values bearing the same letter within column do not differ significantly at $P \leq 0.05$ by the Mann-Whitney U-Test. Data are means ± SE of 3 replicated experiments.

Complete plantlets have been obtained in vitro from encapsulated hairy roots only in *Armoracia rusticana*, but in that work the encapsulated hairy roots contained previously regenerated shoot primordia (Phunchindawan et al., 1997). Nakashimada et al. (1995) also obtained complete plantlets of this plant species after encapsulation of hairy roots containing earlier-regenerated plantlets at different stages of development. Repunte et al. (1995) reported regeneration of *Armoracia rusticana* plantlets from cell aggregates derived from hairy roots encapsulated in 1% sodium alginate, MS medium and 2% sucrose, cultured under light.

Encapsulated *C. erythraea* shoot buds were less suitable for transgenic plantlet formation than root segments. After 6 weeks, 20 synthetic seeds containing single shoot buds produced 7–11 plantlets, depending on the alginate bead composition. However, it only required a one-stage procedure to obtain healthy plants from those synthetic seeds. This made the procedure less time- and labor-consuming.

Besides explant types, factors such as the duration of storage at 4°C and the alginate matrix composition affected the ability of synthetic seeds to convert to transformed *C. erythraea* plantlets. The effects of storage period were similar in both types of explants. In most cases, storage of synthetic seeds significantly reduced the frequency and number of shoots regenerated from responsive hairy root segments, as well as conversion of shoot buds to plantlets. The reduction may be related to oxygen deficiency in the gel capsule and its rapid drying during storage (Redenbaugh et al., 1991; Rai et al., 2009). The decline in viability of synthetic seeds during storage was much lower when the capsule matrix included nutrient medium, 3% sucrose and growth regulator: HR synthetic seeds showed high shoot regeneration (68%), and SB synthetic seeds

showed good plantlet conversion (40%) even after 14-week storage period. They remained viable longer than encapsulated nontransformed *C. erythraea* shoot buds (7% after 14 weeks; unpublished data), and also longer than other plant species such as *Morus* spp. (8–22% after 2–3 months; Pattnaik et al., 1995), *Punica granatum* (12–16% after 45 days; Naik and Chand, 2006), *Withania somnifera* (35% after 2 months; Singh et al., 2006) and *Psidium guajava* (0% after 45 days; Rai et al., 2008). None of the encapsulated *C. erythraea* HRs or SBs survived after 14 weeks of storage when only water with 3% sucrose was used as the solvent in sodium alginate beads. Many authors have demonstrated the advantages of adding nutrients to the capsules to improve survival of encapsulated buds or somatic embryos in storage. In *Camelia* spp., for example, Ballester et al. (1997) reported that adding BAP and IAA to alginate beads increased the survival rate (percentage of cultures capable of shoot proliferation) as well as the number of new shoots from encapsulated shoot tips. Adding BAP to the alginate matrix enhanced shoot development from encapsulated nodal cuttings of *Manihot esculenta* (Danso and Ford-Lloyd, 2003), and adding the auxin α -naphthalenacetic acid (NAA) to alginate beads increased conversion of encapsulated *Punica granatum* nodal segments to plantlets (Naik and Chand, 2006). According to Arun Kumar et al. (2005) the alginate matrix serves as artificial endosperm and should provide nutrients for the "germination" and further growth of encapsulated explants.

In our study there were no evident differences in the number of regenerated shoots per hairy root segment between the various storage periods and capsule formulations. The values ranged from 8.5 to 11 and were always significantly lower than for non-stored synthetic seeds (15–18 shoots per root segment). Lower developmental parameters were noted

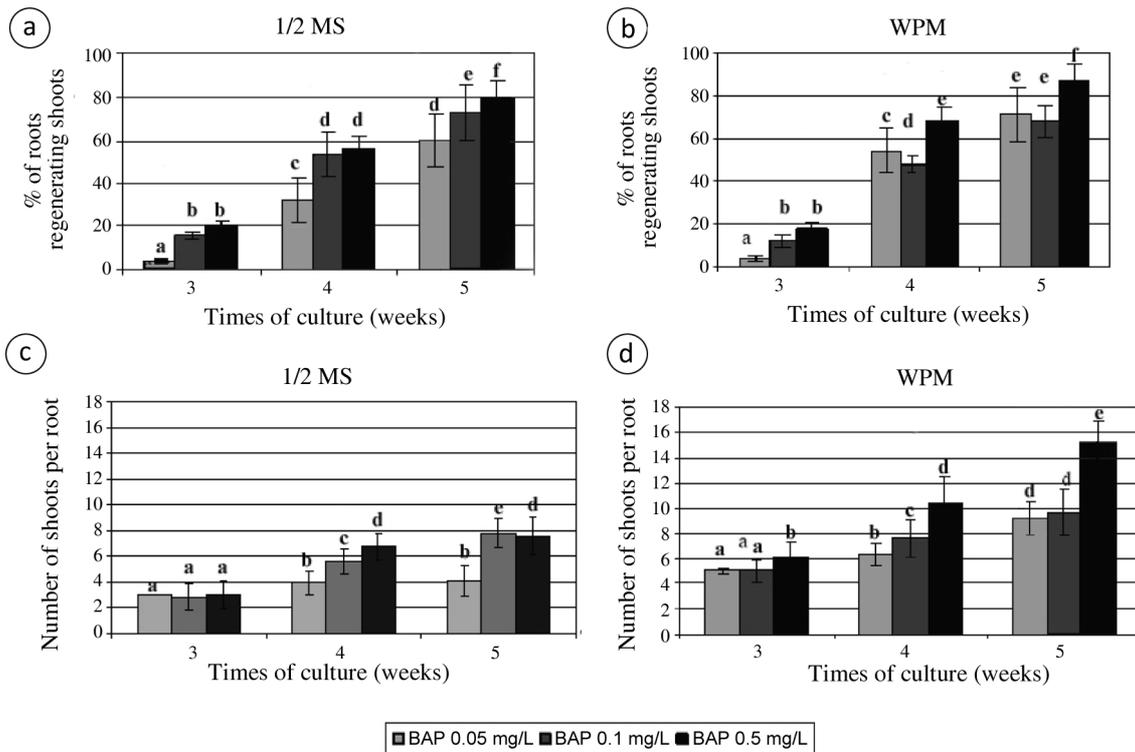


Fig. 2. Effect of BAP concentration (0.05, 0.1, 0.5 mg/L) in regeneration media on shoot development from encapsulated *C. erythraea* hairy root segments during three subsequent weeks of culture: percentage of encapsulated hairy roots regenerating shoots on 1/2 MS agar medium (a) and WPM (b), mean number of shoots per encapsulated root on 1/2 MS agar medium (c) and WPM (d). The alginate bead coat contained 3% sodium alginate with 3% sucrose. Bars indicate standard error (SE) of the mean of three replicated experiments. Means bearing the same letters within each diagram do not differ significantly at $P < 0.05$ by the Mann-Whitney U-Test.

by Thiruvengadam et al. (2012) for encapsulated *Momordica dioica* shoot tips after storage at 4°C, and Verma et al. (2010) showed the same for *Solanum nigrum* shoot tips. They suggested that storage has a negative effect on shoot meristem development. Earlier, Janeiro et al. (1997) observed that 25% of encapsulated somatic embryos of *Camelia japonica* showed only radicle elongation after 60 days of cold storage at 4°C.

Here we described a simple, reproducible and efficient procedure for production of *C. erythraea* synthetic seeds containing transformed tissues – hairy root fragments and shoot buds. The procedure can be useful for multiplication and propagation of Ri-transformed regenerants of *C. erythraea*. The synthetic seeds provide an effective vehicle for storage and exchange of hairy root lines.

REFERENCES

ARUN KUMAR MB, VAKESWARAN V, and KRISNASAMY V. 2005. Enhancement of synthetic seed conversion to seedlings

in hybrid rice. *Plant Cell Tissue and Organ Culture* 81: 97–100.

BALLESTER A, JANEIRO LV, and VIEITEZ AM. 1997. Cold storage of shoot cultures and alginate encapsulation of shoot tips of *Camelia japonica* L. and *Camelia reticulata* Lindley. *Scientia Horticulturae* 61: 67–78.

DANSO KE, and FORD-LLOYD BV. 2003. Encapsulation of nodal cuttings and shoot tips for storage and exchange of cassava germplasm. *Plant Cell Reports* 21: 718–725.

GONGOPADHYAY M, DEWANJEE S, CHAKRABORTY D, and BHATTACHARYA S. 2011. Role of exogenous phytohormones on growth and plumbagin accumulation in *Plumbago indica* hairy roots and conservation of elite root clones via synthetic seeds. *Industrial Crops and Products* 33: 445–450.

GRZEGORCZYK I, and WYSOKINSKA H. 2011. A protocol for synthetic seeds from *Salvia officinalis* L. shoot tips. *Acta Biologica Cracoviensia, Series Botanica* 53: 80–85.

GUILLOIN S, TREMOUILLAUX-GUILLER J, PATI PK, RIDEAU M, and GANTET P. 2006. Hairy root research: recent scenario and exciting prospects. *Current Opinion in Plant Biology* 9: 341–346.

JANEIRO LV, BALLESTER A, and VIEITEZ AM. 1997. in vitro response of encapsulated somatic embryos of camellia. *Plant Cell Tissue and Organ Culture* 51: 119–125.

- KIKOWSKA M, and THIEM B. 2011. Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review. *Herba Polonica* 57(4): 45–57.
- KUMARASAMY Y, NAHAR L, COX PJ, JASPARS M, and SARKER SD. 2003. Bioactivity of secoiridoid glycosides from *Centaurium erythraea*. *Phytomedicine* 10: 344–347.
- LLOYD G, and MCCOWN B. 1980. Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot-tip culture. *The International Plant Propagators Society* 30: 421–427.
- MANDAL J, PATNAIK S, and CHAND PK. 2000. Alginate encapsulation of axillary buds of *Ocimum americanum* L. (hoary basil), *O. basilicum* L. (Sweet basil), *O. gratissimum* L. (shrubby basil), and *O. sanctum* (sacred basil). *In Vitro Cellular and Developmental Biology – Plant* 36: 287–292.
- MURASHIGE T, and SKOOG F. 1962. A revised medium for rapid growth and bioassay with tobacco culture. *Physiologia Plantarum* 15: 473–497.
- NAIK SK, and CHAND PK. 2006. Nutrient-alginate encapsulation of in vitro nodal segments of pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. *Scientia Horticulturae* 108: 247–252.
- NAKASHIMADA Y, UOZUMI N, and KOBAYASHI T. 1995. Production of plantlets for use as artificial seeds from horseradish hairy roots fragments in a blender. *Journal of Fermentation and Bioengineering* 79: 458–464.
- NAKASHIMADA Y, UOZUMI N, and KOBAYASHI T. 1996. Efficient culture method for production of plantlets from mechanically cut horseradish hairy roots. *Journal of Fermentation and Bioengineering* 81: 87–89.
- PATNAIK SK, SAHOO Y, and CHAND PK. 1995. Efficient plant retrieval from alginate-encapsulated vegetative buds of mulberry trees. *Scientia Horticulturae* 61: 227–239.
- PHUNCHINDAWAN M, HIRATA K, SAKAI A, and MIYAMOTO K. 1997. Cryopreservation of encapsulated shoot primordial induced in horseradish (*Armoracia rusticana*) hairy root cultures. *Plant Cell Reports* 16: 469–473.
- PIĄTCZAK E, WIELANEK M, KRÓLICKA A, ŁOJKOWSKA E, and WYSOKIŃSKA H. 2004. Hairy root cultures *Centaurium erythraea* Rafn). *Biotechnologia* 2: 29–36. [In Polish].
- PIĄTCZAK E, KRÓLICKA A, and WYSOKIŃSKA H. 2006. Genetic transformation of *Centaurium erythraea* Rafn. by *Agrobacterium rhizogenes* and the production of secoiridoids. *Plant Cell Reports* 25: 1308 – 1315.
- PIETROSIUK A, FURMANOWA M, and LATA B. 2007. *Catharanthus roseus*: Micropropagation and in vitro techniques. *Phytochemistry Reviews* 6: 459–473.
- RAI MK, JAISWAL VS, and JAISWAL U. 2008. Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. *Scientia Horticulturae* 118: 33–38.
- RAI MK, ASTHANA P, SINGH SK, JAISWAL VS, and JAISWAL U. 2009. The encapsulation technology in fruit plants – A review. *Biotechnology Advances* 27: 671–679.
- REDENBAUGH K, FUJI JA, SLADE D, VISS PR, and KOSSLER ME. 1991. Artificial seeds – encapsulated embryos. In: Bajaj YPS [ed.], *Biotechnology in Agriculture and Forestry*, vol. 17. *High-Tech and Micropropagation III*, 395–416. Springer-Verlag Berlin, Heidelberg.
- REPUNTE VP, TAYA M, and TONE S. 1995. Preparation of artificial seeds using cell aggregates from horseradish hairy roots encapsulated in alginate gel with parafilm coat. *Journal of Fermentation and Bioengineering* 79: 83–89.
- SINGH AK, VARSHNEY R, SHARMA M, AGARWAL SS, and BENSAL KC. 2006. Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *Journal of Plant Physiology* 163: 220–223.
- THIRUVENGADAM M, PRAVEEN N, and CHUNG IM. 2012. Plant regeneration from alginate-encapsulated shoot tips of *Momordica dioica* for short term storage and germplasm exchange and distribution. *Plant Omics Journal* 5: 266–270.
- UOZUMI N, and KOBAYASHI T. 1997. Artificial seeds – production through hairy root regeneration. In: Doran P [ed.], *Hairy Roots. Culture and Applications*, 113–121. Harwood Academic Publisher.
- VÁGNEROVA H. 1992. Micropropagation of common centaury (*Centaurium erythraea* Rafn). In: Bajaj YPS [ed.], *Biotechnology in Agriculture and Forestry*, vol. 19. *High-Tech and Micropropagation III*, 388–398. Springer-Verlag Berlin, Heidelberg.
- VERMA SK, RAI MK, ASTHANA P, JAISWAL VS, and JAISWAL U. 2010. In vitro plantlets from alginate-encapsulated shoot tips of *Solanum nigrum* L. *Scientia Horticulturae* 124: 517–521.
- WEST TP, RAVINDRA MB, and PREECE JE. 2006. Encapsulation, cold storage, and growth of *Hibiscus moscheutos* nodal segments. *Plant Cell Tissue and Organ Culture* 87: 223–23.
- ZYCH M, FURMANOWA M, KRAJEWSKA-PATAN A, ŁOWICKA A, DREGER M, and MENDLEWSKA S. 2005. Micropropagation of *Rhodiola kirilowii* plants using encapsulated axillary buds and callus. *Acta Biologica Cracoviensia, Series Botanica* 47: 83–87.