Germination of Encapsulated Somatic Embryos of *Cyclamen persicum*

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Abstract. Somatic embryos of cyclamen [*Cyclamen persicum* Mill.] were produced using a liquid culture system. Two encapsulation techniques, conventional alginate beads and alginate hollow beads, were tested for globular cyclamen somatic embryos with the aim of developing synthetic seeds. Final germination from alginate beads was as high as observed for non encapsulated control embryos (97 %), but germination was delayed. In contrast, germination from hollow beads was lower (71 %) and occurred later. In hollow beads somatic embryos developed within the capsule, and outgrowth seemed to be more difficult than from alginate. Storage at 4 °C for four weeks resulted in a reduction of viability for controls as well as for encapsulated embryos. Incorporation of medium into the capsules improved the speed of germination for both capsule types. However, somatic embryos were not able to germinate on a medium-free support, even if encapsulated in beads containing medium.

Cyclamen (*Cyclamen persicum* Mill.) is conventionally propagated from seeds. However, this generative propagation method is connected to problems due to inbreeding depressions, inhomogeneity in some cultivars, and manual labor for seed production (emasculatation and pollination). This results in high prices for seeds, given as 0.05/seed in open-pollinated cultivars and up to 0.20/seed in case of F₁-hybrids (Schwenkel, 2001). The economic importance of cyclamen as ornamental potted plants becomes obvious from the at least ≈140 million plants being produced worldwide per year (Bongartz, 1999).

It has been shown that in vitro somatic embryogenesis can be applied successfully for the vegetative propagation of cyclamen (Schwenkel and Winkelmann, 1998; Winkelmann et al., 1998, 2000). Genetic analyses revealed that the ability for regeneration of somatic embryos was inherited dominantly by two major genes (Pueschel et al., 2003). Using liquid and bioreactor cultures proliferation of embryogenic suspensions and high propagation rates have been achieved (Hohe et al., 1999a, 1999b, 2001).

Somatic embryos differ from their zygotic counterparts in some aspects. Zygotic embryos are protected by a seed coat, they undergo desiccation resulting in reduced moisture content and they have access to storage products being accumulated in the embryo itself (albuminous species) or in the endosperm (nonalbuminous species). With the aim of producing synthetic seeds, research has to be done in the fields of developing desiccation treatments, accumulation of storage compounds either in the embryo

or applied as artificial endosperm, and in encapsulation methods.

The idea of encapsulating somatic embryos to create synthetic seeds and thus combining the advantages of vegetative propagation (uniformity) with those of seeds (storability, easy handling, use of sowing equipment, barrier in transmission of diseases) goes back to the vision of Murashige (1977) and Redenbaugh et al. (1986). Thereafter mainly in the model species like carrot and alfalfa in which highly efficient systems for somatic embryogenesis are existing research has been done aiming at artificial seeds (Fujii et al., 1989; Onishi et al., 1994). More recently reports on encapsulation of somatic embryos in other species have been published, like pelargonium (Gill et al., 1994), asparagus (Mamiya and Sakamoto, 2001), citrus (Nieves et al., 1998), and *Camellia japonica* (Janeiro et al., 1997).

The synthetic seed coat should not only protect the embryos physically, it also can carry nutrients and plant protection means and enables the handling as seeds using conventional planting equipment (Gray, 1989). Sodium alginate was most commonly used and dropped into calcium solution where polymerisation results in alginate beads. Kitto and Janick (1985) tested eight chemical compounds for being used as synthetic seedcoats from which Polyox WSR-N750 (polyethylene oxide homopolymer) turned out to be suitable due to its ability to form a film after drying, not to support growth of contaminants, to be readily dissolved in water after desiccation and to be nontoxic to the embryos. Pharmaceutical capsules containing medium were suggested as coating systems and were tested successfully with carrot somatic embryos (Dupuis et al., 1994), although the practical application of this system has not been reported so far. A new form of encapsulation for living material proposed by Patel et al. (2000) are alginate hollow beads with a liquid centre. These authors showed that callus, shoot tips and embryos of potato and carrot can be grown and germinated using this

type of capsules. Although the term “hollow” is not precisely chosen, since it is defined as being empty, in the present study it will be used in accordance to Patel et al. (2000).

The objectives of this study were 1) to compare two encapsulation methods, conventional alginate beads versus hollow beads (Patel et al., 2000), for cyclamen somatic embryos, 2) to analyse their effects on germination before and after storage at 4 °C, and 3) to test the influence of adding nutrients to the capsules on somatic embryo development on medium or medium-free supports.

Materials and methods

Somatic embryo production for encapsulation. Somatic embryos were produced in liquid cultures. Embryogenic callus was induced following the protocol of Schwenkel and Winkelmann (1998) from ovules of the genotype 3738-14 (a seedling of ‘Purple Flamed’). Suspension cultures were established and maintained according to Winkelmann et al. (1998). Fourteen days after subculture the size fraction 500 to 1000 µm was transferred to liquid hormone-free medium (Hohe et al., 2001) at a density of 1% PCV (packed cell volume). Somatic embryos differentiated within 2 to 3 weeks cultured at 120 rpm and 23 to 25 °C in darkness and were collected on a sieve of 500 µm. These embryos mainly at globular stage were used for the encapsulation experiments.

Encapsulation in conventional alginate beads. Somatic embryos were singly sucked up within 50 µL of a sodium alginate solution using a micropipette with a cut tip to provide a wider opening. Initial tests using sodium alginate concentrations of 1.0%, 1.5%, 2.0%, and 2.5% showed that 1.5% sodium alginate solution gave the best result. The 50-µL droplets each containing one selected somatic embryo were dropped into a calcium chloride solution (68 mM), which was stirred for 20 min to induce polymerisation and bead formation. After two washes in deionized water of 10 min each alginate beads were collected on a sieve (1000 µm) and used for storage or germination tests (see below). All solutions used for encapsulation were autoclaved (Patel et al., 2000).

Encapsulation in hollow beads. Somatic embryos were suspended in 1.5% carboxymethylcellulose solution (containing 1 g CaCl₂ × 2 H₂O in 100 mL) in a petri dish. Single embryos were taken up within 35 µL of this solution using a micropipette (as described for alginate beads above) and dropped into 0.8% sodium alginate solution under slow stirring. After 20 min the alginate solution was diluted by adding the double amount of water. After another 10 min the alginate solution was discarded and the beads were washed for two times in water (5 min each). Finally the surface of the beads was hardened by replacing the water with CaCl₂ (68 mM) (Patel et al. 2000). The further steps were same as for alginate beads.

Experimental setup. To test the two different encapsulation methods regarding development and germination of somatic embryos the first experiment was performed. Somatic embryos encapsulated into either conventional alginate

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beads or hollow beads were compared to control (nonencapsulated) embryos.

To test the storability of control and encapsulated somatic embryos they were kept for four weeks at 4 °C in sealed petri dishes on moist filter paper (under sterile conditions). The somatic embryos for these capsules as well as for controls were derived from the same liquid cultures that had been used for the first experiment.

Aim of the third experiment was to investigate the effect of incorporating nutrients into the capsules. For this purpose the solutions in which the embryos were sucked up for encapsulation were prepared with Ca-free hormone-free ½ MS medium instead of water. Alginate beads and hollow beads containing nutrients or not were then cultured on the normal cyclamen differentiation medium (½ MS with 30 g·L⁻¹

sucrose and 2 g·L⁻¹ glucose, Schwenkel and Winkelmann, 1998) or on water solidified with 8.0 g·L⁻¹ agar (SERVA 11396).

Ten embryos each either control or encapsulated were placed in 6-cm plastic petri dishes containing hormone-free differentiation medium (modified ½ MS medium as described by Schwenkel and Winkelmann, 1998) and cultivated at 20 °C in complete darkness. Development of somatic embryos and germination were evaluated in 4-week intervals. Somatic embryos were scored as germinated, when the cotyledon came out of the capsule, since in cyclamen incomplete germination resulting in formation of only roots and sometimes small tubers was observed. Each experiment was

repeated twice with three to six petri dishes per treatment.

Germination is presented in the figures in percentages and the means and standard deviations between the treatments were compared.

Results

Initial experiments revealed that alginate at a concentration of 1.5% and higher resulted in spherical and solid beads (Fig. 1B), while at 1.0% the shape of the capsules was irregular and handling with forceps not easy. Therefore, for all subsequent experiments alginate beads were prepared using 1.5% sodium alginate solution. During the preparation of hollow beads it was observed that in some experiments the beads were destroyed during the last steps of production making this method difficult for practical application. In these cases only half of the capsules could be included in the

Fig. 1. Encapsulated somatic embryos of cyclamen (C to H in 6-cm petri dishes). (A) Hollow beads directly after preparation (the bar = 1 cm). (B) Alginate beads directly after preparation bar = 1 cm). (C) Germinating control embryos (not encapsulated) after 2 months of culture. (D) Germination from in alginate beads after 2 months of culture. (E) Germination from hollow beads after 2 months of culture. (F) Germinating control embryos (not encapsulated) after 4 months of culture. (G) Germination from alginate beads after 4 months of culture. (H) Germination from hollow beads after 4 months of culture.

experiments. Since this problem could not be overcome, research in standardizing the structure and consistency of the beads seems to be necessary.

When the two types of capsules were compared, it was observed that somatic embryos were often not centrally placed in alginate beads (Fig. 1B), while in hollow beads they were totally covered (Fig. 1A). In hollow beads the formation of tubers, roots and cotyledons took place within the capsule in contrast to conventional alginate beads where somatic embryos expanded very early and soon grew out of the capsules (Fig. 1D and E). Germination was asynchronous in all variants. For control embryos the final germination of about 97% was reached late, after 24 weeks (Fig. 2). However, this high percentage points to the fact that somatic embryos of good quality have been produced in the liquid culture system (Fig. 1C and F). Somatic embryos encapsulated in conventional alginate beads were not negatively affected by the encapsulation, since they also reached final germination percentages of 97%, but their development was slower (Fig. 1D and G and Fig. 2). Often they formed bigger tubers with many shoot buds. In contrast, embryos in hollow beads did not reach final germination percentages as high as control or alginate encapsulated ones (Fig. 2). Their development was much slower and after the first increase in size many embryos turned brown and did not break through the capsules (Fig. 1E and H). In addition the variation between the two replications was pronounced in case of hollow beads.

To test the ability of somatic embryos to germinate after four weeks of storage at 4 °C the second experiment was performed. Four weeks after the embryos had been placed at 20 °C for germination first control embryos showed formation of cotyledons (Fig. 3). However, their development was slower than that of unstored embryos and only 68% were able to germinate. Again alginate beads lead to similar final germination percentages as compared to controls, but these were reached with a delay (Fig. 3). In contrast, somatic embryos in hollow beads only expressed very poor germination after cool storage.

To test the effect of additional nutrients and carbohydrates within the capsules liquid hormone-free medium has been integrated into the capsules. For both, alginate beads as well as hollow beads, the medium capsules resulted in better and earlier germination when compared to capsules without medium. This becomes obvious from the steeper rise of the curves and the higher final germination percentages observed for the medium-containing beads in Fig. 4.

The final aim for application of synthetic seeds is the direct sowing into soil. Therefore we tried to germinate encapsulated embryos on water agar (0.8% agar dissolved in water) containing neither nutrients nor carbohydrates. The control embryos did not germinate without medium neither did somatic embryos encapsulated without medium. Also embryos in capsules prepared with medium did not show any development, but turned brown and died indicating that the small amount of nutrients

within 50 or 35 µL of medium (see materials and methods) did not support growth.

Discussion

In this study it was shown, that somatic embryos of cyclamen can be encapsulated in alginate beads without losing germination ability. Only a minor delay in germination was observed probably due to the mechanical barriers. Likewise in some other plant species alginate has proven to be suitable for encapsulating somatic embryos leading to germination similar to that of control embryos, e.g., in *pelargonium* (Gill et al., 1994) or *Camellia japonica* (Janeiro et al., 1997). Castillo et al. (1997) reported on pronounced differences between two types of sodium alginate derived from different manufacturers and demonstrated that for *Carica papaya* alginate beads the time of hardening in CaCl_2 was important as well. The composition of alginate can vary and especially the ratio of guluronic and mannuronic acids (Redenbaugh et al., 1993) is important for the applicability in plant cell culture. Since alginate is a product of natural origin, impurities having phytotoxic effects can not be ruled out.

There are only few reports available dealing with alternative coating materials. One recent comprised hollow beads for encapsulation of plant cell material and embryos (Patel et al., 2000). In our studies one advantage of hollow beads was the complete protection of the embryos that were more centrally placed in the capsules. The first steps of development took place within the capsules, which, with regard to the final aim of synthetic seeds, is positive as well. But since some embryos turned brown and were not able to break through the outer capsule wall, this type of encapsulation has to be improved for cyclamen. In the study of Patel et al. (2000) carrot seeds germinated at 100% within the hollow beads, which was

better than control seeds, but only 13% were able to grow out of the capsules.

Storage of cyclamen somatic embryos at 4 °C resulted in a decrease in the germination rates for both control and encapsulated ones (Fig. 3). The cool temperatures may be one reason for this, the lack of nutrients during storage could be another. However, in other species somatic embryos encapsulated in alginate beads were able to tolerate storage at low temperatures better than controls. In *Carica papaya* capsules were stored at 10 °C for 85 days and some embryos survived, while naked somatic embryos did not (Castillo et al., 1997). Similar observations were made for *Camellia japonica* where storage at 4 °C reduced the germination frequency stronger in nonencapsulated somatic embryos (Janeiro et al., 1997). Synthetic seeds of *Clitoria ternatea* could be stored at 2 °C for 30 d without reduction in viability, whereas control embryos only showed 4% viability after cool storage (Malabadi and Nataraja, 2002). One of the main problems in storing alginate capsules in the hydrated form is the poor respiration of encapsulated tissues (Redenbaugh et al., 1993). Therefore storage of alginate encapsulated somatic embryos in a dried form has been suggested and was carried out successfully for carrot (Liu et al., 1992). Timbert et al. (1996) tested different additives to alginate, which resulted in higher water activities in the synthetic seeds during dehydration. Addition of kaolin or gellan gum lead to slower dehydration being beneficial for the somatic embryos. With regard to the rehydration process the authors stress that rehydration has to be slow, but sufficient to promote regrowth. In case of cyclamen first results on desiccation of naked somatic embryos are promising (Winkelmann et al., 2003), so that a combination of encapsulation and dehydration should be tested in this species as well.

The use of alginate and carboxymethylcellulose dissolved in nutrient medium resulted

Fig. 2. Germination of control (not encapsulated) somatic embryos and embryos in conventional alginate beads and in hollow beads (average and standard deviation of two experiments).

Fig. 3. Germination of control (not encapsulated) somatic embryos and embryos in conventional alginate beads and in hollow beads after 4 weeks storage at 4 °C (average and standard deviation of two experiments).

Fig. 4. Germination of somatic embryos in different capsule types prepared with (+) or without (–) medium in comparison to controls (all variants were cultured on hormone-free medium for germination).

in better germination (Fig. 4). On the other hand this integration of medium into the capsules was not sufficient to promote growth on water agar. Somatic embryos of tangerine placed on medium free supports also did not germinate, even if they had been encapsulated with medium (Nieves et al., 1998). The fact that embryos encapsulated without medium but cultured on medium did germinate shows that diffusion of nutrients through the capsules takes place. Therefore it could also be possible that nutrients are lost from the capsules with medium by diffusion into the water agar.

If the technique of synthetic seeds is going to be integrated in horticultural practice the encapsulated embryos should be capable to

germinate in the soil. Two strategies can be applied to reach this aim. On the one hand a kind of artificial endosperm can be developed which supports germination and is protected against leaching. Possible supplements for an artificial endosperm could be sucrose in form of microcapsules (Mamiya and Sakamoto 2001; Onishi et al., 1994) or amino acids (Nieves et al., 1998). On the other hand culture conditions can be modified so that encapsulatable units are produced, which are able to germinate without external supply of carbohydrates and nutrients. The concept of developing encapsulatable units has been applied for celery and carrot (Onishi et al., 1994) and for asparagus (Mamiya and Sakamoto, 2001).

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