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Germination of encapsulated embryos of interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.)

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ABSTRACT

Interior spruce (*Picea glauca engelmannii* complex) and black spruce (*Picea mariana* Mill.) cotyledonary somatic embryos were encapsulated in sodium alginate. Somatic embryo viability was retained, but germination occurred at a reduced frequency compared with the equivalent zygotic embryos. The addition of 0.5% (w/v) activated charcoal to the alginate capsule significantly enhanced root development and germination for somatic embryos but not for zygotic embryos. The possibility of developing an artifical endosperm was also investigated, by addition of Litvay (Litvay et al. 1981) nutrients with or without 90 mM sucrose to the alginate-charcoal capsule. This treatment significantly enhanced root development for all embryo categories with the exception of black spruce somatic embryos. Encapsulated and non-encapsulated somatic embryos survived one month cold storage at 4 °C without reduction in germination frequency.

INTRODUCTION

Encapsulation of embryos with alginate, as reported by Redenbaugh *et al.* (1984, 1986, 1987a), might serve as a potential method for delivery of fragile conifer somatic embryos from tissue culture through mechanical handling to automated planting. Regeneration of plants from encapsulated embryos has been reported for several agronomic species e.g. *Solanum melongena* (Rao and Singh 1991), *Medicago sativa* (Zhong and Wang 1989), and *Hordeum vulgare* (Datta and Potrykus 1989). With woody tree species, the only example of plant recovery after somatic embryo encapsulation is for *Santalum album* (Bapat and Rao 1988, 1992). Plant recovery after somatic embryo encapsulation for conifer species has thus far not been reported (see Gupta and Durzan 1987; Durzan and Gupta 1988).

Alginate capsules have been used as artificial endosperms to deliver the nutrients necessary for embryo germination in agronomic species (Zhong and Wang 1989; Rao and Singh 1991). Selection of the proper nutrient composition in the alginate capsule might be used to obtain a reliable germination frequency in conifer species. In addition, the hydrated state of alginate capsules requires a storage method to retain embryo viability and capsule integrity. Cold storage (4 °C) was a method investigated by several authors (Bapat and Rao 1988; Datta and Potrykus 1989), but with variable success.

This paper examines the development of an encapsulation matrix for interior and black spruce somatic and zygotic embryos and reports on the germination of those embryos *in vitro*. The effect of 4 °C cold storage on encapsulated and non-encapsulated embryos was also examined.

MATERIALS AND METHODS

Source material

Interior spruce (*Picea glauca engelmannii* complex) seeds (Lot No. 1208-03-71-02) were obtained from the British Columbia Ministry of Forests and Lands, Surrey, B.C. Black spruce (*Picea mariana* Mill.) seeds (Lot No. 08582) were obtained from the Saskatchewan Department of Parks and Renewable Resources (Prince Albert, Sask.). Interior spruce is a naturally occurring hybrid between *Picea glauca* (Moench) Voss and *Picea engelmannii* Parry from the interior of British Columbia (Owens and Molder, 1984) where their ranges overlap. Seeds of each species were surface sterilized and zygotic embryos were aseptically removed following methods described by Tautorus *et al.* (1990). These embryos were used for comparative purposes during encapsulation experiments.

Immature somatic embryos (stage 1, von Arnold and Hakman 1988) of interior spruce (line IS-W70) and black spruce (line BS-D) were maintained in suspension cultures as previously described by Tautorus *et al.* (1992). Somatic embryo maturation followed the method of Dunstan *et al.* (1988, 1991), in which liquid suspension culture aliquots, containing immature somatic embryos, were inoculated onto filter discs (Millipore, AABG04750) placed over 25 ml autoclaved LP medium (von Arnold and Eriksson 1981) with 1 μM indole-butyric acid (IBA), 102 mM sucrose, 0.54% (w/v) agar (A7002, Sigma) (pH 5.8), contained in Petri dishes (100 x 15 mm). (±)-abscisic acid (ABA) (A1012, Sigma), was prepared as a 1 mM stock solution in acetone/water (1:9, v/v) and was added to the medium after filter-sterilization for a final concentration of 40 μM. Such filter disc cultures of interior and black spruces were incubated under a 16 h photoperiod with an incident photosynthetically active radiation (Krizek, 1982) of 3 μE m^{-2} s^{-1}, from 40-W cool white fluorescent lights, 24 ± 2 °C. After approximately 35-55 days, cream-coloured cotyledonary stage embryos (stage 3, von Arnold and Hakman 1988) had developed.

Encapsulation Procedure

Zygotic embryos, and cotyledonary stage 3 embryos collected from maturation plates, were placed into previously autoclaved 3% sodium alginate (medium viscosity) dissolved in water, based

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on the method of Slade *et al.* (1989). Single embryos in alginate were drawn up into Pasteur pipettes and added dropwise into 100 ml of 50 mM $CaCl_2 \cdot 2H_2O$, contained in 250 ml DeLong flasks. On the basis of preliminary results, these flasks were agitated on a gyrotary shaker at 75 rpm for either 20 min (BS-D) or 30 min (IS-W70). The calcium chloride solution was then decanted and the alginate capsules were rinsed twice with sterile distilled water, with the exception that capsules made with Litvay medium (Litvay *et al.*,1981), were rinsed twice with liquid Litvay medium lacking sucrose. Encapsulated embryos were then placed into Petri dishes (100 x 15 mm) containing phytohormone-free half-strength GMD medium (½GMD) (Mohammed *et al.* 1986) with 15 mM sucrose and 0.54% (w/v) agar, for germination and plant development. These cultures were incubated under a 16 h photoperiod of an incident 40-50 $\mu E\ m^{-2}\ s^{-1}$, from 40-W cool white fluorescent lights, at 24 ± 2 °C. After 8 weeks hypocotyl and root elongation were evaluated.

Experimental conditions
To the sodium alginate [alg] were added one of the following: 0.5% (w/v) activated charcoal (neutralized, Sigma No. C-3790) [designated: alg + ch]; 0.5% (w/v) activated charcoal and Litvay medium lacking sucrose (termed here: Litvay nutrients) [designated: alg + ch + LV]; or 0.5% activated charcoal and Litvay medium with 90 mM sucrose [designated: alg + ch + LV + suc]. For comparison, 0.5% activated charcoal was added directly to the ½GMD germination medium [designated: alg on ch]. Non-encapsulated somatic or zygotic embryos were placed directly onto ½GMD germination medium.

For cold storage experiments with each species, zygotic embryos, and cotyledonary stage 3 embryos from maturation plates, were either encapsulated or not encapsulated, and then stored for 1 month in darkness at 4 °C, on ½GMD medium in sealed Petri dishes. After storage, embryos were transferred to fresh ½GMD medium under the germination conditions described earlier.

Statistical analysis
All experiments were performed at least three times with a minimum of 10 replications (Petri dishes) per treatment (7 embryos per dish). The germination frequency (%) was based on radicle elongation greater than 2 mm. Treatments were arranged in a completely randomized design and analysis of variance was performed to determine significant differences among treatments. Separation of means was done by LSD test at the 5% level of significance. Statistical evaluations used Statgraphics, version 5.0 (Statistical Graphics Corp., Rockville, Md., USA). Data are presented as mean values with the standard error of each mean.

RESULTS AND DISCUSSION

Interior Spruce
Interior spruce somatic embryo encapsulation, and subsequent plant development is shown in Fig. 1-6.

Cultured zygotic embryos of interior spruce had a germination frequency of 97% when not encapsulated (Fig. 7). Encapsulation of zygotic embryos resulted in a reduction of the germination frequency, the amount of which depended on the treatment. For example, encapsulation in alginate when used alone resulted in a germination frequency of 27% (Fig. 7). The addition of charcoal to the capsule, or to the germination medium did not substantially improve this frequency (Fig. 7). The addition of Litvay nutrients to the alginate-charcoal capsule significantly improved the germination frequency to about 50% ($P<0.05$). The best combination for encapsulation of zygotic embryos, was when the alginate capsules contained charcoal, Litvay medium and 90 mM sucrose, resulting in a germination frequency of 66%.

Germination frequency of interior spruce cotyledonary somatic embryos when not encapsulated, was similar to zygotic embryos (Fig. 8). Encapsulation of the somatic embryos with alginate (Fig. 1-3) resulted in a significant reduction ($P<0.05$) in germination frequency (Fig. 8). In contrast to zygotic embryos, the germination frequency was significantly improved ($P<0.05$) to about 50% by the addition of charcoal to the capsule (Fig. 4), or to the germination medium. In the latter case there was a tendency for the somatic embryos to become vitrified however. The improved germination frequency could result from increased gas diffusion, supportive of respiration during encapsulation and germination (Redenbaugh *et al.* 1987b; Zhong and Wang 1989). The addition of Litvay nutrients to the alginate-charcoal capsule further enhanced the germination frequency, in comparison to encapsulation with charcoal alone. As with the encapsulated zygotic embryos, the addition of 90mM sucrose to the alginate-charcoal/Litvay capsule provided further improvement to 62% germination ($P<0.05$) (Fig. 8).

Black Spruce
Cultured zygotic embryos of black spruce showed a germination frequency of about 75% when not encapsulated (Fig. 9). As with interior spruce zygotic embryos, encapsulation with alginate reduced the germination frequency significantly (Fig. 9). The addition of charcoal to the capsule, or to the medium, failed to improve the germination frequency. However, addition of Litvay nutrients to the capsule significantly ($P<0.05$) enhanced germination. This was irrespective of whether the capsule also contained 90 mM sucrose or not, for which germination frequencies of 49% (with sucrose) and 55% (without sucrose) were obtained (Fig. 9).

Germination frequency of black spruce cotyledonary somatic embryos when not encapsulated, was about 88% (Fig. 10), which was significantly ($P<0.05$) better than the comparable zygotic embryos. Similarly to interior spruce somatic embryos, encapsulation with alginate alone reduced the germination frequency, whereas the addition of charcoal, and charcoal with Litvay nutrients, improved the germination frequency ($P<0.05$) (Fig. 10). When 90 mM sucrose was added to the alginate-charcoal/Litvay capsule there was a further slight improvement in germination frequency. The germination frequency of encapsulated BS embryos under the latter condition was about 40%, less than half that of the equivalent non-encapsulated somatic embryos (Fig. 10).

Cold Storage
After storage at 4 °C for one month, cultured zygotic embryos of interior spruce showed a slight reduction in germination frequency, when not encapsulated (compare Figs. 7,8 with 11). In contrast, zygotic embryos encapsulated in alginate with charcoal showed a higher germination frequency (50%), than the equivalent embryos that had not been cold stored (30%). Germination frequency of interior spruce cotyledonary somatic embryos when not encapsulated, was 100% after one month cold storage. The equivalent encapsulated embryos had a germination frequency of 52%, which is similar to the germination frequency for encapsulated somatic embryos that had not been cold-stored (compare Figs. 7,8 with 11).

Cultured black spruce zygotic embryos showed a sharp reduction in germination frequency after one month cold storage, whether encapsulated or not (compare Figs. 9,10 with 12). The

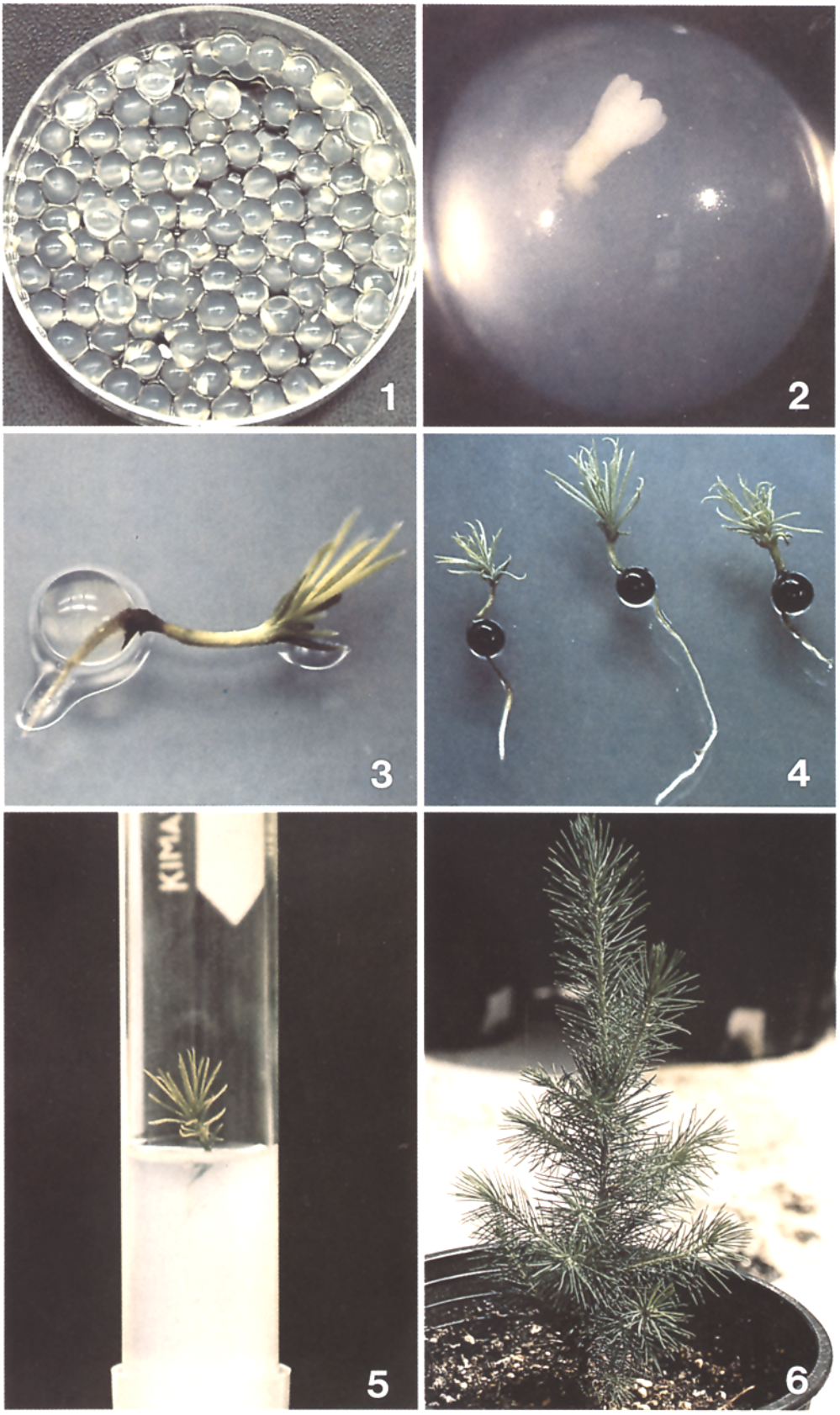

Fig. 1-6. Interior spruce somatic embryo encapsulation, and subsequent development of plants. (1,2) Embryos encapsulated in sodium alginate (1, x 0.8; 2, x 11). (3) Developing plantlet from alginate-encapsulated treatment (x 2). (4) Developing plantlets from alginate-charcoal encapsulated treatment (x 0.8). (5) Continuing development of previously encapsulated embryos after transfer to test tube (x 0.8). (6) Growth in soil under greenhouse conditions (x 0.3).

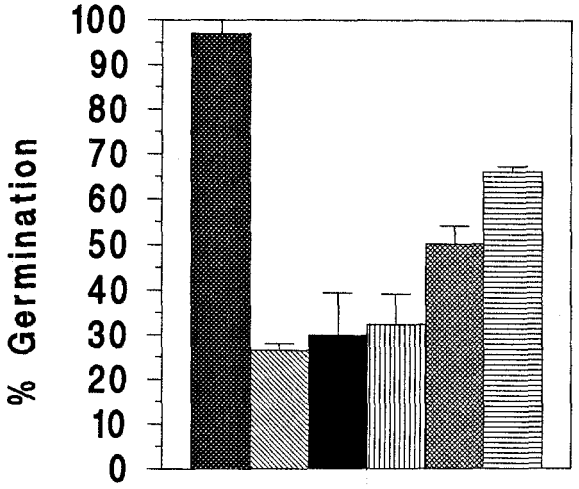

Fig. 7. Effect of encapsulation and nutrients in capsule, on percent germination of cultured zygotic embryos of interior spruce (IS). non-encap, alg, alg + ch, alg on ch medium, alg + ch + LV, alg + ch + LV + suc

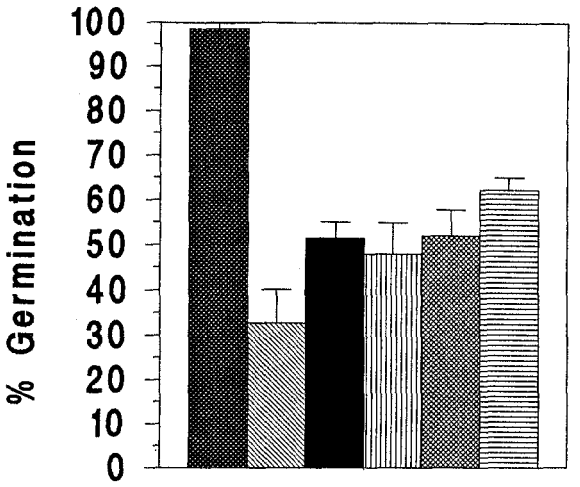

Fig. 8. Effect of encapsulation and nutrients in capsule, on percent germination of somatic interior spruce line IS-W70. Labels as shown in Fig. 7.

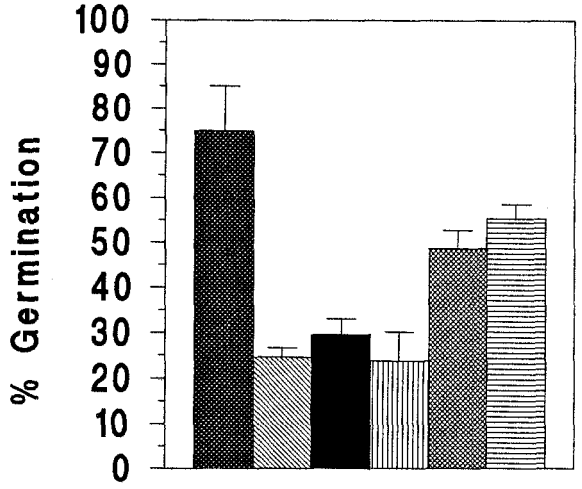

Fig. 9. Effect of encapsulation and nutrients in capsule, on percent germination of cultured zygotic embryos of black spruce (BS). Labels as shown in Fig. 7.

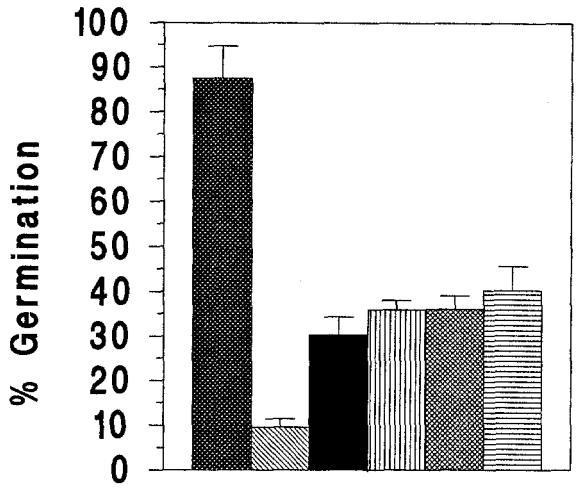

Fig. 10. Effect of encapsulation and nutrients in capsule, on percent germination of somatic black spruce line BS-D. Labels as shown in Fig. 7.

comparable somatic embryos did not show a similar reduction in germination frequency after one month cold storage (compare Figs. 9,10 with 12).

Bapat and Rao (1988) examined the effect of a 45-day 4 °C cold storage period on germination of encapsulated somatic embryos of *Santalum album*, they reported a reduction in germination frequency. Datta and Potrykus (1989) also reported a reduction in germination frequency from 65% to 37.5%, for encapsulated microspore-derived barley embryos after 6 months of 4 °C storage. However, embryos that had not been encapsulated failed to survive (Datta and Potrykus 1989).

A successful encapsulation procedure must ensure that somatic embryos retain their viability and are able to germinate. Although germination frequencies of over 60% were obtained with encapsulated interior spruce somatic embryos, the encapsulation condition reduced the germination frequency, indicating that conditions within the capsule were not optimal and need further improvement. Such improvement may be achieveable by altering the capsule composition, to reflect the storage product composition of seed containing mature zygotic embryos. For example, the megagametophyte and the zygotic embryo of a conifer seed can contain storage proteins (Gifford and Tolley 1989; Roberts *et al.* 1989; Joy *et al.* 1991), and lipids and polysaccharides (Joy *et al.* 1991). Future experiments could also test increases in charcoal concentration, to determine if oxygen supply might be a limiting factor, and further investigation with capsule nutrient supplementation could be attempted, similar to the work of Rao and Singh (1991), and Zhong and Wang (1989).

Survival of somatic embryos during 4 °C cold storage indicates that this method could be used to preserve desirable genotypes over a short-term. The survival of encapsulated embryos during cold storage could also be improved by using an optimized capsule matrix. Alginate capsules provide a useful experimental system for creation of artificial endosperms.

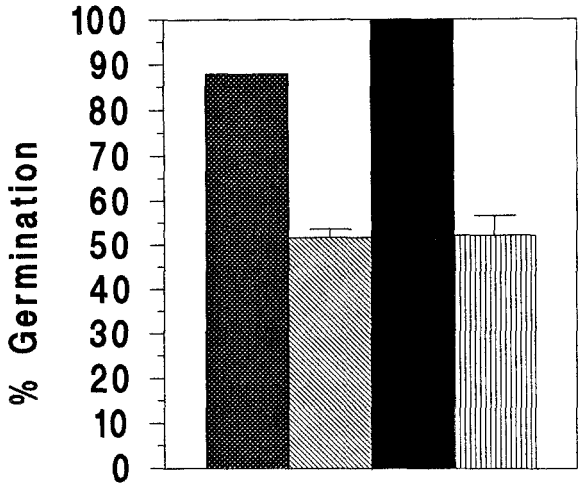

Fig. 11. Effect of cold storage on percent germination of cultured interior spruce zygotic [IS (zyg)] and somatic embryos [IS-W70], either encapsulated or not. ■ IS(zyg) non-encap, ▨ IS (zyg) alg+ch, ■ IS-W70 non-encap, ▥ IS-W70 alg+ch

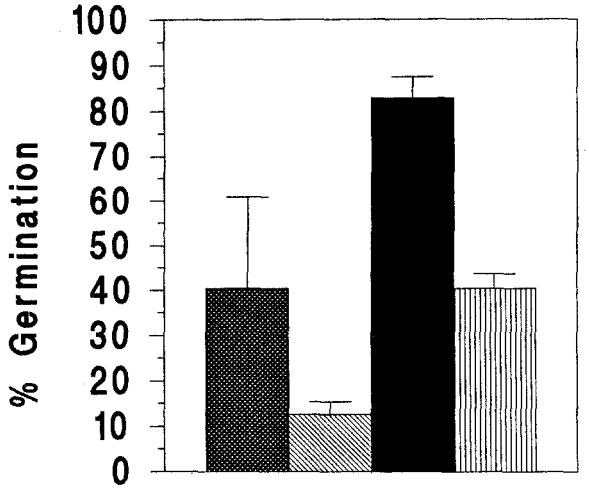

Fig. 12. Effect of cold storage on percent germination of cultured black spruce zygotic [BS (zyg)] and somatic embryos [BS-D], either encapsulated or not. Labels are the equivalent of Fig. 11.

However, use of the hydrated alginate capsule could present difficulties in the production of desiccated synthetic seed, or when used to encapsulate previously desiccated embryos. Our future research will also involve investigations of alternatives to the alginate capsule.

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