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Enhanced post-germinative growth of encapsulated somatic embryos of Siberian ginseng by carbohydrate addition to the encapsulation matrix

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Abstract This experiment was carried out to enhance conversion and ex vitro survival of encapsulated somatic embryos of Siberian ginseng (*Eleutherococcus senticosus*). Cotyledonary somatic embryos were encapsulated with 3.0% sodium alginate; 96% of the encapsulated embryos converted to plantlets with well-elongated epicotyls in Perlite containing sucrose as a carbon source. However, although they germinated, post-germinative growth of encapsulated embryos was suppressed on Perlite that did not contain sucrose. Instead of sucrose addition to Perlite, addition of carbon sources to the encapsulation matrix enhanced post-germinative growth of encapsulated embryos. In the encapsulation matrix with 2% sucrose, post-germinative growth of encapsulated embryos was more than twice (23.5%) that of the control capsules without sucrose (10.0%). Embryos encapsulated with both 2% sucrose and 1% starch powder showed the highest post-germinative growth percentage (42.1%). Iodine staining and analysis of starch content in the encapsulation matrix revealed that starch in the encapsulation matrix decomposed during embryo germination. This result indicates that carbohydrate treatment in the encapsulation matrix enhanced post-germinative growth of encapsulated embryos of Siberian ginseng.

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Introduction

Siberian ginseng (*Eleutherococcus senticosus*) is a woody medicinal plant that grows only in cold regions of Asia (Lee 1979). The cortical tissue of roots and shoots is used for various medicinal purposes (Brekhman 1960; Brekhman and Dardymov 1969). This plant has become an endangered species in several countries due to overexploitation of natural populations. Conventional propagation is very difficult because long-term stratification is required to induce maturation and germination of the zygotic embryos, and propagation by stem cuttings is also very difficult (Isoda and Shoji 1994).

In vitro culture can be an efficient means for mass propagation, and micropropagation though somatic embryogenesis in Siberian ginseng has been reported (Choi et al. 1999a,b). Propagation of plants by tissue culture requires enormous time and labor for plant regeneration and soil transfer. If somatic embryos can be treated as seeds, transportation, handling, storage and planting will be facilitated (Redenbaugh et al. 1988). The use of artificial seeds for obtaining plants has been reported for several crops of agricultural interest (Choi and Jeong 2002; Kitto and Janick 1985a,b; Redenbaugh et al. 1986). However, direct sowing of artificial seeds in the field for practical use remains a limitation of artificial seed application because of low soil survival (Redenbaugh et al. 1986; Kamada 1985).

Redenbaugh et al. (1986) suggested that two research hurdles must be overcome: (1) development of an encapsulation matrix that has many nutrients, growth control agents, and chemicals for embryo-to-plant development; and (2) production of high quality somatic embryos. A large difference between artificial seeds and true seeds is the presence of endosperm in the seeds. In true seeds, reserve materials accumulated in the endosperm play an important role in supplying energy for germina-

tion and seedling growth of zygotic embryos (Bewley 1997; Cardemil et al. 1982). Alginate capsules have been used as artificial endosperms to deliver the nutrients necessary for germination of embryos. However, there is little information on the role of the addition carbon sources in the germination and post-germinative growth of artificial seeds, although addition of sucrose to the encapsulation matrix (Lulsdorf et al. 1993; Ganapathi et al. 2001) or medium (Ghosh and Sen 1994; Ara et al. 1999) has been described.

In this study, we evaluated the effects of carbon source supplementation on germination and subsequent post-germinative growth of encapsulated embryos of Siberian ginseng.

Materials and methods

Production of somatic embryos from embryogenic cells

Embryogenic cells of *E. senticosus* Rupr. & Maxim were induced from excised zygotic embryos as described by Choi et al. (1999a). Embryogenic cells were maintained in MS (Murashige and Skoog 1962) liquid medium supplemented with 4.4 μM 2,4-D in 250 ml Erlenmeyer flasks with 2-week subculture intervals. To induce embryos, 50 mg embryogenic cells were transferred into 250 ml Erlenmeyer flasks containing 75 ml MS liquid medium lacking 2,4-D. The medium was adjusted to pH 5.7 prior to autoclaving at 121°C for 15 min. All cultures were maintained at 22±2°C in 16 h photoperiods with light supplied by white fluorescent tubes at an intensity of 24 μmol m^{-2} s^{-1}. After 2 months, cotyledonary stage embryos were used for encapsulation.

Culture of encapsulated embryos on solid medium

Cotyledonary embryos were soaked in sterilized 3% sodium alginate (low viscosity; Sigma, St. Louis, Mo.), with or without half-strength MS medium and 2% sucrose. Each embryo was picked up using a 0.5 and 0.8 cm internal diameter pipette and dropped into a 100 mM $CaCl_2 \cdot 2H_2O$ solution, where they were left to stand for 10 min on a magnetic stirrer for gelling (50 rpm). Capsules were washed using half-strength MS liquid medium with or without 2% sucrose as per different treatments and then transferred to sterilized filter paper to blot the remaining water. Encapsulated embryos were transferred to a 750 ml polypropylene plastic culture chamber with a round bottom (Gaooze 9; KSTI, Seoul, Korea) containing 200 ml half-strength MS solid (0.3% Gelrite) medium with or without 2% sucrose. The chamber was covered with a plastic cap with a 1-cm membrane filter (0.45 μm pore) in the center; each chamber contained about 30 capsules. Thirty capsules were used per treatment and the experiment was repeated three times. Germination and post-germinative growth of encapsulated somatic embryos were scored after 2 weeks and 2 months of culture, respectively. Post-germinative growth is defined as germinated embryos with epicotyls at least 10 mm in length.

Culture of encapsulated embryos in Perlite

To investigate the encapsulation effect, encapsulated embryos and naked embryos were cultured on sterilized Perlite (250 ml) containing half-strength MS liquid medium (80 ml) plus 2% sucrose in a 750 ml plastic culture chamber. Thirty capsules and 30 naked embryos were used per treatment and the experiment was repeated three times. Germination was recorded after 2 weeks of culture and post-germinative growth of encapsulated embryos recorded after 2 months of culture.

To determine the effect of sucrose addition to the Perlite, embryos in capsules not containing sucrose were cultured in sterilized Perlite containing half-strength MS medium, either with or without 2% sucrose in a plastic bowl. Thirty capsules were used per treatment and the experiment was repeated three times. Germination and post-germinative growth of encapsulated embryos were recorded after 2 weeks and 2 months of culture, respectively.

Effect of carbohydrate addition in Ca alginate capsules

To evaluate the effect of different carbon sources on germination and post-germinative growth, encapsulated embryos with 2% sucrose, 1% starch or 1% starch plus 2% sucrose were cultured on sterilized Perlite in plastic bowls. All encapsulated embryos contained half-strength MS medium with 14.4 μM gibberellic acid (GA_3), which promotes germination of somatic embryos of Siberian ginseng, as described by Choi and Jeong (2002). Two different capsule sizes were used: small (85 mm^3: made by a 0.5 cm internal diameter pipette) and large (290 mm^3: made by a 0.8 cm internal diameter pipette). The perlite did not contain any carbohydrate, but was moistened with sterilized distilled water. Germination and post-germinative growth were scored after 2 weeks and 6 weeks of culture, respectively.

Analysis of starch degradation in the encapsulation matrix during germination

To confirm the utilization of starch during germination of encapsulated embryos, iodine staining (Chester 1968) was carried out after 0 days and 30 days of culture. After 2.5% glutaraldehyde fixation for 24 h, the encapsulated embryos were soaked in 1% iodine solution and rinsed with sterilized distilled water. The stained encapsulated embryos were observed with a dissecting microscope.

Extraction and determination of starch content in the encapsulation matrix (with 1% starch) during the culture period were performed according to Lustinec et al. (1983). Germinated embryos were removed from the encapsulated beads after 0, 15 and 30 days of culture and the empty beads subjected to analysis. One gram of beads without embryos was ground in liquid nitrogen and the materials mixed with 4 ml 80% ethanol to remove the soluble sugar and then centrifuged at 3,000 g for 10 min. After discarding the supernatant, pellets were digested in 2 ml 32% (v/v) $HClO_4$ for 1 h at room temperature; 1 ml distilled water and 4 ml 1.5 M H_2SO_4 were added to the digested samples and then incubated at 100°C for 30 min. The sugar released from the starch was analyzed using the phenol-sulfuric acid procedure (Dubois et al. 1956) with glucose standards.

Statistical analysis

Experiments were carried out in a randomized design and data were subjected to the analysis of variance procedures using SAS statistical software. Statistically significant mean differences were determined by the Duncan's multiple range test at $P<0.05$

Results and discussion

Effect of carbon source on germination and post-germinative growth of encapsulated embryos on MS medium

Cotyledonary stage embryos formed after 2 months of culture (Fig. 1a) and somatic embryos developed individually and in a synchronized state without any special synchronization treatment of the embryogenic cells.

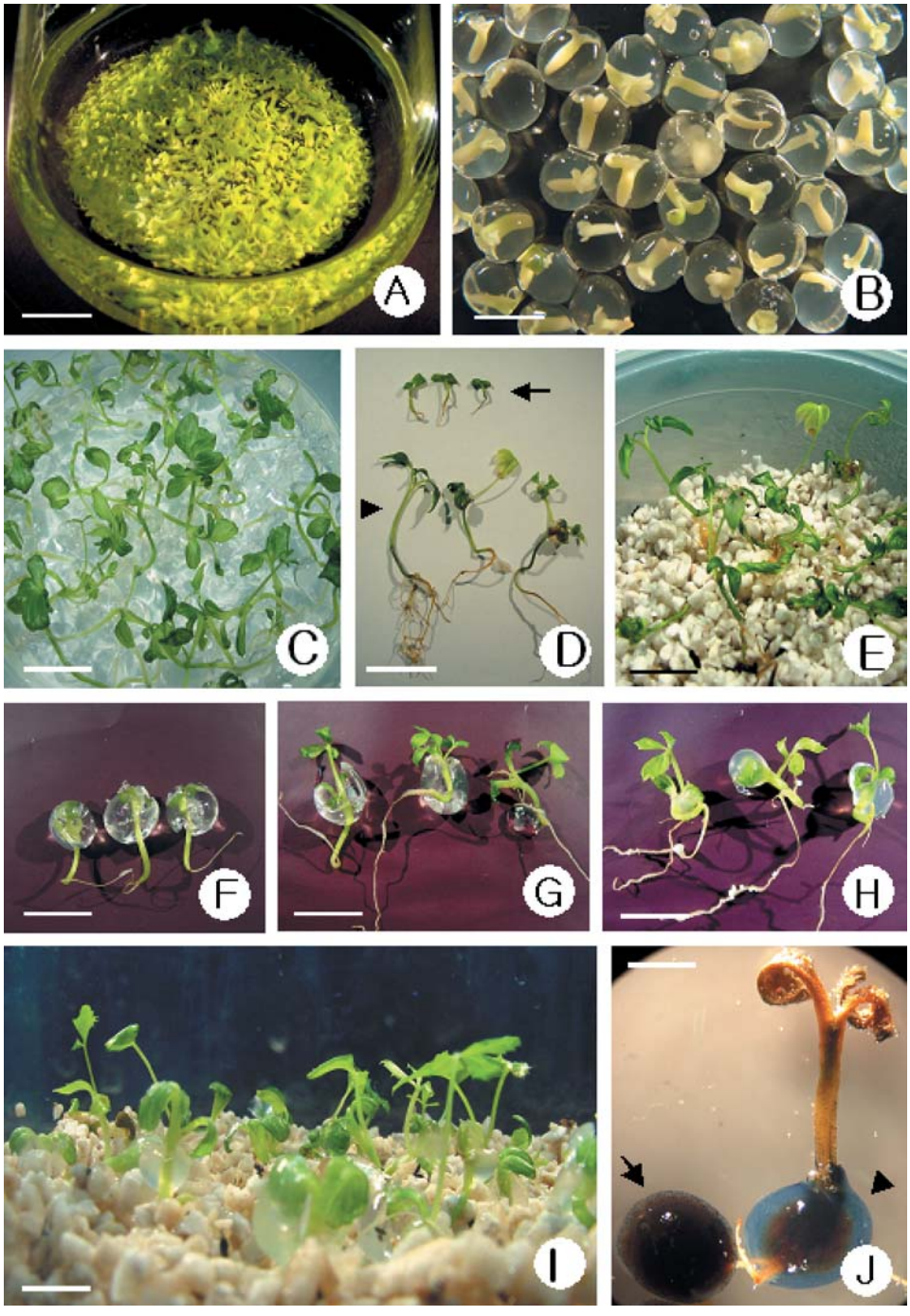

Fig. 1a–j Germination and post-germinative growth of encapsulated embryos of *Eleutherococcus senticosus*. **a** Production of cotyledonary somatic embryos in 250 ml Erlenmeyer flask after 2 months of culture. **b** Somatic embryos encapsulated in 3% sodium alginate. **c** Germination of encapsulated embryos in plastic culture chamber containing half-strength MS solid medium with 2% sucrose. **d** Comparison of post-germinative growth of naked (*arrow*) and encapsulated (*arrowhead*) embryos on Perlite containing half-strength MS medium with 2% sucrose. **e** Conversion of encapsulated embryos on Perlite containing half-strength MS liquid medium with 2% sucrose after 2 months of culture. **f–h** Influence of carbohydrate addition in encapsulation matrix on germination of encapsulated embryos. Growth of encapsulated embryos without carbohydrate (**f**), with 2% sucrose (**g**), and with both 1% starch and 2% sucrose (**h**) after 6 weeks of culture. **i** Growth of plantlets from encapsulated embryos on Perlite containing 1% starch and 2% sucrose. **j** Iodine staining of encapsulated embryos containing 1% starch powder at start (*arrow*) and after 1 month of culture (*arrowhead*). *Bars* **a** 1 cm, **b** 0.8 cm, **c–h** 1.5 cm, **i** 1.2 cm, **j** 0.5 cm

Encapsulated embryos with 3% sodium alginate germinated at a rate of 100% on half-strength MS solid medium with 2% sucrose after 2 weeks of culture (Fig. 1b) and then subsequently grew to plantlets with well-developed epicotyls (post-germinative growth) after 2 months of culture (Fig. 1c). However, while encapsulated embryos on medium without sucrose germinated normally, epicotyl growth failed to reach 10 mm in length, probably due to limitation of carbon source supply. In Siberian ginseng, the high germination rate of encapsulated embryos means that the embryos are of high quality, similar to zygotic embryos. However, the carbon source supply may be a critical factor for the post-germinative growth of embryos.

Encapsulation effect of somatic embryos on germination in Perlite

To investigate the encapsulation effect, naked embryos and encapsulated embryos were cultured on sterilized Perlite containing half-strength MS liquid medium with 2% sucrose. Both encapsulated and naked embryos germinated at 100% (Table 1). However, naked somatic

Table 1 Germination and conversion of encapsulated and naked somatic embryos of *Eleutherococcus senticosus* on sterilized Perlite containing half-strength MS medium with 2% sucrose after 2 months of culture. Post-germinative growth is defined as encapsulated embryos producing epicotyls more than 10 mm in length. Data represent the mean values ± SE of three independent experiments. Means followed by same letter are not significantly different at $P<0.05$

	Germination (%)[c]	Post-germinative growth (%)[d]	Total length (cm)
Naked	100	0 b	2.1±0.86 b
Encapsulated	100	96±3.27 a	8.2±0.82 a

[c] Germination of encapsulated embryos scored after 2 weeks of culture
[d] Post-germinative growth of encapsulated embryos scored after 2 months of culture

Table 2 Effect of sucrose addition to sterilized Perlite on germination and conversion to plantlets of *E. senticosus* from artificial seeds. Post-germinative growth is defined as encapsulated embryos producing epicotyls more than 10 mm in length. Data represent the mean values±SE of three independent experiments. Means followed by same letter are not significantly different at $P<0.05$

	Germination (%)[d]	Post-germinative growth (%)[e]	Total length (cm)
Water	100	0 b	3.1±0.56 b
1/2 MS	100	0 b	1.1±0.23 c
1/2 MS + 2% sucrose	100	96±5.29 a	8.2±0.82 a

[d] Germination of encapsulated embryos scored after 2 weeks of culture
[e] Post-germinative growth of encapsulated embryos scored after 2 months of culture

embryos on Perlite failed to reach the required level of subsequent post-germinative growth, although they were cultured under cover (Fig. 1d). However, 96% of encapsulated embryos converted to plantlets with well-developed shoots on Perlite containing sucrose after 2 months of culture (Table 1, Fig. 1d). This suggests that encapsulation of somatic embryos prevents dehydration and facilitates the absorption of nutrients from Perlite, which stimulates post-germinative growth of the encapsulated embryos. Ara et al. (1999) reported that the percentage germination of encapsulated somatic embryos of mango was higher than that of naked embryos on solid medium. They suggest that the high germination rate may be attributed to the protection provided by the capsules as well as by the presence of nutrients in the gel matrix, which served as a nutrient bed around the somatic embryos to facilitate growth and survival. Contrary to our results, Ghosh and Sen (1994) reported that the conversion frequency was higher (45%) for non-encapsulated asparagus somatic embryos than for encapsulated embryos (28–34%) on MS medium. This difference in plantlet conversion might be due to different culture substrates.

Effect of sucrose addition to Perlite

Encapsulated embryos were cultured on Perlite containing half-strength MS liquid medium with and without 2% sucrose. Germination of encapsulated embryos occurred regardless of sucrose addition on perlite within 2 weeks of culture. However, a high rate of post-germinative growth (96%) to form plantlets with stems occurred only on Perlite with 2% sucrose after 2 months of culture (Table 2, Fig. 1e). Plantlets germinated from encapsulated embryos on Perlite containing half-strength MS medium with 2% sucrose were more than twice (8.08 cm) the length of those (3.10 cm) on Perlite with only water. Encapsulated embryos on Perlite with half-strength MS salts and without sucrose browned immediately after germination. These results indicate that exogenously provided energy sources were required not only for subsequent post-germinative growth, but also for the ex vitro survival of plants.

Effect of carbohydrate addition to the encapsulation matrix

Addition of sucrose to the Perlite was important for stimulating the growth of encapsulated embryos. However, addition of sucrose directly to the Perlite is not the ideal procedure, as compared to including it in the encapsulation matrix. To test the effect of carbohydrate addition to the encapsulation matrix, encapsulated embryos with 1% starch, 2% sucrose, or both 1% starch and 2% sucrose were cultured on Perlite for 6 weeks. In a previous paper, germination of somatic embryos of Siberian ginseng was promoted by treatment with GA_3 (Choi and Jeong 2002). Therefore, in all treatments, 14.4 μM GA_3 was added to the encapsulation matrix, together with half-strength MS salts to stimulate germination of somatic embryos. Post-germinative growth of encapsulated embryos was much higher (36.8%) for capsules containing 1% starch than those not containing any carbohydrate (10.0%) or those containing 2% sucrose (23.5%) (Table 3, Fig. 1f–h). In encapsulated embryos containing no carbohydrate, all embryos germinated. However, the subsequent growth of the germinated embryos was suppressed (Fig. 1f) and only about 10% of the encapsulated embryos converted to plantlets (Table 3). In encapsulated embryos with 1% starch powder and 2% sucrose (Fig. 1h), hypocotyl elongation of plantlets was slow, but epicotyl emergence was more rapid than for those with 2% sucrose (Fig. 1g). For large capsules

Table 3 Effect of carbohydrate addition to alginate capsules on germination and conversion of the encapsulated embryos of *E. senticosus*. Encapsulated embryos were cultured on sterilized Perlite containing distilled water. All the alginate capsules contained half-strength MS salt and 14.4 μM gibberellic acid (GA_3). Post-germinative growth is defined as encapsulated embryos producing epicotyls more than 10 mm in length. Data represent the mean values ±SE of three independent experiments with 30 capsules per treatment. Means followed by the same letter are not significantly different at $P<0.05$

	Germination (%)[e]	Post-germinative growth (%)[f]		Total length (cm)	
		S[g]	L[h]	S	L
Water	100	5.2±0.67 d	10.0±2.24 c	3.1±0.62 c	3.1±0.65 d
1% starch	100	11.1±1.18 c	36.8±4.51 a	3.5±0.70 c	3.9±0.63 c
2% sucrose	100	15.0±1.02 b	23.5±2.15 b	4.4±0.86 b	5.2±1.27 b
2% sucrose + 1% starch	100	36.8±3.19 a	42.1±3.46 a	5.3±1.13 a	5.9±0.95 a

[e] Germination scored after 2 weeks of culture
[f] Post-germinative growth scored after 6 weeks of culture
[g] Small bead, 85 mm^3
[h] Large bead, 290 mm^3

(290 mm^3), further growth of the germinated embryos was faster than for smaller capsules (Table 3). Thus, larger capsules appeared better for promotion of post-germinative growth, possibly due to the larger energy source. Encapsulated embryos containing both 1% starch and 2% sucrose gave the highest post-germinative growth (42.1%) (Table 3, Fig. 1h,i). These results indicate that carbohydrate may act as an energy source to stimulate post-germinative growth. In black spruce, addition of salts and sucrose to the encapsulation matrix enhanced the germination rate of encapsulated somatic embryos, although they were also cultured on MS medium containing sucrose (Lulsdorf et al. 1993). Janeiro et al. (1997) reported that artificial seeds without sucrose in the capsule showed lower germination rates than embryos encapsulated with alginate containing MS medium with sucrose.

Sucrose can escape from the encapsulation matrix in hydrated soil because it is water-soluble. In *Cedrela odorata* L., sucrose in the encapsulation matrix might have diffused into the soil, so that while a high germination frequency was achieved, the amount of nutrients remaining in the encapsulation matrix was not sufficient to support conversion (Maruyama et al. 1997). In contrast to diffusible sucrose, starch powders in beads are not lost in hydrated soil because they are water-insoluble. This indicates that starch powder in the encapsulation matrix might be used as an effective carbon source or as a substitute for sucrose.

Analysis of starch degradation during germination

To determine if the starch content decreased in the encapsulation matrix during germination, the capsules were stained with iodine (Chester 1968). After 30 days of culture, capsules with 1% starch powders stained faint blue color and had smeared blue spots (Fig. 1), while at day zero of culture, the capsules with 1% starch powders were clear, except for dark-blue starch spots (Fig. 1j). This indicates that the somatic embryos decomposed the starch powder.

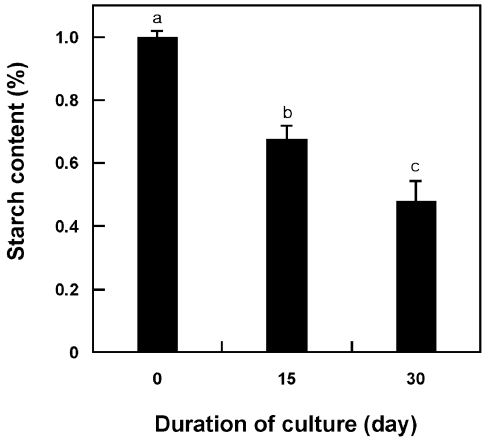

Fig. 2 Changes of starch content in encapsulation matrix containing 1% starch powder during the culture measured as glucose following acid hydrolysis. Columns with the same letter are not significantly different at $P<0.05$. *Bars* Standard error

The starch content in the encapsulation matrix decreased markedly during germination of the somatic embryos. After 30 days of culture, the starch content in the encapsulation matrix was 48% of that at day zero (Fig. 2), while in the encapsulation matrix without somatic embryos, degradation of starch powder was not detected as the duration of culture proceeded (data not shown). This result indicates that starch powder was degraded by somatic embryos and utilized as an energy source for the germination and further post-germinative growth of somatic embryos.

In conclusion, we have shown that carbon source addition to the medium or encapsulated matrix markedly enhances the conversion of encapsulated embryos. However, sucrose addition in soil substrates may be detrimental to the artificial seeds because of the growth of microorganisms. Therefore, carbohydrate addition to the encapsulation matrix may be suitable. In Siberian ginseng, the addition of carbon sources to both the encapsulation matrix and the culture medium did not enhance the germination percentage of encapsulated somatic embryos but did enhance the post-germinative growth of the encapsulated somatic embryos. This indicates that car-

bohydrate addition plays an important role in the further growth of germinated somatic embryos. The addition of starch powders to encapsulation matrix might contribute to new artificial endosperm for providing energy to the post-germinative growth of encapsulated embryos and may stimulate microbial growth less than sucrose.

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