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Encapsulation of Somatic Embryos of Carrot and Promotive Effects on the Growth of Plantlets *in vitro*

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Abstract
Encapsulation of somatic embryos is considered to be an effective method for mechanical handling of fragile somatic embryos. Encapsulation method of somatic embryos using sodium alginate and calcium chloride solutions and growth of plantlets from encapsulated embryos were investigated. It was concluded that the optimum hardness of the capsule was 2.0 N/cm^2 based on the germination percentage of embryos, growth of plantlets and ease of handling. The growth of the carrot plantlets was promoted by encapsulation of somatic embryos compared with the plantlets from naked, unencapsulated embryos due to the removal of the water and nutrient stress to which embryos are readily exposed at the early stage of development.

Discipline: Horticulture
Additional key words: capsule hardness, encapsulation method, germination percentage, sodium alginate

Introduction

Mass propagation of plants using tissue culture techniques has many advantages; it enables to propagate plants with a low level of fertility or very low germination percentage, to propagate or to maintain parents of F_1 hybrids and plants not fixed genetically yet, to reduce the duration of the breeding period, etc. However, mass propagation is not practically useful except for some expensive ornamental plants because conventional culture methods of plantlets *in vitro* require laborious transplanting operations which result in the increase of the cost of the plantlets. Therefore, we attempted to develop a labor-saving and growth-promotive technique for the production of plantlets *in vitro*.

At present, although plantlets *in vitro* are mainly produced by meristem culture, from the viewpoint of mass propagation, somatic-embryo culture seems to be more suitable. The somatic embryo, however, is so fragile that it is difficult to manipulate it intact without inducing damage through mechanical handling. To solve this problem, an embryo was encapsulated in a gel.

Encapsulation of somatic embryos as an artificial seed was suggested by Murashige[2], followed by several studies by other researchers. Kitto et al.[1] coated the embryos with polyethylene oxide (Polyox) as a gelling compound, and after drying treatment they observed that some of the embryos survived. However, in this case, as Polyox became a flat wafer containing many embryos, it became difficult to use it as an artificial seed. In addition, for the purpose of determining the survival percentage of the coated embryos, embryos were recovered from the suspension in which the wafer was redissolved and then were placed and cultured on a filter paper in a petri dish. Therefore, this system appeared to be meaningless as an encapsulation system. Polyox was thus considered to be unsuitable as a gelling compound.

Redenbaugh et al.[3,4] tested a large number of hydrogels for their ability to form a hydrated capsule around somatic embryos. They mentioned that

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sodium alginate (Na-ALG) was one of the most useful compounds for encapsulation, due to various properties. Na-ALG, which can be dissolved in water at room temperature, does not require heat to produce a gel, and it gelatinizes easily when it comes into contact with relatively nontoxic, divalent metal salts such as calcium chloride ($CaCl_2$). They investigated the minimum concentration of the $CaCl_2$ solution which enabled the formation of a capsule, and the relation between the capsule hardness and soaking time of Na-ALG drops in the $CaCl_2$ solution. However, they did not examine the relation between capsule hardness and the germination percentage and growth rate of the embryos.

In this paper, the authors investigated the relation between the capsule hardness and 1) methods of encapsulation, as well as 2) germination percentage and growth of embryos of carrots. In addition, the promotive effect of encapsulation of embryos on the growth of carrot plantlets *in vitro* was analyzed.

Materials and methods

1) Induction of somatic embryos

Somatic embryos of carrot (*Daucus carota* L. cv. "T-gou sanzun") were induced as follows. Carrot seeds sterilized with 70% of ethyl alcohol for 1 min. and then with sodium hypochlorite containing 2% of chlorine for 10 min were sown on Murashige and Skoog's (MS) gelrite (0.2%) medium containing 3% of sucrose. A hypocotyl removed from the 2-week old aseptic seedling was cultured on MS gelrite (0.2%) medium containing 0.4 mg/*l* of 2.4-D for 2 months. Callus obtained was then suspended in liquid MS medium with 0.4 mg/*l* of 2.4-D for 2 weeks. Cell clumps 53–75 μm in diameter obtained from the suspension were resuspended in hormone-free MS medium and cultured for 2 weeks to induce somatic embryos.

2) Encapsulation of somatic embryos

Somatic embryos were encapsulated basically according to the method developed by Redenbaugh et al.[4] under aseptic conditions. Equal volumes of a Na-ALG aqueous solution and MS liquid medium (3% sucrose) which was twice as concentrated as the standard one were mixed (1.5% sucrose finally) after sterilization in an autoclave independently. Somatic embryos were suspended in this solution, and then the suspension was dropped into a sterilized $CaCl_2$ aqueous solution with a pipette to form a capsule containing one embryo, followed by a rinse with sterilized water after soaking in the solution for the prescribed time to harden it.

3) Regulation of the capsule hardness and determination of hardness

We regulated the capsule hardness by changing the concentrations of the Na-ALG solution (0.5, 1.0 and 1.5%, w/v after mixing with MS medium), gelling compound, and $CaCl_2$ (1.0, 2.0 and 3.0% w/v), hardener, as well as soaking time of the capsule in the $CaCl_2$ solution (1, 5 and 30 min). Hardness of the capsules was determined with an apparatus for the measurement of sarcocarp hardness (Texturometer: Zenken Co., Ltd.).

4) Relation between capsule hardness and germination percentage and growth of embryos

Two pieces of medium-support made of polyester wool ($30 \times 30 \times 60$ mm, manufactured by Toyobo Co., Ltd.) were put into a ready-made culture vessel ("Plant box"; $60 \times 60 \times 100$ mm) followed by sterilization, and about 100 m*l* of MS liquid medium (1.5% sucrose) was poured in the vessel. Then capsules of a somatic embryo with varying degrees of hardness were placed on the supports and cultured, and several days later the germination percentage and height of the plantlets were measured.

5) Promotive effect of encapsulation on the growth of plantlets

For this test, we used a culture vessel made of polycarbonate where the environmental conditions could be controlled ($210 \times 320 \times 120$ mm) (Fig. 1) and which had been developed in this institute[5]. By using the culture vessel we were able to achieve forced ventilation and to replace the culture medium in the course of cultivation. Eighteen pieces (6×3 pieces) of the medium-support described above were put in the vessel and soaked in MS medium. In a vessel, the 18 pieces of medium-support were divided into three blocks. Encapsulated embryos, single naked (unencapsulated) embryos, and about 20 naked embryos in a cluster were placed on supports in the respective blocks. Placement densities of the embryos in the respective methods were as follows: In the first and the second cases 6 embryos were placed

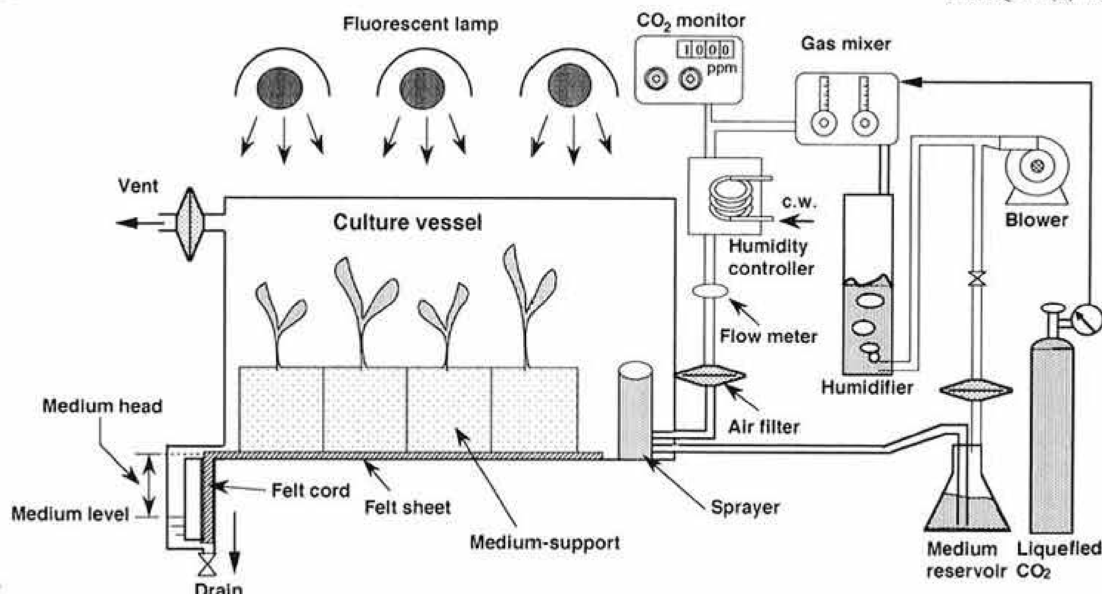

Fig. 1 Culture system for controlling environmental conditions

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on a support, and in the last case about 40 embryos.

Ventilation of the culture vessel with humidified air through a membrane filter, which removed miscellaneous microorganisms, was started 1 week after the placement of the embryos. Flow rate of the air was 3 *l*/min.

Culture conditions were as follows: intensity of light was 10–15 klx, photoperiod; 12 hr, temperature; 23°C (dark) – 25°C (light).

Results and discussion

1) Encapsulation

The Na–ALG solution of the capsule material changes into Ca–ALG and gelatinizes when it comes into contact with the $CaCl_2$ aqueous solution, and the shape and hardness of the gel vary with the changes in the concentration of the solution and the soaking time. If the gel is too soft to form a capsule, it can not coat and embed an embryo. On the contrary, a very hard capsule may induce the depression of the germination and growth of embryos. Accordingly, optimum concentrations of Na–ALG and $CaCl_2$ solution for the encapsulation of embryos and optimum soaking time of a Na–ALG drop into the $CaCl_2$ solution were examined.

First, the shape of the gels was observed when

Table 1. Effect of concentration of sodium alginate and calcium chloride on the shape of the gel

Conc. of $CaCl_2$ aqueous solution (%)	Conc. of Na–ALG solution (%)		
	0.5	1.0	1.5
1	– [a]	+ [b]	+
2	–	+	+
3	–	+	+

a): Non-spherical shape.
b): Spherical shape, suitable for encapsulation.

various concentrations of Na–ALG drops were soaked into several concentrations of a $CaCl_2$ solution for 10 min. The Na–ALG concentration affected the shape of the gels, unlike the $CaCl_2$ concentration (1.0–3.0%) (Table 1). When the concentration of Na–ALG was 0.5%, the gels lost their spherical shape and assumed irregular forms although they were able to coat the embryos. In the case of 1.0% and 1.5% Na–ALG they became spherical. Based on the shape of the gel it appeared that the optimum concentration for encapsulation was 1.0% for $CaCl_2$ and 1.0–1.5% for Na–ALG.

Then the relationship between the capsule hardness and soaking time of Na–ALG drops into the $CaCl_2$ solution was investigated. Examinations were made at Na–ALG concentrations of 0.5, 1.0 and

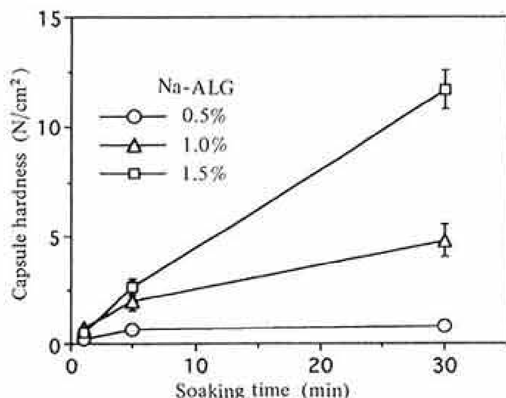

Fig. 2. Relationship between capsule hardness and soaking time of Na-ALG in 1% $CaCl_2$ solution

Vertical axis indicates the strength at which a capsule is broken by compression.
Vertical lines represent standard deviations.

1.5% and at a $CaCl_2$ concentration of 1.0%. As shown in Fig. 2, the capsule hardness increased with the increase of the Na-ALG concentration and generally with the increase of the soaking time. In the case of 0.5% Na-ALG, however, the capsule hardness increased with the soaking time up to 5 min, and then hardly increased any further.

2) Relation between capsule hardness and germination and growth of embryo

Fig. 3 shows the effect of the capsule hardness on the germination percentage of somatic embryos of carrot. The germination percentage at 3 days after placement increased with the decrease of the capsule hardness. At 6 days after placement, the percentage was almost 100% (higher than 97.5%) when values of the capsule hardness were 0.70, 2.00 and 2.62 N/cm^2. On the other hand, the percentage did not exceed 82.5% when the value of the capsule hardness was 4.78 N/cm^2. The relation between the capsule hardness and plant height of carrot at 6 days after placement is shown in Fig. 4. Carrot plantlets grew well with the decrease of the capsule hardness, which may have promoted early germination.

Therefore, the growth of the somatic embryos was promoted by the decrease of the capsule hardness. Taking into account the shape and ease of handling of capsules, it was considered that a hardness value

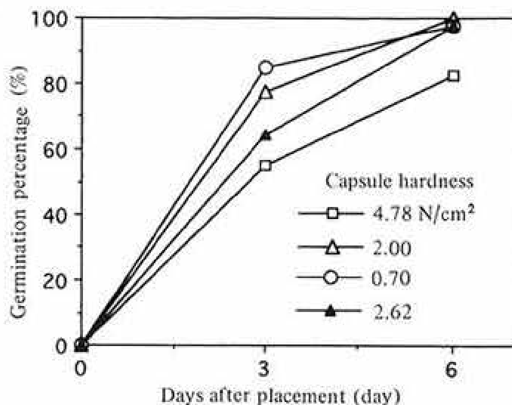

Fig. 3. Effect of capsule hardness on the germination percentage of the encapsulated somatic embryos

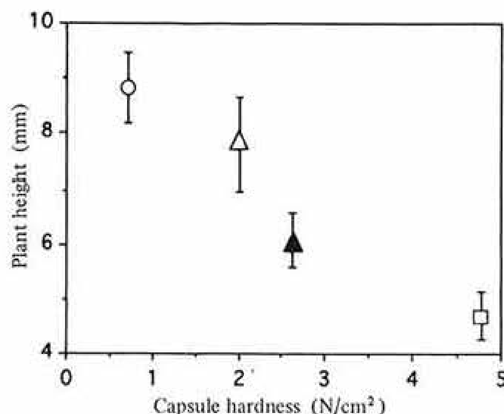

Fig. 4. Effect of capsule hardness on the growth of carrot plantlets

The measurements were performed at 6 days after placement.
Symbols are the same as in Fig. 3.
Vertical lines represent standard errors.

of 2.0 N/cm^2 was optimum for the encapsulation of somatic embryos. Such hardness level could be obtained when 1% of a Na-ALG solution was dropped and soaked into a 1% $CaCl_2$ solution for 5 min as shown in Fig. 2.

3) Promotive effect of encapsulation on the growth of plantlets

We determined whether the encapsulation of somatic embryos exerted a promotive effect on the growth of plantlets. Capsules with a hardness value

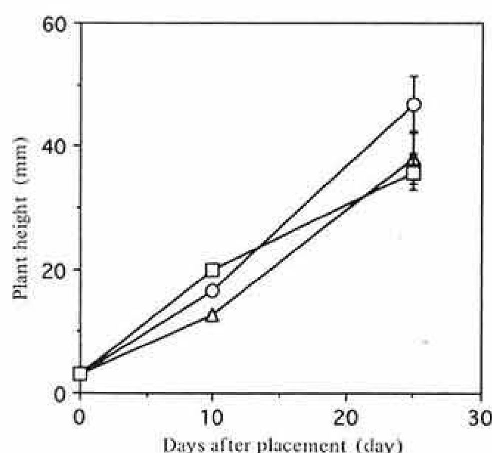

Fig. 5. Relationship between placement methods and the growth of carrot plantlets
○ ; Single encapsulated embryo and △ ; single naked (non-encapsulated) embryo were placed on a support. On each support 8 embryos were placed individually.
□ ; A large number of naked embryos were placed in a cluster. About 40 embryos were placed on a support.
Vertical lines represent standard errors.

of 2.0 N/cm^2 were used. Fig. 5 shows the changes in the height of carrot plantlets after placement of the embryos using the three methods described previously. The height of the plantlets from naked embryos placed in a cluster was the tallest in the three methods at 10 days after placement, but became the shortest at 25 days after placement. In contrast, naked embryos placed individually were the shortest at 10 days after placement and became slightly taller than those placed in a cluster at 25 days after placement. On the other hand, encapsulated embryos grew vigorously next to naked embryos placed in a cluster at the early stage up to the first 10 days, and thereafter they grew up very well and became the tallest in the three methods at 25 days after placement. Table 2 shows the fresh weight, dry weight and dry matter ratio of the top of the plantlets at 25 days after placement. Both fresh and dry weights of the plantlets were maximum in the case of encapsulated embryos, followed by naked embryos placed individually. No differences in the dry matter ratio were observed between these two placement methods. On the other hand, the values of fresh and dry weight and dry matter ratio of the plantlets were the lowest

Table 2. Effect of placement method on the fresh weight, dry weight and dry matter ratio

Placement method	Fresh weight (mg/plant)	Dry weight (mg/plant)	Dry matter ratio (%)
Encapsulated embryo	96.7	9.58	9.91
Single naked embryo	59.2	5.83	9.86
Naked embryos in a cluster	35.7	2.08	5.84

when naked embryos were placed in a cluster.

Thus, it was concluded that the growth of carrot plantlets was promoted by the encapsulation of embryos compared with the placement of naked embryos, for the following reason: As the roots of the embryos had not been developed yet and did not reach the inside of the medium-supports immediately after placement, the embryos became prone to water and nutrient stress. In such a case, it was considered that when embryos were covered with ALG-gel containing the medium, they were not subjected to such a stress, and therefore, the growth of the plantlets was promoted at the early stage. The growth of naked embryos placed individually may be suppressed at the early stage for the reason mentioned above, and their growth could not resume even at a later stage. In the case of cluster placement, the embryos which survived were able to grow well at the early stage. At the later stage, however, the plantlets became spindly because the growth was suppressed due to competition among those in a cluster.

It was concluded that the growth of carrot plantlets was promoted by encapsulation of somatic embryos with Na-ALG and $CaAl_2$ with a hardness value of 2.0 N/cm^2 compared with the growth of plantlets from naked, unencapsulated embryos due to the removal of water and nutrient stress at the early stage when the embryo roots had not yet reached the medium-supports.

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