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High Frequency Plant Regeneration of Garlic (*Allium sativum* L.) Calli Immobilized in Calcium Alginate Gel

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Abstract Calli obtained from a shoot-tip of garlic, *Allium sativum* L., were encapsulated using a calcium alginate gel. Some of the encapsulated calli were cultured on a 1/2 MS medium supplemented with 3% sucrose, 10^{-5} M kinetin, and 5×10^{-6} M NAA, whereas the remainder was stored for 40 days at 4°C. All the naked calli regenerated on the solid medium, while 95% of the encapsulated calli regenerated, and 88% of the encapsulated calli regenerated after 40 days of storage at 4°C. The capsule matrix delayed the germination time of the encapsulated calli, yet activated the shoot formation of the artificial garlic seeds. The shoot length of the encapsulated garlic calli was much longer than that of the naked garlic calli. The encapsulated garlic calli were dried in a laminar airflow cabinet and the conversion frequency of the dried artificial garlic seeds on a 1/2 MS medium remained at 93% with a water loss of less than 50%.

Keywords: artificial seed, callus, garlic, calcium alginate

INTRODUCTION

Since the original concept of artificial seeds was introduced in 1978 [1], artificial seeds have been prepared by various methods, including the fluid drilling method [2], somatic embryo dehydration method [3,4], and hydrogel encapsulation method [5,6]. An artificial seed prepared by coating a somatic embryo using a polymer matrix is a true seed analog. An immobilized somatic embryo can germinate under suitable growth conditions and become a complete plant. The polymer matrix protects the somatic embryo during storage, transportation, and planting. It also contains the nutrients necessary for germination and conversion. A new plant created using gene cloning can be converted into a large quantity of seeds without any inherited damage using these methods. However, hydrated artificial seeds require special care as regards aseptic storage, transportation, and sowing. Nonetheless, some of them still regenerate during cold storage. Therefore, dry-type artificial seeds have been developed, although their regeneration yield is low [7].

Despite many reports on preparing artificial seeds by encapsulating the somatic embryos of dicotyledonous plants, such as carrots, celery, and alfalfa, there have been relatively few reports on the preparation of artificial seeds for monocotyledonous plants, such as rice, barley, and garlic plants, due to the difficulty in preparing somatic embryos of monocotyledonous plants. Recently, rice calli immobilized in a polyurethane foam were *in situ* regenerated [8]. This technique involves three steps: the induction of embryogenic calli, adhesion of the calli to a reticulate polyurethane foam, and *in situ* regeneration of the immobilized calli. Patel *et al.* [9] encapsulated embryogenic calli of *Daucus carota*, as well as calli and shoot tips of *Solamum tuberosum* for the production of artificial seeds.

Garlic plants are monocotyledonous and are easily infected by viruses after multiple vegetative propagations and decay due to various disease germs during storage, thereby resulting in a bad garlic harvest [10,11]. Accordingly, the aim of the current study was to develop an artificial seed by encapsulating a garlic callus, induced on a garlic shoot-tip, using a calcium alginate gel in the hope that the encapsulated garlic callus would germinate and become a healthy plant. This process would also eliminate the hormone treatment essential for the transformation of garlic calli into somatic embryos. The effect of cold storage on the germination of the artificial garlic seeds was tested. Plus an attempt was made to create a dry-type artificial garlic seed that would be more convenient to deal with.

MATERIALS AND METHODS

Callus Induction and Subculture

A garlic bulb (*Allium sativum* L.) was sterilized for 30 min with a 10% sodium hypochlorite liquid. One mm of a shoot-tip was cut off after triple washings with sterilized water. The shoot-tip was inoculated onto an MS agar medium containing 9×10^{-6} M 2,4-D, 1×10^{-5} M NAA, and 9.3×10^{-6} M kinetin, necessary for the induction of the callus, and cultured for a photoperiod of 16 h at 22°C. The pH of all media was adjusted to 5.8 prior

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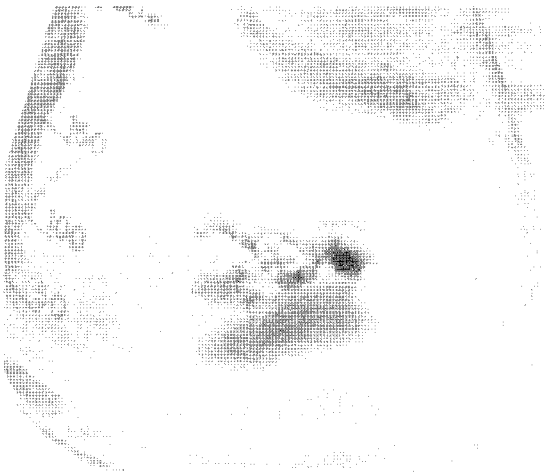

Fig. 1. Compact garlic calli derived from the shoot tip after 4 weeks of culture on modified MS agar medium containing 1 × 10^{-5} M NAA, 9 × 10^{-6} M 2,4-D, and 9.3 × 10^{-6} kinetin at 22°C for a photoperiod of 16 h.

to autoclaving for 20 min at 121°C.

A light-yellow callus began to form on the surface of the shoot-tip after one week, and a hard compact callus was induced at the beginning of the fourth week, as shown in Fig. 1. The induced callus was subcultured for proliferation in an LS agar medium supplemented with 1 × 10^{-6} M 2,4-D and 1 × 10^{-6} M kinetin and then transferred onto a new medium after an interval of 4 weeks. Without screening and proliferation of rapid growing calli [12], the mass-proliferated calli were inoculated for differentiation onto a medium supplemented with 5 × 10^{-6} M NAA and 1 × 10^{-5} M kinetin, and cultured for a photoperiod of 16 h at 22°C. The proliferated calli were cut into pieces of less than 2 mm using a sterilized knife and then cultured in a liquid medium with the same hormone composition for a week with shaking at 90 rpm at 22°C for a photoperiod of 16 h. Thereafter, the calli were placed on a sterilized filter paper and used for the production of artificial seeds.

Encapsulation

The schematic procedure used for the garlic callus encapsulation is shown in Fig. 2. 1.5 wt% sodium alginate was dissolved in a 1/2 MS medium supplemented with 5 × 10^{-6} M NAA and 1 × 10^{-5} M kinetin. The garlic calli were placed in the sodium alginate solution sterilized at 121°C for 20 min. Next, the sodium alginate solution was dropped into a swirling 50 mM $CaCl_2$ solution. Calcium alginate capsules were formed after 30 min and each capsule contained calli. Some of the capsules were inoculated into the 1/2 MS medium, while the remainder was stored at 4°C without photolight.

Dehydration of Capsules

The garlic callus immobilized capsules were dried to

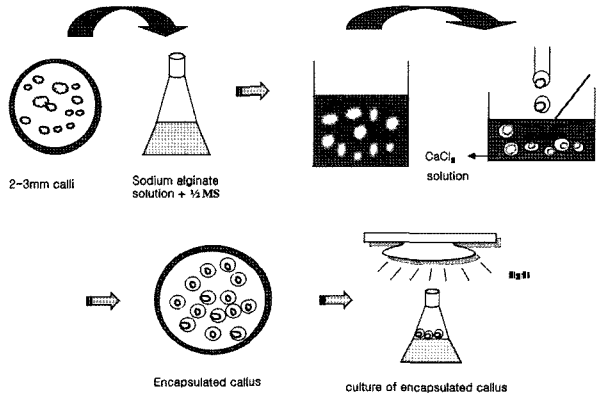

Fig. 2. Schematic presentation of the preparation of encapsulated garlic calli using a calcium alginate gel.

investigate the regeneration frequency of dried garlic artificial seeds according to the amount of water loss. The degree of water loss was defined as the ratio of the weight difference between the original and dried capsules to the weight of an original capsule. The rate of water loss was nearly 0.15-0.2 g/h. The maximum degree of water loss was 90% in this experiment. A dried garlic callus immobilized capsule was inoculated on a 1/2 MS medium supplemented with 4.4 × 10^{-2} M sucrose, 5 × 10^{-6} M NAA, and 1 × 10^{-5} M kinetin and cultured for a photoperiod of 16 h. In the current study, a garlic callus capsule was classified as regenerated if the callus roots pierced through the calcium alginate wall.

RESULTS AND DISCUSSION

Regeneration of Hydrated Garlic Artificial Seed

It has been previously reported that the supply of proper inorganic and carbonic salts is necessary for the regeneration of artificial seeds and their development into a plantlet. An artificial seed needs a proper nutrition supply system analogous to the endosperm of a true seed. Therefore, in the current experiment, a 1/2 MS medium with the proper hormone was immobilized in the calcium alginate matrix by adding nutrients to the sodium alginate solution during the encapsulation step. When encapsulated garlic calli were inoculated onto a solid 1/2 MS medium including the hormone, they regenerated, as shown in Table 1. Meanwhile, the encapsulated garlic calli also regenerated on the solid 1/2 MS medium containing no hormone, however, an excess of nutrients above a certain amount in the MS medium appeared to inhibit the regeneration of the encapsulated garlic callus. This was quite different from the encapsulated embryos of sandalwood and asparagus where the highest germination yields were on a full-strength MS medium [13-15].

Adventitious buds and roots formed on some of the encapsulated calli on the 1/2 MS medium and the cap-

Table 1. Regeneration frequency of encapsulated garlic calli cultured at 22°C for 3 weeks on a solid medium for a photoperiod of 16 h

Culture medium	Regeneration frequency[b] (%)
MS	56
1/2 MS	94
1/2 MS + hormone[a]	95

[a] 5×10^{-6} M NAA and 1×10^{-5} M kinetin
[b] 100 × germinated calli / total calli

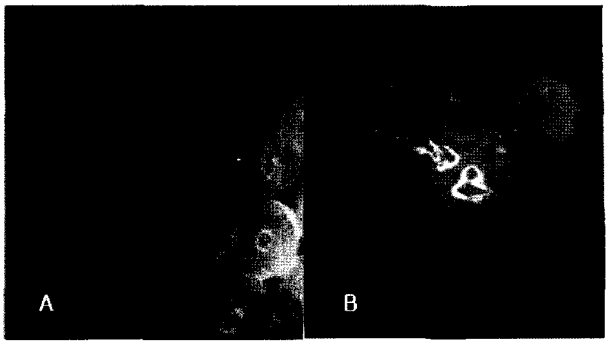

Fig. 3. (A) State of artificial garlic seeds prepared by encapsulating garlic calli using a calcium alginate gel. (B) Regeneration of encapsulated garlic calli after 10 d of culture on a 1/2 MS medium containing 5×10^{-6} M NAA and 1×10^{-5} M kinetin at 22°C for a photoperiod of 16 h.

sules regenerated, as shown in Fig. 3. The regeneration rate of the naked calli was faster than that of the encapsulated calli during the earlier culture period. Only 24% of the encapsulated calli regenerated after 1 week, whereas 62% of the naked calli regenerated on the 1/2 MS medium supplemented with 5×10^{-6} M NAA and 1×10^{-5} M kinetin, as shown in Fig. 4. However, the regeneration rate of the encapsulated calli increased relative to the number of culture days and reached 68% after two weeks, which was close to the 78% with the naked calli. After 3 weeks, 95% of the encapsulated garlic calli regenerated, although roots were formed on all the encapsulated calli, as shown in Table 2. This result reveals that the hormone contained in the calcium alginate matrix was sufficient for the regeneration of the encapsulated calli into a plantlet. After the roots pierced through the capsule matrix, the shoots elongated and became green. The shoot formation yield of the encapsulated garlic calli was 80%, which was much higher than the 44% for the naked calli, as shown in Table 2. As such, the capsule matrix delayed the regeneration time of the encapsulated garlic calli, yet activated the shoot formation of the artificial garlic seeds based on an unknown mechanism. This was very interesting compared to the low germination frequency of embryo-immobilized capsules in which active embryogenesis occurs [16]. Plus the shoot length of the encapsulated garlic calli was much longer than that of the naked garlic calli. This was very similar to a previous report on

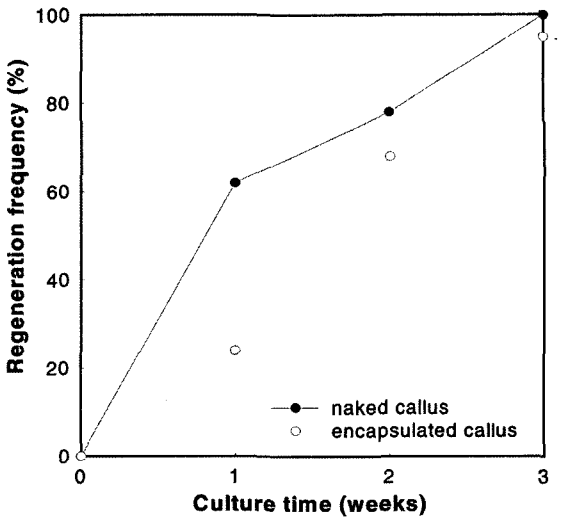

Fig. 4. Regeneration frequency of naked garlic calli on a solid 1/2 MS medium supplemented with 5×10^{-6} M NAA and 1×10^{-5} M kinetin and encapsulated garlic calli on a solid 1/2 MS medium without hormone. The garlic calli were cultured at 22°C for a photoperiod of 16 h.

Table 2. Growth of naked garlic calli on a solid 1/2 MS medium supplemented with 5×10^{-6} M NAA and 1×10^{-5} M kinetin and encapsulated garlic calli on a solid 1/2 MS medium without hormone. The garlic calli were cultured for 3 weeks at 22°C for a photoperiod of 16 h

Calli	Regeneration frequency (%)	Shoot formation (%)	Root formation (%)	Remarks
Naked calli	100	44	100	Multiple short roots, poor shoot growth
Encapsulated calli	95	80	100	Multiple long shoots, good shoot growth, multiple short roots

immobilized rice calli [8]. The shoots of rice calli immobilized in polyurethane foam measured 20 to 40 mm, whereas those of naked rice calli were only 5 mm or less. The regenerated capsules were transferred to an MS medium in a vessel with an air-filter and developed well into plantlets, as shown in Fig. 5. Bulbs were formed after 3 months of culture.

Storage at Low Temperature

There have been many reports that encapsulation is effective in preserving the germination potency of a somatic embryo. The germination frequency of encapsulated buds decreases to 83% after 50 days storage at 5°C, while that of naked buds decreases to 63% [17]. An encapsulated papaya embryo successfully germinated after 85 days storage at 10°C, yet the naked embryo did

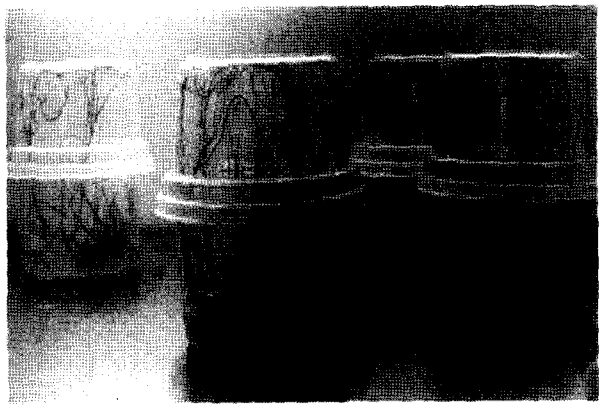

Fig. 5. Well-developed plantlets from encapsulated calli after 3 months of cultivation on a solid MS medium in a vessel with an air filter at 22°C for a photoperiod of 16 h.

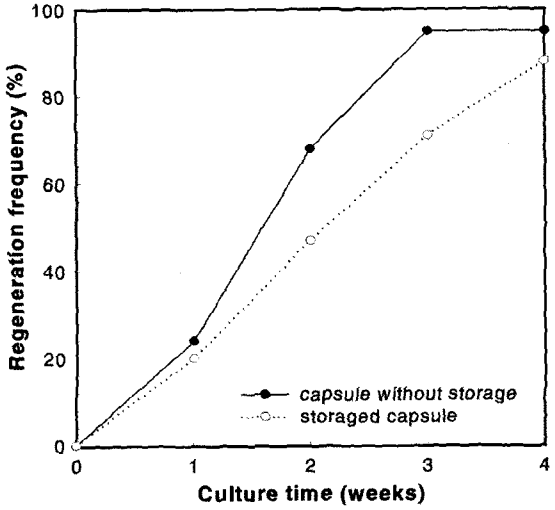

Fig. 6. Regeneration frequency of hydrated encapsulated calli on a 1/2 MS medium and encapsulated calli, that had been stored for 40 d at 4°C, on a 1/2 MS medium supplemented with 5 × 10^{-6} M NAA and 1 × 10^{-5} M kinetin at 22°C for a photoperiod of 16 h.

not [18]. Encapsulated blackberry buds were also found to regenerate after 80 days of storage in an MS liquid medium [19]. Therefore, the current study investigated the effect of cold storage on the regeneration of encapsulated garlic calli.

Garlic calli immobilized capsules were placed in a petri dish, sealed with teflon film, and stored in a refrigerator at 4°C for 40 days. The capsules were then transferred onto a 1/2 MS solid medium supplemented with 5 × 10^{-6} M NAA and 1 × 10^{-5} M kinetin and cultured at 22°C for a photoperiod of 16 h. The regeneration yield of the capsules is shown in Fig. 6. The storage of the capsules at 4°C delayed the germination time by more than one week and decreased the germination yield from 95% to 88%. However, those capsules transferred

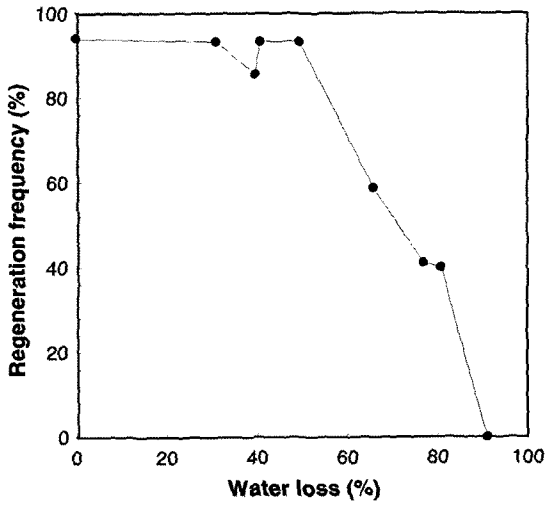

Fig. 7. Regeneration frequency of dry-type artificial garlic seeds relative to the water loss in the capsule. The artificial seeds were cultured on a 1/2 MS medium supplemented with 4.4 × 10^{-5} M sucrose, 5 × 10^{-6} M NAA, and 1 × 10^{-5} M kinetin at 22°C for a photoperiod of 16 h.

onto a solid 1/2 MS medium without any hormone, did not regenerate. This was different from the hydrate capsules without storage, which were able to germinate on a solid 1/2 MS medium containing no hormone. The 88% regeneration yield was not low compared to that of a somatic embryo-immobilized capsule. The germination yield of embryo-immobilized artificial camellia seeds decreased from 90% to 13% after storage at 20-22°C for one month. However, the germination yield after storage at 4°C increased to 63%, which was substantially higher than 13% [16]. The frequency of encapsulated *Santalum album* L. stored for 45 days at 4°C was much lower at 18% [15]. None of the garlic calli immobilized capsules prepared in the current study regenerated during the 40-day storage, although there is a previous report that hydrated embryo-immobilized capsules can begin to germinate after 1 week during storage [7].

Dehydration of Encapsulated Garlic Calli

For the successful commercialization of artificial seeds, problems such as pollution during storage and transportation and a high seed vigor before planting need to be overcome. The regeneration frequency of the encapsulated garlic calli relative to the amount of water loss in the capsule is presented in Fig. 7. The curve was found to be similar to a typical survival curve of a microorganism under stress and agreed well with seed survival curve by Mathews and Powell [20]. Table 3 shows the relationship between the total water loss and the water loss in the calli(micropores). According to the drying theory in a porous system, water drying in a micropore follows fast drying in the macropore. In the current study, the pores in the capsule membrane ma-

Table 3. Relationship between the total water loss in capsules and water loss in the garlic calli

Total water loss (%)	Water loss in calli(micropores) (%)
0	-
30.98	-
39.58	9.82
40.75	11.57
49.45	24.55
65.84	49.0
76.88	65.49
80.8	71.34
91.0	86.57

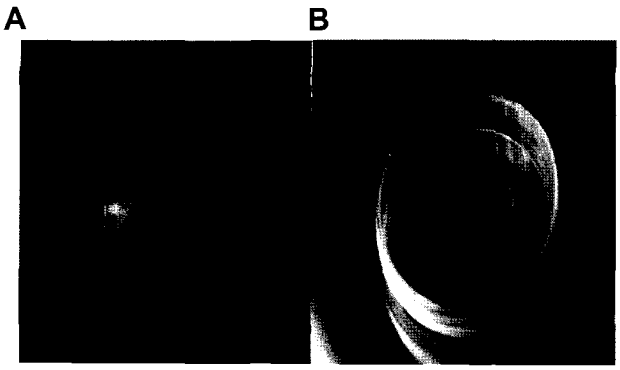

Fig. 8. Regeneration of dry-type artificial garlic seeds *in vitro*. A; Dehydrated capsules (50% water loss), B; Regeneration of dehydrated artificial seeds.

trix were considered as the macropores, while the garlic calli were considered as the micropores. From calculations using the experimental data, 33% of the total water in the capsules was found to be equal to the water contained in the membrane matrix. Thus, a 50% total water loss implied the drying of all the water in the capsule membrane and 25% of the water contained in the calli, as shown in Table 3. Up to a 50% total water loss, the regeneration frequency of the encapsulated garlic calli remained at 93%, as shown in Fig. 7. However, above this value, the regeneration frequency decreased linearly relative to the water loss and reached zero at a 91% total water loss (86.6% water loss in the calli). Although the minimum moisture content in the capsules required for the constant drying rate is assumed to be 67%, the critical water content in the capsules for the maximum plateau value of regeneration frequency of encapsulated calli is 50%. The states of a garlic callus immobilized capsule with a 50% total water loss and an artificial seed that regenerated on a 1/2 MS medium are shown in Fig. 8.

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