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Plant regeneration from alginate encapsulated somatic embryos of *Asparagus cooperi* baker

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Abstract. Somatic embryos of *Asparagus cooperi* were encapsulated as single embryos approximately 4-6 mm in diameter to produce individual synthetic seeds. The frequency of conversion of artificial seeds to plants was 34%. This frequency was affected by the concentration of calcium chloride, the commercial sources of sodium alginate, and the nutrient medium. The conversion frequency of artificial seeds to seedling plants was 8.3% after storage for 90 days at 2°C.

Abbreviations : NAA, α-Naphthaleneacetic acid; ABA, Abscisic acid ; GA_3, Gibberellic acid; Kn, 6-Furfurylaminopurine; MS, Murashige and Skoog (1962) medium; SH, Schenk and Hildebrandt (1972) medium ; WM, White (1963) medium.

Introduction

Artificial seeds, consisting of tissue culture-derived somatic embryos encased in a protective coating have been suggested as a powerful tool for mass production of elite plant species. Advantages of artificial seeds over somatic embryogenesis for propagation include ease of handling and potential long-term storage. In view of the high volume, low cost of production and subsequent propagation, this technique is considered an effective alternative method of propagation (Redenbaugh *et al.* 1987).

Asparagus cooperi is widely used in Asian countries as a medicinal (Kar and Sen 1986) and horticultural plant (Ghosh and Sen 1992a). It is generally vegetatively propagated but multiplication is limited to 1-3 plants per year from a single stock plant. In addition, this species produces flowers at intervals of 12-15 years and propagation through conventional seed propagation is therefore not very practical. Since somatic embryogenesis has been well characterized in *A. cooperi* (Ghosh and Sen 1989, 1991, 1992b), propagation through use of synthetic seed may be desirable.

Materials and Methods

Source of explant. Asparagus cooperi Baker collected from Chandra Nursery, Sikkim, India, was grown at the experimental garden, University of Calcutta. The apical region (2-2.5 cm from the apex) of 15-20 cm tall young spears was discarded and the subjacent 6 cm section of each spear, denuded of lateral leaves, was cut into small discs (4-6 mm) which were used as explants. The explants were disinfested with 0.1% mercuric chloride for 6 min and washed three times with sterile distilled water.

Culture conditions and experimental media. The pH of MS basal medium was adjusted to 5.8 prior to autoclaving at 121°C, 1.05 kg/cm^2 for 15 min. The growth regulators GA_3, zeatin and ABA were filter sterilized prior to addition to the culture media. Cultures were kept under 16/8 h light/dark cycle at 25°C±1° C under fluorescent 40W tubes providing 3000 lux intensity.

Embryo formation and encapsulation. Callus was induced after four weeks of culture on MS basal medium containing 1 mgl^{-1} NAA and 1 mgl^{-1} kinetin. Small pieces (300-400 mg) of calli were subcultured at 40d intervals in the same medium. After the 3rd subculture, embryogenesis was induced in four month old calli by increasing the concentration of KNO_3 from 1900 mgl^{-1} to 2900 mgl^{-1} in the basal medium (Ghosh and Sen 1989, 1991). Embryogenic calli, which formed after 140-160d, were used to establish suspension cultures. About 750-1000 mg of embryogenic callus was placed in a 150 ml Erlenmeyer flask in 30ml of liquid medium containing 1 mgl^{-1} NAA, 1 mgl^{-1} Kn, 0.2 mgl^{-1} ABA and 2900 mgl^{-1} KNO_3. The flasks were placed on gyratory shaker at 120 rpm in the dark at 25°C to initiate suspension cultures. Suspensions were subcultured every 8d and produced 86-90% embryogenic cells. After 8 weeks of active growth, the suspension cultures consisted of somatic embryos of different

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developmental stages (Ghosh and Sen 1992b). The embryos were isolated, blotted for 1 min on filter paper and dipped for a few seconds in a mixture of 2-6% (w/v) sodium alginate (Sigma, Fluka and BDH) in MS medium supplemented with 1 mgl^{-1} zeatin. The different stages of embryos (2-5 mm size) were picked up using a pipette of different (3-6 mm) internal diameter. Each embryo with 0.09-0.15 ml alginate was then dropped into a solution of $CaCl_2$ $2H_2O$ (25, 50, 75 and 100 mM). Each drop (bead) containing a single embryo was maintained in $CaCl_2$ solution for 40 min on a gyratory shaker (80 rpm) in the presence of 1000 lux light intensity.

After the incubation period, the beads were recovered by decanting the $CaCl_2$ $2H_2O$ solution and washing 4-5 times with MS basal medium. Encapsulated embryos were then cultured on 9 cm filter paper soaked with various complete nutrient media (MS, 1/2 MS, SH, 1/2 SH, WM, 1/2 WM) in Petri dishes. Non encapsulated embryos were also cultured in the same media containing as 1 mgl^{-1} zeatin a control. Some sets of encapsulated and non- encapsulated embryos were stored at 2° and 4°C and subsequently cultured on the various nutrient media at 25±1°C.

Each treatment of all experiments were repeated 3 times.

Results and Discussion

Somatic embryos with distinct shoot and root meristems were the most suitable among the various stages (Figs. 1-2) for encapsulation in different concentrations of sodium alginate. Encapsulated embryos (Fig. 3) cultured on MS basal nutrient did not germinate for the first 5 weeks of culture. At the end of 6 weeks, the shoot and root segments of the embryos pierced through the matrix in 28-34% of the beads. Following transfer of germinated encapsulated embryos to filter paper soaked with fresh MS medium, the shoot and root further elongated (Figs. 4-5) and well-developed plantlets were obtained after 10-11 weeks. The conversion frequency of non-encapsulated embryos was 45%.

Effect of sodium alginate and calcium chloride on conversion frequency

The concentration of sodium alginate needed for encapsulation and optimal conversion varied among different commercial sources. Embryos that were encapsulated in 3.5% sodium alginate from Sigma showed the highest conversion percentage (Table-1) compared to alginate from Fluka and BDH. This may be due to differential purity or different varietal algal resource of alginic acid or the variation in the mannuronic acid : guluronic acid ratio (Redenbaugh *et al.* 1986). The concentration of alginic acid also influenced the frequency of conversion due to the hardness factor of the beads. At a higher percentage of sodium alginate (6-7%), beads were harder which may have suppressed the ability of shoots and roots to emerge. Beads of uniform size and shape were obtained when using 3.5% sodium alginate. Similar reports of a maximum plantlet regeneration frequency with an optimum concentration of 3.2% sodium alginate has been reported in alfalfa and celery (Redenbaugh *et al.* 1986) and 3% for *Solanum melongena* (Lakshmana Rao and Singh 1991).

The concentration of complexing agent, $CaCl_2$ $2H_2O$ (calcium) also affected the frequency of conversion of encapsulated embryos (Table-2). Complexing time was longer (60-65 min) when using 25 mM calcium as compared with a complexing time of 35-40 min for 50 mM. If 75 mM and 100 mM calcium were used, the

Table 1. Conversion frequency of somatic embryo encapsulated in different concentrations of sodium alginate of three commercial sources. Complexing agent $CaCl_2$ $2H_2O$ at 50 mM concentration.

SOURCE OF ALGINATE	ALGINATE (%)	CONVERSION (%)
Fluka	4	22.2 (± 1.7)
	5	26.6 (± 1.7)
	6	17.7 (± 1.8)
Sigma	2	28.8 (± 1.8)
	3.5	34.0 (± 2.0)
	4.5	26.6 (± 3.1)
BDH	2	19.9 (± 1.8)
	3	24.4 (± 1.7)
	4	16.2 (± 2.0)
Non encapsulated embryo		44.9 (± 1.4)

LSD at 5% level = 3.7

Table 2. Conversion frequency of somatic embryo encapsulated in sodium alginate in different concentrations of $CaCl_2$ $2H_20$

ALGINATE	CONCENTRATION $CaCl_2$ $2H_20$ (mM)	CONVERSION (%)
3.5%	25	24.4 (± 1.4)
	50	32.2 (± 1.4)
	75	19.9 (± 2.7)
	100	8.8 (± 1.6)
Non encapsulated embryo		44.2 (± 1.7)

LSD at 5% level = 4.7

Table 3. Effect of media on conversion of non encapsulated and encapsulated embryos.

MEDIUM	CONVERSION FREQUENCY (%)	
	NAKED EMBRYO	ENCAPSULATED
MS	45.0(± 0.8)	34.3 (± 1.2)
½ MS	38.3 (± 0.9)	22.0 (± 1.6)
SH	36.6 (± 0.9)	18.6 (± 1.8)
½ SH	26.6 (± 1.2)	12.6 (± 0.9)
WM	31.3 (± 0.4)	16.6 (± 1.8)
½ WM	22.6 (± 1.2)	8.0 (± 1.6)

LSD at 5% level, for media type (A) = 3.02, state of embryo (B) [encapsulated or non encapsulated] = 8.19 and interaction (A×B) = 4.32.

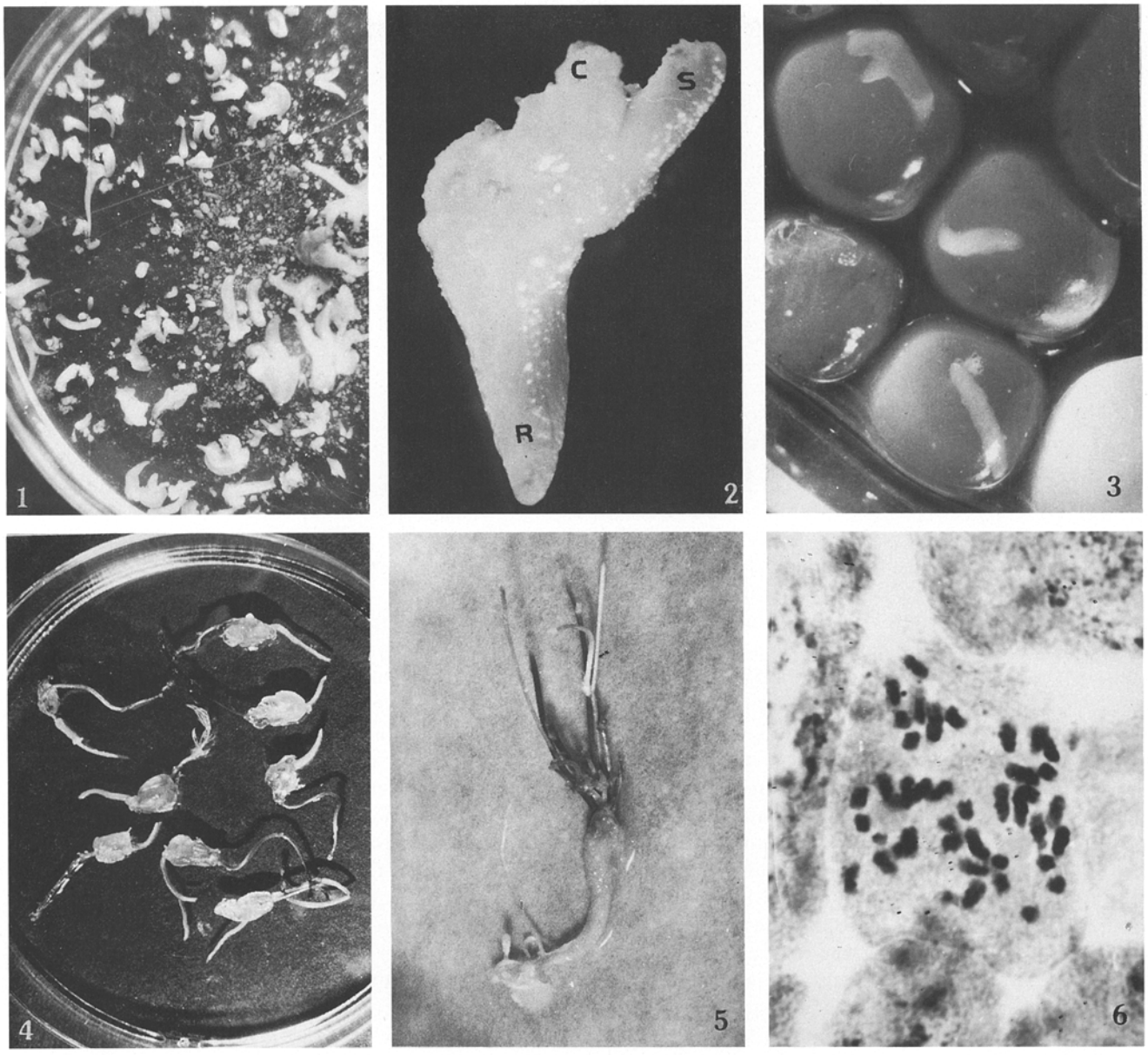

Fig. 1. Somatic embryos of *A. cooperi*, used for encapsulation. **Fig. 2.** Appropriate stage of somatic embryo (S = shoot region, C = cotyledon region, R = root region) for encapsulation and conversion. **Fig. 3.** Artifical seeds. **Fig. 4.** Germination of artificial seeds. **Fig. 5.** A plantlet originating from an artificial seed. **Fig. 6.** Metaphase plate of root tip cell of artificial seed derived plant (2n = 40).

polymerization took place in 15-20 min. The highest plantlet regeneration (32.2%) was obtained with 3.5% sodium alginate and 50mM calcium chloride. This differential response may be due to synergistic effect of alginate and calcium concentration. Both alginate and calcium concentration play a role in complexing time and capsule hardness (Redenbaugh *et al.* 1991). A similar concentration (50 mM) of calcium was used in wheat (Datta and Potrykus 1989). Differential response of conversion with respect to concentration of calcium has also been reported for *Solanum melongena* (Lakshmana Rao and Singh 1991).

Effect of media on conversion frequency

Different basal media were evaluated to increase the

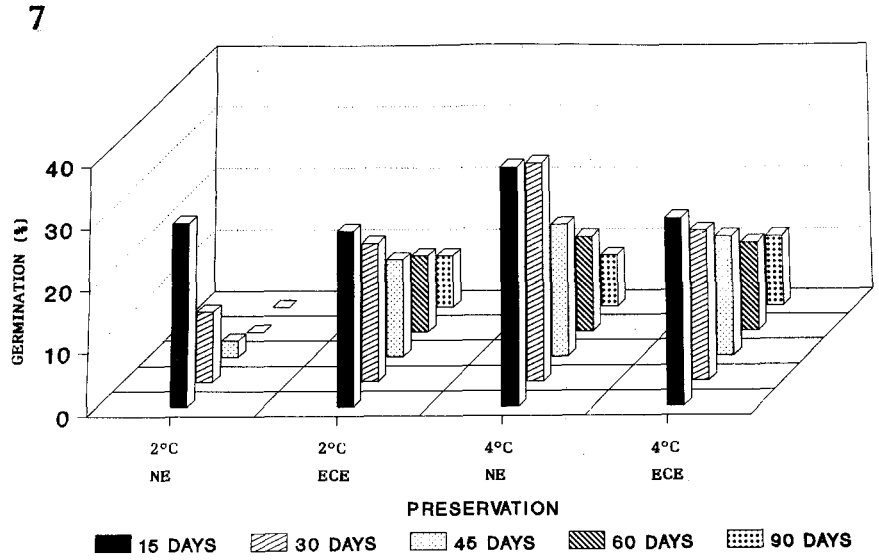

Fig. 7. Effect of temperature and duration of storage on conversion of naked embryo (NE) and encapsulated embryo (ECE). LSD at 5% level, for state of embryo (encapsulated or non encapsulated) (A) = 2.54, for storage temperature (B) = 2.7, for duration of incubation (C) = 1.04 and for interaction (A × B × C) = 2.08.

conversion rate of encapsulated embryos. The conversion frequency of encapsulated embryos and non encapsulated embryos on different conversion media were significantly different (Table - 3). Non encapsulated embryos showed a higher frequency (45%) of conversion of MS basal medium. The lowest conversion rate for non encapsulated embryos (22.6%) was obtained using half-strength White's medium. In comparison to non encapsulated embryo, the conversion frequency for encapsulated embryos was low ; the maximum response was 34.3% on full-strength MS medium and lowest response was 8.0% on half-strength White's medium. Although the percentage of conversion was low (18.6%) on full-strength SH medium, the time required for emergence of shoots and roots was reduced from 6 to 4 weeks. Significant differences were observed in plantlet regeneration from encapsulated and non encapsulated embryos on full and half-strength media. This may be due to the nature of alginate or physical handling of embryos. Non encapsulated embryos are directly in contact with the conversion medium as opposed to an encapsulated embryo. The factor media (A) was characteristically significant for conversion purposes (Table-3). The state of embryo (B) i.e. encapsulated and non encapsulated was also showed a significant role in conversion frequency. Lastly, the interaction between media and state of embryo (A × B) significantly affected the conversion frequency. The use of full-strength MS basal medium for conversion, also gave the highest conversion frequency of artificial seeds in *Solanum melongena* (Lakshmana Rao and Singh 1991) and sandalwood (Bapat and Rao 1983).

Evaluation of storage propagules

The conversion frequency of encapsulated and non encapsulated embryos stored at 2°C and 4°C was variable (Fig. 7). The encapsulated embryos germinated at 30.3% to produce complete plantlets after 15 d storage at 4°C. After 60 and 90 days of storage at 4°C, the conversion frequency was reduced to 14.6% and 11.3% respectively. Non encapsulated embryos converted to whole plants at 38.6%, 15.3% and 8.3% after 15, 60 and 90 days storage respectively at 4°C. Storage of propagules at 2°C however, gave a somewhat reduced conversion frequency. The encapsulated embryos showed 28.3% germination frequency after 15 d storage, 12.3% after 60 d storage and 8.3% after 90 d storage. On the other hand, non encapsulated embryos germinated to produce 29.6% complete plantlets after 15 d storage, 2.6% after 45 d storage and none after 60 d or more. Thus encapsulated embryos could withstand lower temperatures in long term storage compared to non encapsulated embryos (Fig. 7). The encapsulated or non encapsulated state of embryo (A) was significant for germination (Fig. 7). Storage temperature (B) and duration of incubation (C) also had significant effects. Lastly, the cumulative interaction between all three factors (A × B × C) was also highly significant for storage and conversion frequency.

Cytological study

All plantlets derived from both stored and non-stored encapsulated embryos using different conversion media

were morphologically uniform and true to mother plants. Twenty plantlets from stored and 30 plantlets derived from non-stored encapsulated embryos were cytologically stable showing 2n=40 chromosomes (Fig. 6) in somatic cells. Similar stability was also noted in the plants derived from non encapsulated embryos. No structural and numerical variation of chromosomes from the mother plants were noted in all four types, namely stored or non stored, encapsulated and non encapsulated embryo derived plantlets. No noticeable phenotypic variation was observed in the plantlets.

Artificial seed technology for *A. cooperi*, may be useful for propagation to overcome the barrier of seed propagation in this plant.

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