Indian Journal of Experimental Biology
Volume 31, January 1993, pp. 61-64

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Propagation of *Cymbidium giganteum* Wall. through high frequency conversion of encapsulated protocorms under *in vivo* and *in vitro* conditions

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Received 6 January 1992; revised 15 July 1992

Well formed protocorms of an orchid *C. giganteum* Wall. were encapsulated in calcium alginate beads with nutrients of Murashige and Skoog's (MS) medium, growth regulators and antimicrobial agents. Encapsulated protocorms upon transferring either to nutrient medium or directly in sterile sand, and soil mixture, gave rise to healthy plantlets. Conversion frequency was high in both *in vitro* (100%) and *in vivo* (88% in sand and 64% in soil mixture) conditions. The technique described has made it possible to transplant the aseptically grown protocorms directly in the soil, cutting down the cost of raising *in vitro* plantlets and their subsequent acclimatization steps.

The prospect of using plant somatic embryos as artificial seeds has been a subject of great interest[1-4]. Murashige[5] first proposed the concept of encapsulating *in vitro* grown somatic embryos within an artificial seed coat to make it analogous to a true seed. Encapsulation of somatic embryos using various gels have been reported[1-3]. Sodium alginate has been preferred for ease of capsule formation and its low toxicity to the embryo. Though several experiments have been successfully conducted for regeneration of plantlets from encapsulated seeds under *in vitro* conditions, very few soil conversion experiments have been reported using 'artificial seeds'. highest conversion ratio of about 54% was achieved for alfalfa, in soil[6].

Orchids have tremendous horticultural and floricultural importance. Though orchids produce many seeds, less than 5% germinate in nature as the seeds are minute, non-endospermic with reduced embryo (just a mass of cells, not differentiated into cotyledon, root tip and stem tip) and require mycorrhizal association[7]. Moreover, the present day orchids are csomplex hybrids, some of them have genomes of three to four genera. The only viable method for multiplying virus-free orchids at a rapid rate is by mericloning through formation of protocorm-like bodies (plbs) from shoot tips. The present communication describes the plantlet conversion of plbs of *Cymbidium giganteum* Wall. in MS medium or directly in soil following calcium alginate encapsulation.

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Materials and Methods

C. giganteum, a highly ornamental orchid with exquisite long lasting inflorescence of export potential, is predominant in North-East India.

Apical portion from the shoots measuring about 1 cm were cut and washed with teepol (0.05% v/v) and then with running tap water for 1 hr. The explants were surface sterilized with sodium hypochlorite solution (0.2-0.3% available chlorine) for 5 min and washed repeatedly with sterile distilled water. Shoot tips (1-2 mm) were excised aseptically and cultured on Murashige and Skoog's[9] (MS) medium with α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) in combination at 0.5-5 mg/l range of concentration. The cultures were incubated in light (2,000 lux, 14 hr photoperiod) at $25° \pm 2°C$ and 80% RH.

Solutions of 100 m*M* calcium nitrate and 4% sodium alginate were prepared separately in liquid MS medium (without sucrose) containing 1 mg/l each of NAA and BAP. Bactericides, soframycin (0.5 mg/l) and rosebengal (0.1 mg/l) and fungicide, dithane (4 mg/l) were incorporated in the sodium alginate solution individually or in combination. *p*H of the solutions was adjusted to 5.8 before autoclaving at 1.06 kg/cm^2 for 20 min.

Approximately, 90 days old plbs (3-4 mm size) were selected, mixed in sodium alginate solution and dropped in calcium nitrate solution with the help of a small sterilized spoon and gel complexation was allowed to take place for 30 min. The beads were then sieved and plated on (i) MS medium containing 1 mg/l each of NAA and BAP; (ii) sterile sand (0.3 mm size); and (iii) sterile soil mixture (a mixture of sand,

garden soil and cow dung in 1:1:1 proportion mixed with charcoal and a few small brick pieces). Ten beads were placed in each flask/pot. Five replicates were used for each substratum and the experiments were repeated twice. The controls comprised of non-encapsulated protocorms placed on MS medium (with 1 mg/l each of NAA and BAP), sterile sand, and soil mixture. The encapsulated and naked protocorms placed in plastic pots containing sterile sand and sterile oil mixture were supplied with 1/2 MS nutrient salts solutions containing anti-microbial agents, at an interval of 2 days for 2 weeks. The pots were covered with polythene bags to maintain high humidity and water was sprayed twice daily. The pots were kept under culture room conditions described above for 2 weeks and subsequently transferred to glass house maintained at 20°-25°C and 60-70% RH. The development of plbs was divided into six different stages (Table 1).

Results

Shoot tips cultured on MS medium with NAA (0.5 mg/l) and BAP (1 mg/l) produced spherule-like bodies within 10-15 days of culture (unpublished data). A large number of plbs were obtained by cutting and sub-culturing these bodies.

Protocorms mixed in 4% sodium alginate and dropped in 100 mM calcium nitrate solution gave firm, round beads of about 0.6-0.9 cm diam. (Fig.1a). These beads were placed in different substrata for growth and developmental studies.

Table 1—Development of plantlets from non-encapsulated and encapsulated protocorms on different substrata

		Developmental Stages					
		I	II	III	IV	V	VI
		Days required for development					
Nutrient medium	N	10-15	20	35	80	90	110
	E	20-25	30-35	55	70	85	90
Sterile sand	N	Nil	Nil	Nil	Nil	Nil	Nil
	E	8-10	25	60	75	90	120
Sterile soil mixture	N	Nil	Nil	Nil	Nil	Nil	Nil
	E	8-10	25	70	80	110	130

N, non-encapsulated protocorms; E, encapsulated protocorms; Nil, no conversion.

Stages I, protocorms showing pointed vegetative apex (4-5 mm);
II, protocorms with leaf initials (6-8 mm);
III, two leaf stage with root initials (8 mm-1 cm);
IV, three leaf stage with rhizoids (2.0-2.5 cm);
V, four leaf stage (2.5-3.0 cm), and
VI, four leaf stage with well developed leaves (3-3.5 cm)

Under *in vitro* conditions, the encapsulated protocorms required an average of 20-25 days to emerge out of the beads (Table 1) and plantlets of two leaf stage (about 1 cm high) were produced within 55 days (Fig.1b). Sometimes profuse multiplication of plbs from the beads was also observed. Such proliferating protocorms assumed a globular ball like shape. The non-encapsulated protocorms when plated on MS medium, emerged out in 10-15 days and within 35 days plantlets of two-leaf stage were produced. Some of the non-encapsulated protocorms proliferated into numerous protocorms too. The conversion frequencies under *in vitro* condition for both encapsulated and non-encapsulated protocorms were 100% (Fig.2). The plantlets from encapsulated protocorms reached stage IV and V in the medium earlier than the non-encapsulated protocorms. Protocorms of 3 and 4 mm size were suitable for optimum conversion frequency (Fig.3), whereas those of 2 mm size and below either failed to grow or required very long time foremerging out of the capsule. Hence, only big and mature protocorms were selected encapsulation and conversion studies. Under *in vivo* conditions, the

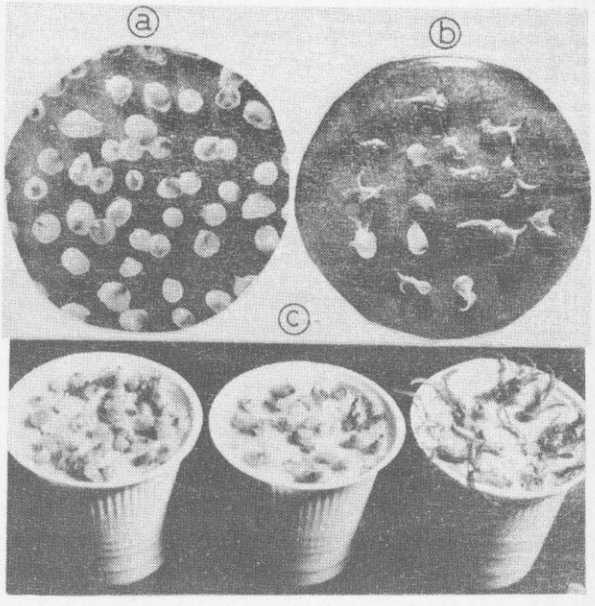

Fig. 1—(a) Protocorms encapsulated in calcium alginate beads; (b) conversion of encapsulated protocorms to plantlets of two leaf stage after 55 days of transfer; (c) conversion of encapsulated protocorms in sterile and (different stages of seedling development)

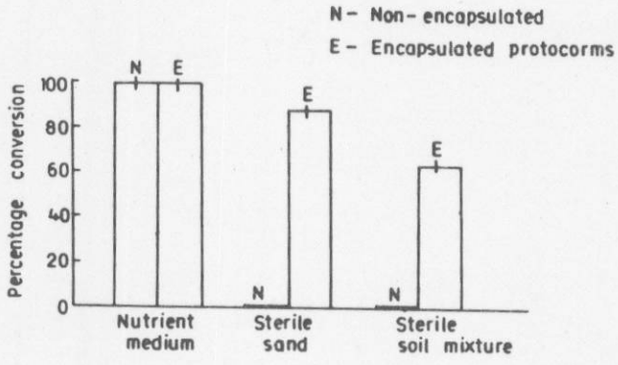

Fig. 2—Conversion frequency of non-encapsulated and encapsulated protocorms in nutrient medium, sterile sand and sterile soil mixture after 55 days of transfer

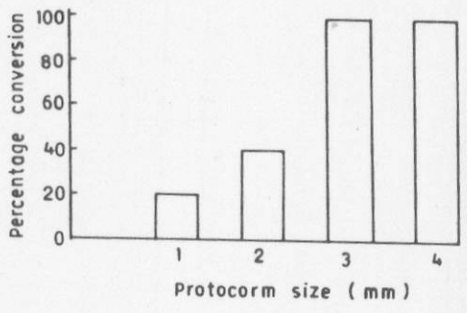

Fig. 3—Effect of size of encapsulated protocorms on conversion frequency on MS media containing both NAA and BAP (each 1 mg/l). Data recorded after 35 days of transfer

non-encapsulated plbs grown in sterile sand or sterile soil mixture did not show any plantlet conversion.

The beads with MS nutrients and sucrose showed severe fungal and bacterial contaimination when placed in sterile sand and soil mixture and did not form plantlets. When sucrose was excluded from the medium the conversion frequency was 44% in sand and 20% in sterile soil mixture (Table 2). However, when antimicrobial agents were added in the medium (MS without sucrose) there was a marked increase in conversion frequency. The multiplication rate of non-encapsulated plbs was not affected by incorporation of antimicrobial agents in the medium. When all the three antimicrobial agents were added together in the matrix of encapsulated plbs, conversion frequencises of 88% in sand and 64% in soil were recorded (Table 2). The capsules placed in sand and soil mixture gradually shrunk and the growing protocorms came out of the capsules in 8-10 days (Table 1, Fig.1c) and attained three-leaf stage (Stage IV) in 75 days in sand and 80 days in soil mixture (Table 1). Forty-six regenerated plants from 'artificial seeds' in soil and sand mixture are growing luxuriantly in the glass-house. The established plantlets have reached a height of 10-12 cm within a period of 1 year.

Table 2—Effect of fungicide and bactericides on conversion of encapsulated and non-encapsulated protocorms under *in vivo* and *in vitro* conditions (+SE)

Fungicide/ Bactericides (mg/l)		% Conversion			
		Media		Sand	Soil Mixture
		N	E	E*	E*
Control (without antimicrobial agents)		100	100	44+0.37	20+0.42
Soframycin	0.1	100	100	54+0.34	35+0.32
	0.2	100	100	48+0.27	40+0.40
	0.3	100	100	60+0.35	48+0.35
Rose bengal	0.1	100	100	58+0.51	42+0.43
	0.2	100	100	42+0.48	26+0.44
	0.3	100	100	40+0.45	28+0.42
Dithane	2.0	100	100	64+0.34	46+0.23
	4.0	100	100	80+0.37	46+0.45
	6.0	100	100	56+0.40	44+0.37
Dithane + Rose bengal + Soframycin (4+0.1+5)		100	100	88+0.12	64+0.16

* non-encapsulated explants failed to survive upon *in vivo* planting

Discussion

The present study is an attempt to plant *in vitro* grown plbs into the soil after encapsulation. The encapsulated protocorms are well protected and the nutrients and hormones in the capsule ensure their ready supply to the growing protocorms. Antimicrobial substances in the capsule provide protection from the microbes.

The 100% conversion frequency and better growth at states IV and V of encapsulated protocorms under *in vitro* conditions indicate that encapsulation is advantageous for the growth of the protocorms. The initial lag (55 days) in protocorm conversion to reach the stage III from the encapsulated protocorms compared with the non-encapsulated ones (30-35 days) may be accounted for the time required for the adjustment of protocorms to the new environment and also for the time taken during the emergence of developing protocorms through the beads. The reason for higher growth rate of encapsulated protocorms may probably be due to the availability of abundant nutrients and growth regulators in the immediate cell surroundings.

The ability of encapsulated protocorms to establish plantlets in soil (conversion frequency 88% in sand and 64% in soil mixture) under culture room and glass house conditions shows promise for direct

field planting of tissue culture grown protocorms. Non-encapsulated protocorms failed to grow in soil despite proper growth conditions and supply of nutrients. The smaller sized encapsulated plbs showed poor conversion frequency due to immature nature of the developing plb which could not withstand encapsulation. This limitation was overcome by taking larger, well-developed protocorms of 3-4 mm diameter.

Inclusion of antimicrobial agents increased conversion frequency in sand and soil mixture. The highest conversion for alfalfa was 53-54% in soil[6]. Earlier Mathur *et al.*[10] reported 64% conversion of *Valeriana wallichi* shoot-tips and axillary buds using rosebengal and bavistin as antimicrobial agents. In the present experiment, we could increase the conversion frequency by incorporating fungicide and bactericide. The combination of rosebengal, soframycin and dithane was most effective than using any of these alone.

Encapsulated protocorms could revolutionize the propagation system for orchids. This technique has made it possible to place the aseptically grown protocorms directly in the soil for plantlet regeneration. The bypassing of *in vitro* plant production and acclimatization step make this technique economically and practically viable which could be exploited by commercial growers. Further, a large number of encapsulated protocorms could be easily transported in low bulk with minimum damage and at much reduced cost.

Acknowledgement

This study was partly supported by a research grant to PT from North-Eastern Council, Ministry of Home Affairs, Government of India.

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