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Production of Synthetic Seeds by Encapsulating Asexual Embryos of Carrot

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Abstract. **Synthetic seed coats were applied to asexual embryos of carrot (*Daucus carota* L.) by mixing equal volumes of embryo suspension and a 5% (w/v) solution of polyethylene oxide (Polyox WSR-N 750) and dispensing 0.2 ml drops of this mixture onto teflon sheets. Drops dried to form detachable wafers consisting of embryo suspension embedded in Polyox. Embryo survival after drying was determined by redissolving wafers in embryogenic medium and culturing the rehydrated embryo suspension on filter paper supports in petri dishes for 2–3 weeks. When dried to constant weight (6.5 hr) 3% of asexual embryos coated with 2.5% Polyox survived encapsulation, whereas survival of uncoated embryos was nil. Pretreating the embryogenic suspension with 10^{-6} M abscisic acid (ABA) during the 14 day embryo induction phase increased coated embryo survival to 40% of the initial number of embryos.**

Asexual embryos can be produced for various crops using in vitro techniques (7). Morphological development of asexual embryos produced in vitro, and of zygotic embryos of the same species produced in vivo is similar (12, 13, 25), although in vitro-produced asexual embryos differ from in vivo-produced seeds by the absence of seed coats.

The proliferation and development of asexual embryos in vitro led us to speculate that embryos of exalbuminous-seed species could be transformed into seeds if a synthetic seed coat could be provided. The objective of this study was to investigate the feasibility of producing synthetic seeds by coating asexual embryos. Carrot was chosen as the test species, because methods for initiating and maintaining cell and asexual embryo cultures were well established (8, 10, 12, 19).

Materials and Methods

Plant material. Two sources of carrot callus were used in these experiments: 'White Belgium' and an unknown cultivar in which callus was initiated from roots using techniques described by Dougall (4).

Culture methods. Basal medium consisted of the inorganic salts of Murashige and Skoog (20) with the following addenda, per liter: pyridoxine·HCl, 0.5 mg; thiamine·HCl, 0.4 mg; nicotinic acid, 0.5 mg; *i*-inositol, 100 mg; and sucrose, 20 g. Callus was initiated on basal medium containing 1 g/liter Difco Bacto agar, 1.0 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/liter 6-furfurylamino purine (kinetin). Callus was maintained in darkness at 25°C on gelled basal medium supplemented with 0.1 mg/liter 2,4-D and 0.2 mg/liter kinetin (maintenance medium) and recultured every 4–8 weeks in petri dishes (60 × 15 mm).

Cell suspensions were initiated from callus using the techniques of Gamborg (9) in a liquid basal medium containing 0.1 mg/liter 2,4-D and 0.2 mg/liter kinetin. Cell aggregates, ranging from 0.15 to 1.00 mm (in diameter), were inoculated at a density of 0.2 g/25 ml; cell suspensions were recultured every 14 days.

Asexual embryos were initiated by culturing 14-day-old stationary-phase cell suspension (0.15 to 1.00 mm) at a density of 0.4 g/25 ml in a hormone-free basal medium. Embryogenic suspension, containing cells, cell aggregates, callus clumps and embryos of varying maturity (Fig. 1), was recultured every 3 days during a 2-week embryo induction phase. Liquid cultures were maintained on a gyratory shaker (120 rpm) at 29°C and a photoperiod of 16 hr (16 μmol $s^{-1}m^{-2}$ of photosynthetically active radiation) from cool-white fluorescent lamps. Media were autoclaved for 30 min at 121° and 124 kPa. ABA, when used, was filter sterilized and added to cooled medium.

Coating material. Polyox WSR-N 750 (Union Carbide Corp., New York) was chosen as a suitable synthetic coating compound for asexual embryos of carrot, based on previous results with in vivo-produced embryos of 'Troyer' citrange (16) and preliminary experiments with in vitro-produced embryos of 'White Belgium' carrot (15). Polyox dried to form a thin, embryo-encapsulating film, was nontoxic, and readily redissolved in aqueous solutions. Polyox-coated embryo suspension dried to form a wafer (Fig. 2) that could be handled with forceps. Drying time necessary for wafer formation differed due to variability in ambient temperature and humidity. Suspensions without Polyox (noncoated controls) did not form wafers when dried and

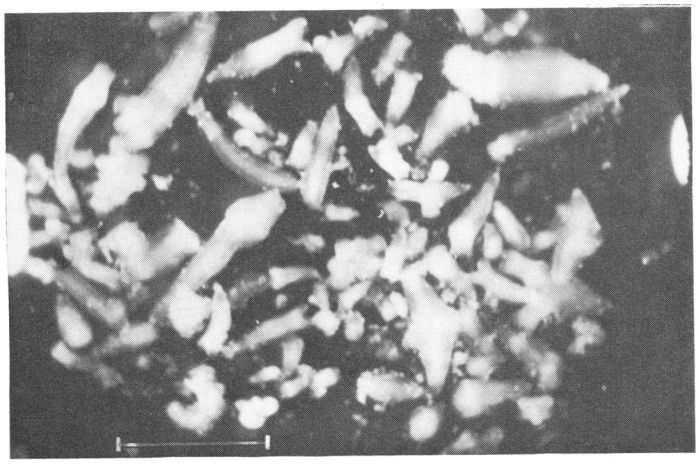

Fig. 1. Asexual embryos of carrot from suspensions prior to coating with Polyox (bar = 1 mm).

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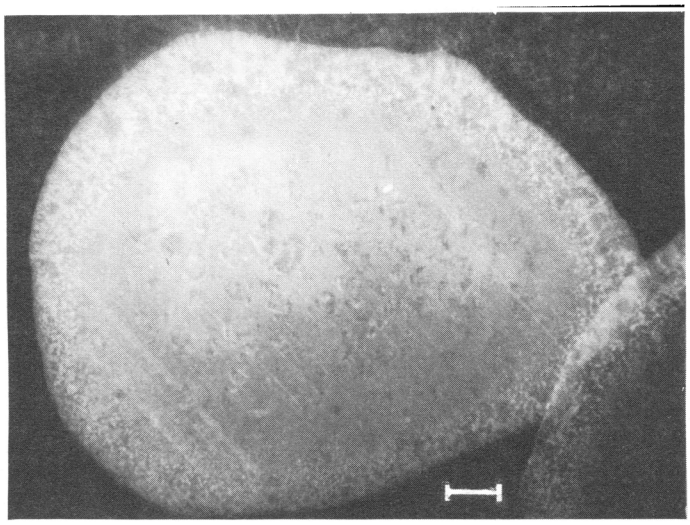

Fig. 2. Polyox wafer containing embryogenic suspension (bar = 1 mm).

were detached from the teflon sheet with a spatula. Polyox was redissolved by suspending wafers in embryogenic medium and agitating for 14 hr. Rehydrated embryo suspensions were collected, cultured on moist filter paper supports, and grown for 3 weeks at 25°C with a 16 hr photoperiod at 22 μmol $s^{-1}m^{-2}$. Initial embryo counts were made before Polyox was added, and final counts were made after the 3-week growth period. The initial embryo numbers for each treatment were estimated by counting globular and more mature stages in two or three 0.1 ml samples. Final embryo counts for reconstituted wafers were made after the 3-week growth period. Counts from reconstituted wafers were the actual total number of embryos observed. The percentage of survival of encapsulated embryos was based on initial embryo counts. The protocol for encapsulating embryos is summarized in Fig. 3.

Results

The most favorable Polyox concentration for embryo encapsulation was determined by mixing an embryogenic suspension of 'White Belgium' in medium with equal volumes of water (control) or Polyox to produce final Polyox concentrations of 0.5%, 1.25%, and 2.5%. Higher concentrations of Polyox were not used because extreme viscosity made them difficult to handle. Polyox-embryo suspension mixtures were dispensed as 0.2-ml drops onto teflon sheets and dried for 0, 4, 5, or 6 hr. Control

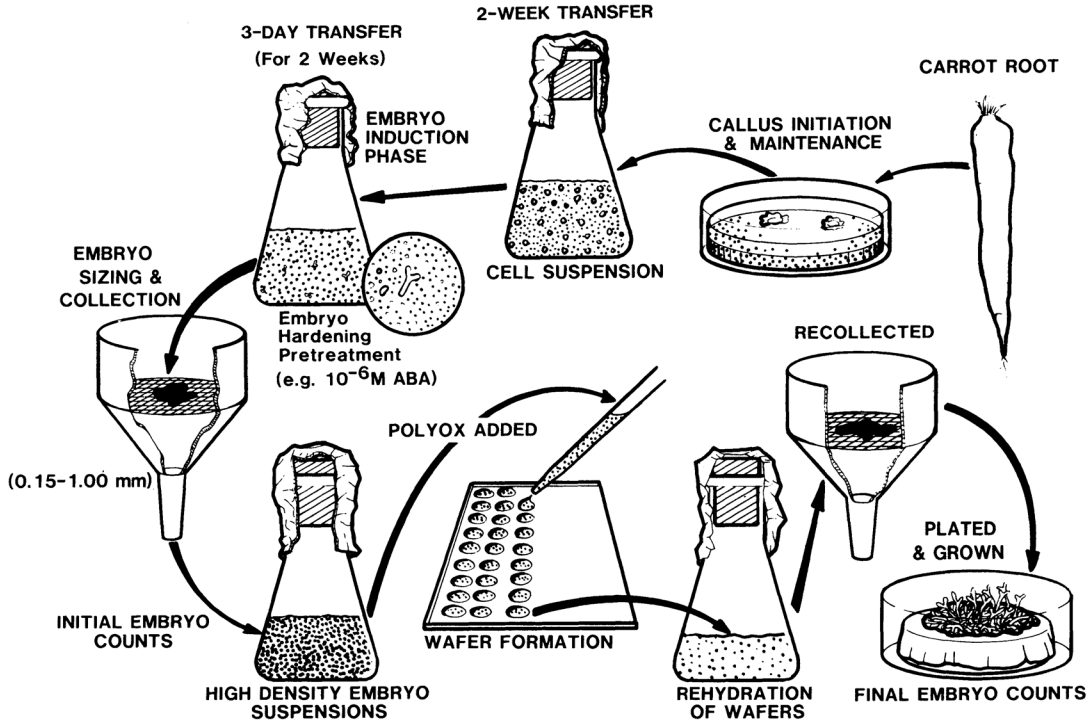

Fig. 3. *Protocol for carrot embryo encapsulation.* Callus induced from secondary phloem of carrot is recultured onto maintenance medium. Cell cultures are initiated from callus and maintained in basal medium containing 2,4-D (0.1 mg/liter) and kinetin (0.2 mg/liter), and transferred to fresh medium every 2 weeks. Embryos are initiated and grown by placing 0.4 g of cells and cell aggregates (0.15 to 1.00 mm) per 25 ml of medium without either 2,4-D or kinetin and reculturing every 3 days for 2 weeks. Embryos can be hardened by pretreating with 10^{-6} M ABA during this induction stage. Embryogenic suspension ranging in size from 0.15 to 1.00 mm is mixed in equal volumes with 5% Polyox to achieve a final concentration of 2.5%. Embryo suspension plus coating compound is dispensed as 0.2 ml drops onto teflon sheets and dried to wafers in a laminar flow hood. Drying time is based on the ability of wafers to separate from teflon sheets. The time necessary to form a dried, detachable wafer varies with humidity and temperature, but is not less than 5 hr. Wafers are placed in embryogenic medium to dissolve the coating compound and to rehydrate embryos. Rehydrated embryo suspensions are collected on screens with 0.15 mm spaces, scooped-up with a spatula, and dispensed on filter paper supports in petri dishes containing 3 ml of medium. Petri dishes are sealed with Parafilm and placed in a growth room.

embryos did not survive drying (Table 1). Although some embryos coated with each concentration of Polyox survived 4 hr of drying, only embryos coated with 2.5% Polyox survived 5 or 6 hr of drying. A final Polyox concentration of 2.5% therefore was used in all subsequent coating experiments.

Embryogenic cell suspensions were initiated from a root of an unknown carrot, and the 3 most embryogenic clones were selected for encapsulation trials. Embryogenic suspension from each clone was coated with water (control) or 2.5% Polyox and dried for 0, 6, or 8 hr. No control embryos survived 6 or 8 hr of drying (Table 2). Polyox-coated embryos from clone 4 did not survive drying, some embryos from clones 1 and 5 survived 6 hr of drying, whereas a few embryos from clone 5 survived 8 hr of drying. Clone 5 was chosen for all further encapsulation tests.

Embryo survival during 13 hr of drying was compared for noncoated and Polyox-coated embryos (Fig. 4). Control embryos had an increased death rate, with no embryos surviving 7 or more hours of drying. Survival of Polyox-coated embryos decreased with increased drying time, but some embryos survived as long as 13 hr. Similar death curves were obtained when drying time was extended to 32 hr (Fig. 5). The largest drop in viability for both noncoated and Polyox-coated embryos occurred during the first 2 hr of drying. Noncoated embryos did not survive 8 hr of drying, but some Polyox-coated embryos survived 32 hr of drying. The drop in moisture content for both noncoated and Polyox-coated wafers was greatest during the first 2 hr, and constant weight was attained after 4 hr (Fig. 6). When wafers were oven dried (75°C, 24 hr), the additional moisture loss was 4% for noncoated controls and less than 1% for Polyox wafers.

Embryo suspensions of carrot were left untreated or were pretreated with 10^{-7} or 10^{-6} M ABA during the 2-week embryo induction phase in an effort to block precocious germination

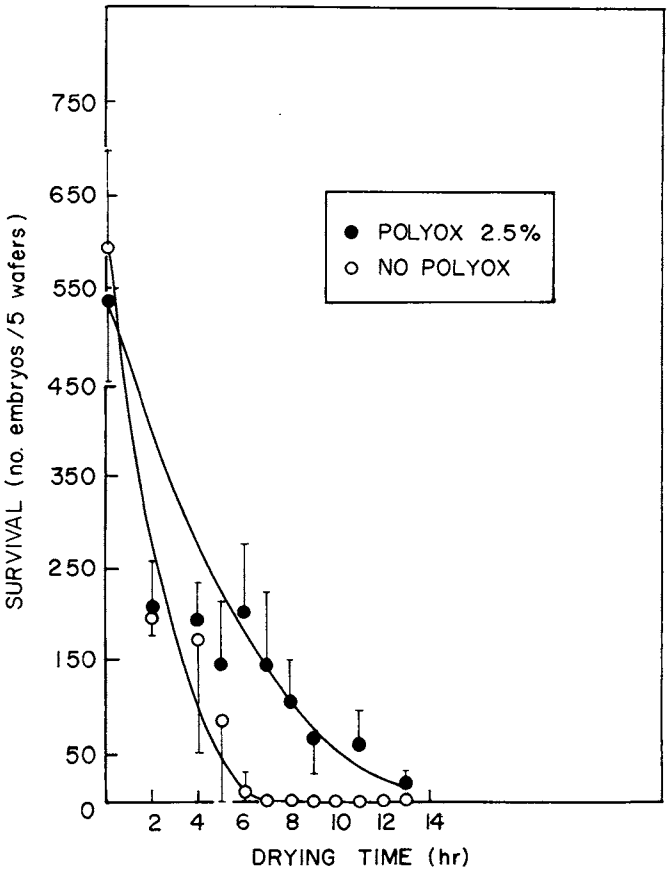

Fig. 4. Effect of Polyox encapsulation on survival of carrot embryos over a 13-hr drying period (± SD).

Table 1. Effect of Polyox concentration on survival of 'White Belgium' carrot embryos.

Polyox concn (%)	Embryo survival (no./30 wafers) Hours of drying 0	4	5	6
0.0	4	0	0	0
0.5	---	2	0	0
1.25	---	1	0	0
2.5	---	4	2	2

Table 2. Survival of asexual embryos from 3 embryogenic clones derived from the same carrot root after Polyox encapsulation.

Coating treatment	Drying time (hr)	Survival (no. embryos/10 wafers) ± SD Clone 4	Clone 1	Clone 5
No Polyox (control)	0	10→85[z]	4→34	31→99
	6	→ 0	→ 0	→ 0
	8	→ 0	→ 0	→ 0
Polyox 2.5%	6	→ 0	→ 7±9	→ 8±5
	8	→ 0	→ 0	→11±8

[z]Initial embryo count → embryo count after 8 weeks (see Fig. 3).

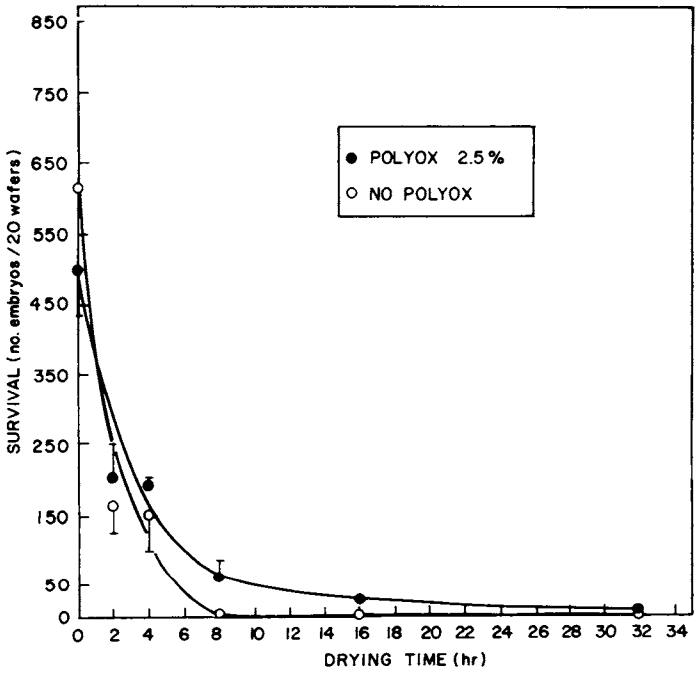

Fig. 5. Effect of Polyox encapsulation on survival of carrot embryos over a 32-hr drying period (± SD).

and increase survival during encapsulation. Fewer embryos were produced as ABA concentration increased from 0 to 10^{-6} M (Table 3). This difference persisted after 3 weeks of growth in petri dishes, although final embryo counts converged.

Noncoated embryos, with or without a 10^{-7} M ABA pretreatment, did not survive 5 or 7.5 hr of drying (Table 3, Expt. 1), and less than 1 noncoated control embryo/10 wafers survived 5 hr of drying when pretreated with 10^{-6}M ABA. Polyox-coated embryos dried for 5 hr did not survive without ABA pretreatment, but 8% survived with 10^{-7} M ABA and 2% survived with 10^{-6} M ABA. No coated embryos survived 7.5 hr of drying. These results suggested that ABA pretreatment increased survival of coated embryos. In a repeat experiment (Table 3, Expt. 2), only coated embryos survived 5 or 6.5 hr of drying. Survival was higher at 5 than at 6.5 hr for 0 and 10^{-7} M ABA concentrations, and at both drying times, survival was higher at 10^{-6} M than at 10^{-7} M ABA. All subsequent ABA pretreatments were carried out at 10^{-6} M.

The influence of ABA-pretreatment time on subsequent survival of encapsulated embryos was examined (Table 4). Embryo suspensions were either untreated or treated with 10^{-6} M ABA for the first 9 days, the entire 15 days, or the last 6 days during the embryo induction phase. Embryos from noncoated controls of each treatment did not survive 6 hr of drying. Embryos from Polyox-coated embryo suspensions, whether pretreated with ABA or not, remained viable during the encapsulation process. Survival was 0.8% with 0 ABA, 3% with ABA for the first 9 days, 1% with ABA for the entire 15 days, or 10% with ABA for the last 6 days (Table 4, Expt. 1). In a repeat experiment (Table 4, Expt. 2), embryos from all noncoated controls did not survive 7.5 hr of drying, but embryos from Polyox-coated embryo suspensions from all 4 treatments survived encapsulation. Survival was 2% without ABA, 3% with ABA for the first 9 days, 11% with ABA for the entire 15 days, or 4% with ABA for the last 6 days. These experiments suggested that ABA treatment was less effective at the beginning than at the end of the embryo induction phase.

Embryogenic suspensions, untreated or pretreated with 10^{-6} M ABA, were sieved into 3 size ranges: (mm ± SD) 0.2 ± 0.1, 0.4 ± 0.2, and 0.6 ± 0.4, in order to determine embryo size that best survived encapsulation. Embryo suspension from each size range was resuspended in medium at a density of 0.29 g/ml and encapsulated with 2.5% Polyox. Embryo suspensions pretreated with 10^{-6} M ABA were better able to survive Polyox

Table 3. The effect of Polyox-coating and a 14-day ABA pretreatment on survival of carrot embryos (clone 5).

Coating treatment	Drying time (hr)	Survival (no. embryos/10 wafers) ± SD		
		ABA		
		0 M	10^{-7} M	10^{-6} M
Expt. 1				
No Polyox (control)	0	215→379[z]	127→300	93→282
	5	→ 0	→ 0	→ 0.7±1 (0.8%)[y]
	7.5	→ 0	→ 0	→ 0
Polyox 2.5%	5	→ 0	→ 10±5 (8%)	→ 2.0±1 (2%)
	7.5	→ 0	→ 0	→ 0
Expt. 2				
No Polyox (control)	0	102→190	98→158	78→165
	5	→ 0	→ 0	→ 0
	6.5	→ 0	→ 0	→ 0
Polyox 2.5%	5	→ 10.3±7 (10%)	→ 11.0±10 (11%)	→ 21.0±3 (27%)
	6.5	→ 3.3±4 (3%)	→ 2.7±0.6 (3%)	→ 31.0±14 (40%)

[z]Initial embryo count → embryo count after 3 weeks (see Fig. 3).
[y]Percentage of survival in parentheses based on initial embryo count.

Table 4. Survival of asexual embryos of carrot pretreated with ABA for different lengths of time during a 15-day induction-growth cycle and encapsulated with Polyox.

Coating treatment	Drying time (hr)	Survival (no. embryos/10 wafers) ± SD			
		Time grown with 10^{-6} M ABA			
		0 days	First 9 days	15 days	Last 6 days
Expt. 1					
No Polyox (control)	0	370→197[z]	358→211	590→242	483→323
	6	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	6	→ 3±3 (0.8%)[y]	→ 12±8 (3%)	→ 6±4 (1%)	→ 50±7 (10%)
Expt. 2					
No Polyox (control)	0	975→475	685→762	765→664	765→699
	7.5	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	7.5	→ 19±7 (2%)	→ 19±5 (3%)	→ 83±18 (11%)	→ 28±18 (4%)

[z]Initial embryo count → embryo count after 3 weeks (see Fig. 3).
[y]Percentage of survival in parentheses based on initial embryo count.

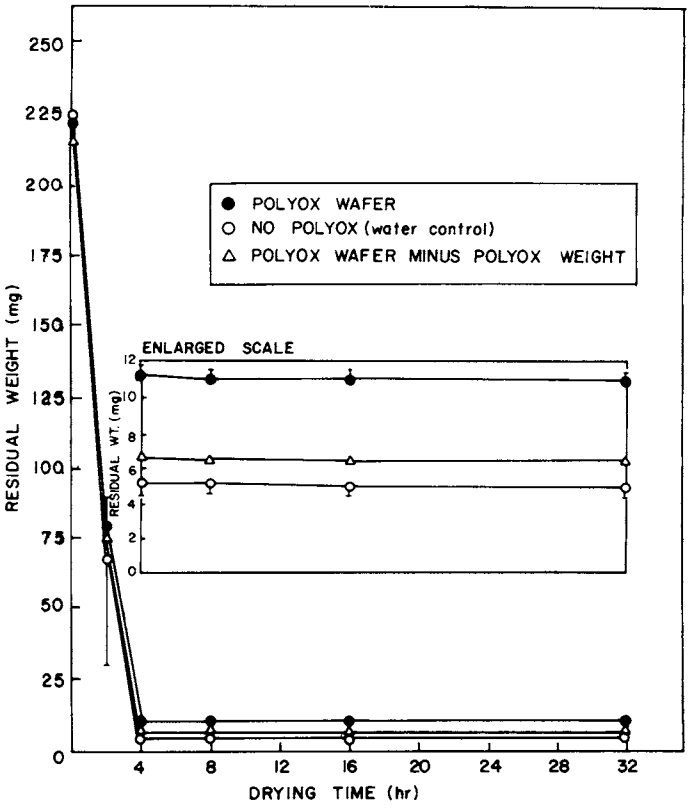

Fig. 6. Effect of Polyox encapsulation on weight loss of carrot embryo suspension over a 32-hr drying period (± SD).

coating and 7 hr of drying than nonpretreated suspensions (Table 5). The largest embryos had the greatest survival whether they were pretreated with ABA or not. The data from 0 and 10^{-6} M ABA were combined, and relative survival, based on the observed freqency of control embryos, was calculated. Relative survival efficiency of large embryos was 9 times that of medium embryos and 12 times that of small embryos.

Discussion

The low but consistent survival of asexual carrot embryos encapsulated with Polyox was the most significant result of this study. The coating results achieved with in vitro-produced asexual embryos of carrot support the data obtained with in vivo-produced citrus embryos (16) and verify the potential of Polyox as a synthetic coating compound. Polyox-coated carrot embryos survived for at least 32 hr (Fig. 2). Polyox seems to provide embryo protection by forming a thin film, perhaps preventing lethal embryo desiccation. Encapsulated embryos, like seed, surely must retain a minimum moisture content in order to remain viable.

Although Polyox coating consistently enabled asexual embryos of carrot to withstand desiccation, survival was low. Survival was improved by ABA pretreatment. ABA had 2 obvious effects: 1) a reduction in the number of embryos produced with increasing concentration, and 2) increased survival of encapsulated embryos. The increase in the percentage of survival, however, varied widely from one experiment to another.

Reduction in asexual embryo number with increasing ABA concentration (Table 3) has been reported previously (8, 12, 13). Reduction in frequency of individual developmental stages indicated that ABA retarded embryo development, however, but did not inhibit embryo initiation. Although fewer embryos were produced by embryo suspensions pretreated with ABA, in comparison to those not pretreated, a greater percentage of those embryos survived Polyox encapsulation (Table 3). The physiological basis for this enhancement of survivability can be inferred from the hardening effect attributed to ABA (14, 26, 27). ABA regulates many activities late in embryogeny associated with maturation. An increased endogenous ABA level is associated with a reduction in precocious germination in carrot (13) and cotton (5). Exogenous ABA blocks precocious germination of embryos produced in vivo (22, 23) and in vitro (2, 13), which suggests that ABA induces embryo quiescence. ABA-treated embryos, while quiescent, continue to synthesize specific proteins associated with embryonic growth and seed maturation (3, 24). Increased survival of encapsulated ABA-treated carrot embryos may be due to the imposition of a developmental arrest (quiescence) during which time embryos mature and develop desiccation resistance.

During maturation, embryos produced in vivo acquire a resistance to desiccation (1) that has been associated with high endogenous ABA levels (6, 17). Long (18) has shown that immature zygotic embryos of *Phaseolus* acquired desiccation resistance during a 3-day exposure to 10^{-5} M ABA while embryos were in liquid medium and under no apparent water or nutrient stress.

Carrot embryos germinated and grew after encapsulation and rehydration, indicating that the growth-retarding effect of ABA

Table 5. Effect of embryo size and ABA pretreatment on embryo survival after encapsulation with Polyox.

Coating treatment	Drying time (hr)	Initial mean embryo size (mm)	Survival (no. embryos/10 wafers) ± SD		
			ABA 0	ABA 10^{-6} M	Mean
No Polyox (control)	0	0.6	940→ 720[z]	640→784	790→752
		0.4	1140→1002	888→972	1014→987
		0.2	560→ 536	620→840	590→688
Polyox 2.5%	7	0.6	→ 5.0±3.7 (0.5%)[y]	→ 9.4±7.5 (1.5%)	→ 7.2 (0.9%)
		0.4	→ 0.8±0.8 (0.1%)	→ 1.2±1.1 (0.1%)	→ 1.0 (0.1%)
		0.2	→ 0.0 (0%)	→ 0.8±0.8 (0.1%)	→ 0.4 (0.1%)

[z]Initial embryo count → embryo count after 2.5 weeks (see Fig. 3).
[y]Percentage of survival in parentheses based on initial embryo count.

is reversible in the present system. This reversible effect of exogenous ABA on embryo development has been reported previously (2, 21).

The results of this study demonstrate that asexual embryos can be encapsulated with a water soluble resin and survive desiccation. The production of synthetic seed by encapsulating asexual embryos might have practical applications in agriculture. These include the production of hybrid seed for those species where conventional techniques are difficult or as a delivery system for genetically-engineered genotypes produced by in vitro techniques.

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