J. AMER. SOC. HORT. SCI. 110(2):283–286. 1985.

Hardening Treatments Increase Survival of Synthetically-coated Asexual Embryos of Carrot

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Additional index words. *Daucus carota*, tissue culture, embryogenesis, polyethylene oxide, sucrose, chilling, inoculum density, storage

Abstract. **Asexual embryos of carrot (*Daucus carota* L.) were initiated from cell suspensions and hardened by treatments with high sucrose (12% as compared to 2%), high inoculum density (0.8 as compared to 0.4 g/25 ml), or chilling (4°C the last 3 days of a 2-week embryo induction phase otherwise carried out at 29°), individually or in combination with 10^{-6} M abscisic acid (ABA). Carrot embryos either were encapsulated with a water-soluble resin (Polyox WSR-N 750) or nonencapsulated before drying to constant weight. Nonencapsulated embryos did not survive drying. All hardening treatments increased survival of encapsulated embryos. However, survival of embryos treated with ABA plus each of the other 3 hardening treatments was less than expected, assuming additivity. Encapsulated embryos pretreated with ABA and chilling survived 16 days of storage in darkness at 4°.**

Asexual embryos of carrot encapsulated with polyethylene oxide (Polyox WSR-N 750, Union Carbide Corp., N.Y.) survived desiccation to constant weight to produce "synthetic seeds" (16). Although a low percentage of embryos, usually less than 4%, survived Polyox encapsulation, survival was increased by hardening embryos with 10^{-6} M ABA during embryo initiation and development. The purpose of this study was to examine other treatments, alone or in combination with ABA, as hardening agents and to determine the storage life of encapsulated embryos.

Materials and Methods

Cell suspensions from the root of an unknown carrot cultivar designated clone 5 were initiated and maintained as described by Kitto and Janick (16). Control embryos were initiated by inoculating 0.15 to 1.00 mm diameter cell aggregates at a density of 0.4 g/25 ml into medium containing 2% (w/v) sucrose at 29°C. Three hardening treatments were applied to these embryogenic suspensions alone or in combination with 10^{-6} M ABA during the 14-day embryo-induction phase. Presumptive hardening treatments included a high inoculation density of 0.8 g/25 ml, a high sucrose concentration of 12%, or chilling at 4° the last 3 days of the embryo induction phase. Cultures were maintained under a 16 hr photoperiod (16 μmol $s^{-1}m^{-2}$ of photosynthetically active radiation from cool-white fluorescent lamps); during the 3-day chilling period cultures received constant light at 2.6 μmol $s^{-1}m^{-2}$ from tungsten lamps. When provided, ABA was filter sterilized and added to cooled medium.

Each of the 3 hardening treatments was tested in 2 experiments separated over time; within an experiment, each treatment had 5 replicates, except for the 1st experiment with sucrose, which had 4 replicates. The percentage of survival of encapsulated embryos was based on initial embryo counts. Initial embryo counts were made before Polyox was added, and final counts were made after the 3-week growth period.

Received for publication 11 June 1984. Journal Paper No. 9919 of the Purdue Univ. Agr. Expt. Sta. This research was supported by a grant from the Amer. Seed Trade Assn. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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The protocol for encapsulating and rehydrating asexual embryos has been described previously (16). Embryo suspension plus coating compound was dispensed as 0.2 ml drops onto teflon sheets that dried to wafers in a laminar flow hood. The drying time necessary to form a detachable wafer varied because of humidity and temperature differences.

In the wafer storage experiments, Polyox-encapsulated embryos were placed in petri dishes and stored in darkness at the temperature and time periods specified. In the 1st experiment, embryos were prechilled at 4°C in the dark and stored for 0, 24, 36, 48, 74, or 98 hr. In the 2nd experiment embryos were both prechilled and treated with 10^{-6}M ABA and stored for 0, 1, 2, 4, 8, or 16 days.

Results

Inoculum density. Embryo suspensions inoculated at 0.8 g/25 ml produced fewer embryos than at 0.4 g/25 ml, with or without the addition of ABA in both experiments (Table 1). Nonencapsulated embryos initiated at either density, with or without ABA treatments, did not survive drying. Either high inoculum density or ABA increased survival of encapsulated embryos. However, survival of embryos receiving both high inoculum density and ABA was less than expected had the treatments been additive. In the 2nd experiment, survival of embryos receiving both treatments was less than from either treatment alone.

Sucrose. Embryo suspensions grown in medium with 12% sucrose with or without 10^{-6}M ABA produced fewer embryos than did control suspensions grown with 2% sucrose in both experiments (Table 2). Noncoated embryos of all treatments failed to survive drying. Without ABA, survival of Polyox-coated embryos was 2–3 times higher with 12% than with 2% sucrose. Survival of embryos grown with 2% sucrose was increased by ABA in both experiments, but survival was much higher in Expt. 2 than in Expt. 1. Survival of embryos initiated both with high sucrose and ABA was less than that of the control in Expt. 1 and less than 10^{-6} M ABA alone in Expt. 2.

Chilling. Embryo suspensions treated either with ABA, chilling, or both, had fewer initial embryos than did nontreated controls (Table 3). Once again, nonencapsulated embryos did not survive 7 hr of drying (Table 3, Fig. 1). Embryo survival was increased markedly in both experiments (42- and 13-fold) when suspensions were chilled prior to encapsulation. Increased survival was obtained with or without ABA. In both experiments,

Table 1. Survival of asexual embryos of carrot initiated at high or low inoculum density with or without ABA and encapsulated with 2.5% Polyox.

Coating treatment	Drying time (hr)	Survival (no. embryos/10 wafers) ± SD			
		No ABA		10^{-6} M ABA	
		Density		Density	
		0.4g/25 ml	0.8g/25ml	0.4g/25ml	0.8g/25ml
Expt. 1					
No Polyox (control)	0	2600→ 1020[z]	760→ 1360	1200→ 1360	620→ 1220
	11.5	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	11.5	→ 28 ± 15 (1.1% a)[y]	→ 127 ± 22 (16.7% c)	→ 132 ± 38 (11.0% b)	→ 129 ± 27 (20.1% c)
Expt. 2					
No Polyox (control)	0	930→ 1180	340→ 870	1150→ 1215	320→ 1050
	7	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	7	→ 212 ± 21 (22.7% a)	→ 175 ± 21 (51.5% c)	→ 499 ± 95 (43.4% b)	→ 85 ± 12 (26.6% a)

[z]Initial embryo count → embryo count after 3 weeks.
[y]Percentage of survival in parentheses based on initial embryo count. Mean separation in rows by Duncan's multiple range test, 5% level. Analysis of percentage data based on arc sine transformation.

Table 2. Survival of asexual embryos of carrot pretreated with 12% vs. 2% sucrose with or without ABA during a 2-week induction-growth cycle and encapsulated with 2.5% Polyox.

Coating treatment	Drying time (hr)	Survival (no. embryos/10 wafers) ± SD			
		No ABA		10^{-6} M ABA	
		Sucrose		Sucrose	
		2%	12%	2%	12%
Expt. 1					
No Polyox (control)	0	495→ 101[z]	392→ 114	318→ 65	333→ 86
	6	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	6	→ 16 ± 11 (3.2% a)[y]	→ 42 ± 16 (10.7% b)	→ 16 ± 17 (5.0% a)	→ 7 ± 4 (2.1% a)
Expt. 2					
No Polyox (control)	0	940→ 736	900→ 380	400→ 480	610→ 290
	7	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	7	→ 29 ± 5 (3.1% a)	→ 57 ± 11 (6.3% b)	→ 232 ± 35 (58.0% d)	→ 99 ± 24 (16.0% c)

[z]Initial embryo count → embryo count after 3 weeks.
[y]Percentage of survival in parentheses based on initial embryo count. Mean separation in rows by Duncan's multiple range test, 5% level. Analysis of percentage data based on arc sine transformation.

there was no influence of ABA alone. The combination of ABA and chilling gave inconsistent results; no effect was observed in Expt. 1 but survival improved in Expt. 2.

Wafer storage and embryo viability. The influence of wafer storage time on embryo viability is shown in Tables 4 and 5. In Expt. 1, survival of encapsulated embryos declined with storage time; however, some encapsulated embryos within wafers remained viable after 98 hr of storage (Table 4). In Expt. 2, Polyox wafers containing embryo suspension pretreated both with 10^{-6} M ABA and chilling were stored in darkness at 26° or 4°C for up to 16 days. Embryos within wafers survived up to 4 days storage at 26° and at least 16 days at 4° (Table 5).

Discussion

Hardening carrot embryo suspensions by exposure during the induction phase to high inoculum density, high sucrose, or chilling increased survival of embryos after Polyox encapsulation, relative to nonhardened embryos (Table 6). ABA pretreatment improved survival of asexual embryos after Polyox encapsulation in 5 of the 6 experiments reported (Tables 1–3), although the magnitude of the response was highly variable, confirming previous findings (16). Exogenous ABA affects in vivo and in vitro embryo development by inhibiting precocious germination (1, 4) and increasing desiccation resistance (17), thereby allowing the completion of maturation.

Carrot embryo suspensions inoculated at high density, with or without ABA, produced fewer embryos initially than at low density (Table 1), but there was little difference in total number of embryos between high or low density cultures after the 3-week growth period. Inoculation density seems to affect embryo development rather than embryo initiation (10). In the present study, embryos from suspensions initiated at a high density and then encapsulated with Polyox were better able to survive drying

Table 3. Survival of asexual embryos of carrot pretreated with chilling (4°C) vs. nonchilled during the last 3 days of a 2-week induction-growth cycle with or without ABA and encapsulated with 2.5% Polyox.

Coating treatment	Drying time (hr)	Survival (no. embryos/10 wafers) ± SD			
		No ABA		10^{-6}M ABA	
		Nonchilled	Chilled	Nonchilled	Chilled
Expt. 1					
No Polyox (control)	0	630→ 439[z]	487→ 406	540→ 255	177→ 187
	7	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	7	→ 8±5 (1.3% a)[y]	→ 140±41 (28.7% b)	→ 7±3 (1.3% a)	→ 51±31 (28.8% b)
Expt. 2					
No Polyox (control)	0	1220→ 504	980→ 502	1160→ 562	680→ 526
	7	→ 0	→ 0	→ 0	→ 0
Polyox 2.5%	7	→ 3±5 (0.2% a)	→ 25±5 (2.3% b)	→ 3±1 (0.3% a)	→ 54±22 (7.9% c)

[z]Initial embryo count → embryo count after 3 weeks.
[y]Percentage of survival in parentheses based on initial embryo count. Mean separation in rows by Duncan's multiple range test, 5% level. Analysis of percentage data based on arc sine transformation.

stress. Apparently, high inoculum density influenced embryo maturation by creating a favorable developmental environment that may be related to medium conditioning (8, 9).

Sucrose plays 2 roles in vitro: as a carbon source and as an osmotic agent. Increased osmotic stress has been associated with cell plasmolysis (18), cell growth inhibition (23), and the elevation of endogenous ABA levels (18, 23) when applied in vitro. Exposure of embryos to high sucrose has been linked to maturation (6), inhibition of precocious germination (2), and acquisition of desiccation tolerance (19). Thus, high osmotic potential, imposed by 12% sucrose (0.35 M), could increase endogenous ABA as well as cause dehydration of asexual embryos of carrot.

Chilling plant tissue may mimic some effects of cold hardening that plants acquire outdoors as temperatures decrease in the fall. The 1st measurable events occurring during cold acclimatization are cessation of growth (13), accumulation of carboyhydrate (24), and reduction of moisture content (11).

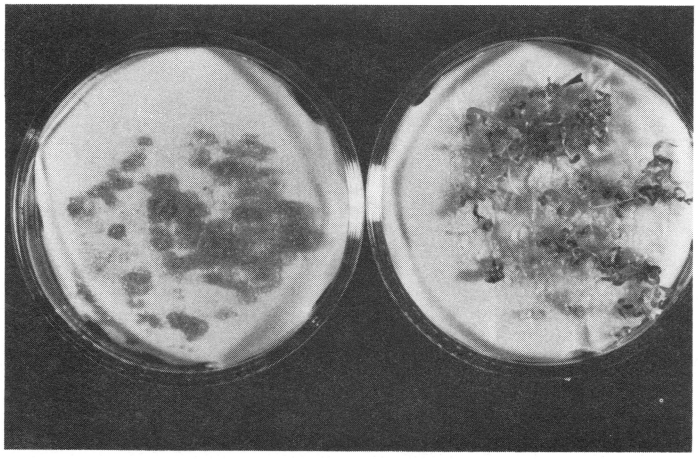

Fig. 1. Encapsulation increased survival of chilled asexual embryos of carrot dried to constant weight. (**Right**) Three-week-old carrot "seedlings" grown from prechilled embryos encapsulated with Polyox WSR-N 750. (**Left**) Chilled but nonencapsulated embryo suspension did not survive drying.

Associated with the reduction in intracellular water is an increased tolerance of the protoplasm to dehydration (3). Cold hardening is associated with ABA in some species. *Phaseolus vulgaris* plants exposed to 5°C had increased ABA levels (7), and the morphological appearance of alfalfa seedlings was similar either when cold-hardened or ABA-treated (20).

Although there is little published information to explain high inoculum density effects on embryos, there is evidence that high sucrose and chilling act indirectly through elevation of endogenous ABA. Both high sucrose concentration and cold hardening affect ABA synthesis through increased osmotic pressure (22, 23). Increased ABA levels prevent precocious germination,

Table 4. Survival of prechilled, encapsulated carrot embryos dried for 7 hr and stored for up to 98 hr in darkness at 4°C.

Storage at 4°C (hr)	Survival/14 wafers	
	No.	%
0	35	100[z]
24	2	6
36	3	8
48	8	23
74	1	3
98	1	3

[z]Represents 4% survival from initial embryo count (nonencapsulated, nondried).

Table 5. Survival of encapsulated carrot embryos pretreated with 10^{-6}M ABA plus chilling, dried for 7 hr, and stored in darkness up to 16 days at 26° or 4°C.

Storage (days)	Survival/20 wafers			
	26°C		4°C	
	No.	%	No.	%
0	7	100[z]		
1	0	0	2	29
2	1	14	1	14
4	2	29	1	14
8	0	0	1	14
16	0	0	2	29

[z]Represents 1% survival from initial embryo count (nonencapsulated, nondried).

Table 6. Summary of survival of embryos pretreated with ABA, high density, high sucrose, or chilling and encapsulated with Polyox.

ABA (M)	Mean survival[z] by hardening treatment (%)	
	Density	
	0.4g/25ml	0.8g/25ml
0	11.9	34.1
10^{-6}	27.2	23.3
	Sucrose	
	2%	12%
0	3.2	8.5
10^{-6}	32.0	9.2
	Chilling	
	0 day	3 day
0	0.8	15.5
10^{-6}	0.8	18.4
	Mean of hardening treatments	
	Low	High
0	5.3	19.4
10^{-6}	20.0	17.0

[z]The 2 experiments in each hardening series were averaged.

suggesting that embryos become quiescent. Quiescent embryos continue to synthesize proteins associated with embryonic growth and seed maturation (5). Individual hardening treatments imposed on asexual embryos of carrot may suspend growth, cause dehydration, but allow maturation. "Mature" asexual embryos encapsulated with Polyox were better able to survive drying than "immature" embryos (15). Encapsulated carrot embryos grew after rehydration, indicating that the growth-retarding effect of the hardening treatments was reversible.

Although individual hardening treatments increased embryo survival, the combinations tested (ABA + high inoculum density, high sucrose, or chilling) resulted in lower embryo survival than would be expected if there had been a positive, additive interaction (Table 6). ABA at high concentrations (10^{-5} to 10^{-4} M) inhibited the formation and growth of asexual embryos of caraway (1) and carrot (14), and when combined with a 2nd hardening agent reduced plant hardiness of maple (12) and alfalfa (20). One possible explanation for the absence of additivity in the present study (Table 6) is that asexual embryos of carrot treated with ABA in combination with another hardening regime have supra-optimal ABA levels.

Embryos within wafers dried to constant weight survived storage in darkness for as long as 16 days at 4°C compared to 4 days at 26° (Table 5). Orthodox seeds remain viable over long periods if both seed moisture content and storage temperature are reduced (21). More research is needed to define the storage characteristics of encapsulated carrot embryos and to determine the precise relationship between viability and time limits of the system. The fact that encapsulated embryos survived is encouraging and indicates that they behaved similar to true seeds.

The successful encapsulation and storage of embryos has a number of implications for the seed industry. Additional studies will be needed to better define hardening treatments, coatings, storage conditions, and to determine applicability to a range of germination environments.

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