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Cryopreservation of immature spring wheat zygotic embryos using an abscisic acid pretreatment *

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Abstract. Spring wheat (Triticum aestivum L.) zygotic embryos were successfully cryopreserved, without the addition of exogenous cryoprotectants, using only an abscisic acid (ABA) pretreatment. Optimum survival was obtained when embryos were cultured in vitro for 10 days on semi-solid Murashige and Skoog (MS) nutrient medium supplemented with 0.5 mg/L (±) ABA prior to cryopreservation. The embryos resumed growth within three days when returned to MS medium devoid of ABA but containing 2mg/L 2,4-dichlorophenoxyacetic acid. The embryogenic calli produced from these embryos exhibited normal plant regeneration on auxin-free media. Changes in dw/fw ratio, as well as the esterified fatty acid and sucrose concentrations correlated positively with the development of tolerance to cryopreservation.

Key words: cryopreservation, *Triticum aestivum*, abscisic acid.

Introduction

In the past vegetatively propagated plants and valuable natural accessions were the chief candidates for cryopreservation. Now, with the rapid progress in plant transformation, cryopreservation will find wide use in preserving the experimental material of primary transformed tissue, stably transformed cell lines and secondary cultures derived from desirable transformed phenotypes. Preservation of transgenic germplasm in this way will ensure that these genetic resources will be available should cultural practice result in the loss of a transformed phenotype.

Increasingly with cereals, the primary tissue used for transformation studies is the zygotic embryo (Chibbar et al. 1991, Christou et al. 1991, Kartha et al. 1989, Vasil et al. 1992). It is totipotent, genetically uniform, highly regenerative and culture conditions are well established. These features also render zygotic embryos excellent candidates for cryopreservation.

For cryopreservation a priori requirements are that survival be at an acceptable level and, that surviving cells or organs undergo faithful plant regeneration. In conventional cryopreservation protocols, the biological samples are initially treated with cryoprotectants and the samples frozen in a cryoprotective solution in order to reduce intracellular water and enhance intracellular solute concentrations (Kartha 1985). One of the difficulties with this approach is that the types and concentrations of cryoprotectants must be empirically determined for each species (Kartha 1985).

Abscisic acid (ABA) has been shown to reduce cellular water and enhance solute accumulation while inducing freezing tolerance in cultures of a number of winter annual and perennial species (Tanino et al. 1990; Reaney et al. 1989; Keith and McKersie 1986; Chen and Gusta 1983; McLaren and Smith 1976). ABA has also been shown to confer tolerance to cryopreservation in cell cultures derived from winter wheat (Triticum aestivum L. cv. Norstar, 16% survival, Chen et al. 1985) and bromegrass (Bromus inermis Leyss cv. Manchar, 80% survival, Reaney and Gusta 1987). However, these species are known to possess an inherent ability for cold acclimation to very low temperatures. The use of ABA has not been reported for less hardy species such as spring wheat.

This laboratory has recently shown (Qureshi et al. 1989) that ABA enhances somatic embryogenesis in cultures of spring wheat (Triticum aestivum L.) embryos harvested between 15 and 25 days after anthesis. The stress tolerance related properties of the somatic embryos have not been investigated. However, Senaratna and co-workers report that somatic embryos, induced by ABA in alfalfa suspension cultures, exhibit a high degree of tolerance to desiccation stress (Senaratna et al. 1989). We have noted that, in spring wheat, ABA treated embryos have excellent regeneration activity and are therefore ideal candidates for cryopreservation. These observations prompted us to investigate whether ABA treatment could induce an adequate level of freezing protection for cryopreservation.

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Materials and methods

Plant material. The spring wheat (Triticum aestivum L.) breeding line, SWP9302, was a gift from the Saskatchewan Wheat Pool (Saskatoon, Canada). Growth conditions for the plants as well as procedures for the isolation and culture of immature embryos have been described previously (Qureshi et al. 1989).

Cryopreservation. Five embryos, forming an experimental unit, were aseptically transferred to 5 cm plates and placed in a CRYO-MED programmable freezer where they were cooled to -35 °C at 60 °C/h. The plates containing the embryos were then transferred to liquid nitrogen for one hour. The tissue was thawed at room temperature before being returned to semi-solid MS medium devoid of ABA but containing 2 mg/L 2,4-D (2MS). Viability was assessed on the basis of regrowth after 3 weeks.

Freezing tolerance. Experimental units were formed from 5 embryos aseptically transferred to 5 cm plates. The plates were placed in a CRYO-MED programmable freezer where they were cooled at 6 °C/h. Three plates were removed at 2 °C intervals and the embryos permitted to thaw at room temperature. The embryos were incubated overnight on 2MS medium before being assayed for viability.

The viability assay was adapted from Steponkus and Lamphear (1969). Each experimental unit was weighed and then transferred to a 15 mL centrifuge tube containing 3 mL of 0.6% triphenyltetrazolium chloride in Sodium-Potassium phosphate buffer pH 6. After 20h, the cells were washed in distilled water, homogenized and the reduction product, formazan, extracted using 7 mL of 95% ethanol. Survival was calculated from the normalized (per gram fresh weight) absorbance (530 nm) values of the extracts from frozen embryos, compared to non-frozen controls. The LT_{50} values refer to the temperature where 50% of an experimental unit was killed.

Sugar analysis. Embryos or calli (ca. 0.05 g) were washed in distilled water to remove superficial sucrose, frozen in liquid nitrogen, then ground to a fine powder using a chilled pellet homogenizer (Mandel Scientific, Canada). The sugars were extracted, derivitized and analysed by GLC on a DB1701 column (J&W Scientific) as described previously (Adams et al.,1990).

Lipid analysis. Embryos or calli (ca. 0.05g/sample) were powdered as described above. Lipids were extracted into chloroform:methanol (2:1; 3 X 0.5 mL) containing margaric acid (0.5 umoles) and cholestane (0.5 umoles) as internal standards. The extracts were partitioned with 0.5mL (0.6%) sodium chloride, dried under helium and the residues taken up in 1.0 mL chloroform:methanol (2:1). The fatty acid and sterol compositions of the samples were determined by gas-liquid chromatography as described previously (Kendall and McKersie 1989).

Results

Spring wheat zygotic embryos harvested 15 to 25 days after anthesis, were highly susceptible to the severe stress of cryopreservation (programmed cooling at 60°C/h to -35°C followed by immersion in liquid nitrogen, Table 1). At lower cooling rates (6°C/h) these embryos had demonstrated an LT_{50} near -5°C (Table 2). Their level of sensitivity to cryopreservation did not change significantly during 30 days growth in vitro on MS medium (Table 1) even though considerable callus formation occurred. In contrast, embryos cultured for 7 d in the presence of ABA (0.5 mg/L), developed a high level (83% survival) of tolerance to the cryopreservation protocol (Table 1). This tolerance persisted for an additional 16 days in culture. Ten days incubation in the presence of 0.5 mg/L ABA also reduced the LT_{50} to less than -35°C when cooled at 6°C/h in conventional freeze-thaw tests (Table 2).

Control embryos that survived ultra-low temperature storage often produced watery, non-embryogenic callus. This material did not undergo plant regeneration. In contrast, the ABA treated embryos that survived immersion in liquid nitrogen, produced high quality embryogenic callus. This callus exhibited normal plant regeneration on hormone-free medium.

Table 1. Survival of SWP9302 spring wheat zygotic embryos after cryopreservation with and without abscisic acid pretreatment.

Culture period (days)	No pretreatment	Abscisic acid pretreatment
	% Survival	
0	16a	0a
7	18a	83b
12	20a	98b
16	0a	78b
20	10a	100b
22	0a	100b
30	0a	0a
40	0a	0a

*Values in a row or column followed by the same letter are not significantly different at the 5% level according to the LSD test.

The embryos were analyzed to determine if the development of ultra-low temperature tolerance was accompanied by changes in chemical composition. Freshly plated immature wheat embryos were approximately 27% dry matter (Fig. 1d). After 7 days growth on MS medium, resulting in callus initiation, the dry weight percentage declined to about 12% (Fig 1d).

Exposure to ABA (0.5 mg/L) delayed by nearly two weeks the decline in dry matter (%) and the initiation of callus formation. At 12 d, when the scutella of ABA-treated embryos were just beginning to expand, dry matter was still near 27% (Fig. 1d). At 1 mg/L ABA these effects were not enhanced but persisted longer in the cultures. At still higher ABA levels (4 mg/L) long-lasting inhibition of callus development was observed. Analysis of soluble sugars extracted from freshly harvested embryos revealed that mono- and di- saccharides contributed about 10% to their dry weight.

Sucrose was the major extractable sugar species forming 60% (24 mg/gfw) of the extract (Fig. 1b). While the total soluble sugar level in control embryos did not change significantly during the 30 d observation period, a major change in composition was noted after 5 d (Fig. 1c). At this time and for the balance of the test period sucrose levels declined to roughly 30% (12 mg/gfw) of the total sugars

Table 2. The effect of ABA on the sensitivity of the spring wheat line SWP9302 to freeze-thaw stress.

Growth Medium	Days in Culture	LT_{50}(°C)[b]
1. embryos harvested 10 days post anthesis		
2 MS[a]	0	- 4
2 MS	10	- 6
2 MS + 0.5 mg/L ABA	10	< -35
2. embryos harvested 15 - 20 days post anthesis		
2 MS	0	- 5
2 MS	10	- 8
2 MS + 0.1 mg/L ABA	10	< -35
2 MS + 0.1 mg/L ABA	20	< - 8
2 MS + 0.5 mg/L ABA	10	< -35
2 MS + 0.5 mg/L ABA	20	< -35
2 MS + 0.5 mg/L ABA	30	< - 8
3. embryos harvested 25 - 30 days post anthesis		
2 MS	0	-15
2 MS + 0.5 mg/L ABA	10	< -35

[a]MS: Murashige and Skoog medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid as described in Materials and methods.
[b]LT_{50} values were determined by cooling callus pieces (5 per experimental unit) at 6 °C/h and sampling at 2 °C intervals. Viability was determined from the ability of the calli to reduce tetrazolium chloride. Values are averages from three experiments each with 3 experimental units per temperature.

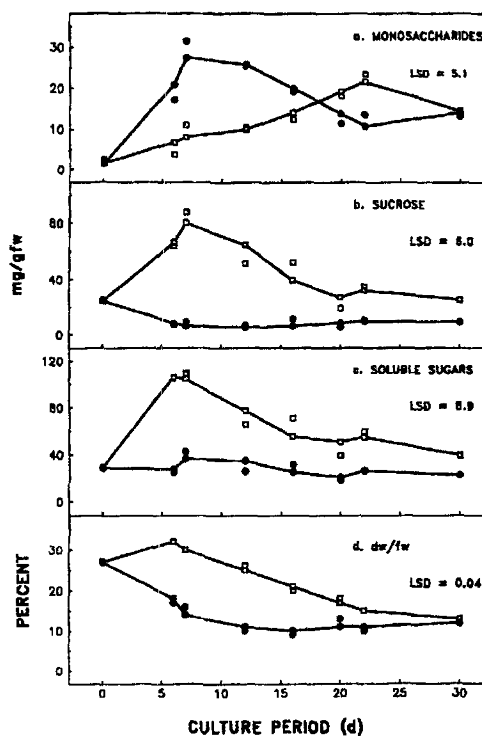

Fig. 1. The effect of abscisic acid (ABA) on soluble sugar levels (mg/gfw) and dry weight (percentage) accumulation in SWP9302 embryos during culture. Curves were fit to the data using a fourth order polynomial smoothing routine. Outlier points, where visible, represent actual data. Closed circles represent control embryos, open squares signify ABA-treated embryos. Values represent the means of triplicate determinations carried out on 15 or 30 embryos. LSD values were calculated for a 5% level of significance.

while the relative levels of fructose and glucose increased to approximately 60% (Fig. 1a).

The ABA treatment profoundly altered sugar deposition. In particular, sucrose accumulation was heightened and prolonged (Fig. 1c). This occurred when the embryos were cultured on media containing either sucrose, maltose, fructose or glucose as the carbon source (Table 3). ABA also promoted the accumulation of maltose when it was substituted for sucrose. By 12 d on MS medium soluble sugars formed 26% (66 mg/gfw) of the dry matter (Fig.1c). Sucrose remained the principal extractable sugar component throughout the test period (Fig.1a,c). After 12 d the total soluble sugar level in the ABA-treated embryos began to decline (Fig. 1c). At the same time the relative proportions of glucose and fructose increased from less than 8% to nearly 30% (Fig. 1a).

After 7 days growth, ABA treated embryos contained roughly twice (93.52 moles/gfw) the concentration of fatty acids as did control embryos (40.51 moles/gfw, Table 4). When calculated on a percentage basis, the relative abundance of saturated and unsaturated fatty acids remained constant over the test period. The distribution of fatty acids, palmitate (21%), stearate (2%), oleate (7%), linoleate (59%) and linolenate (10%), was typical for non-photosynthetic tissue. Sterol levels were relatively higher in ABA-treated embryos, compared with controls, at 7 days but not at 17 days (Table 4). Campesterol (24%), stigmasterol (10%) and beta-sitosterol (66%) were the major sterol species identified in the extracts. ABA-treatment did not alter the relative distribution of free sterol species. Sterol levels in treated and control embryos increased relative to fatty acid levels between 7 and 17 days (Table 4).

Discussion

Cryopreservation is in many cases the method of choice for long-term storage of valuable germplasm (Kartha 1985). However, despite recent advances in cryopreservation technology (Langis et al. 1989, Sakai et al. 1990) the necessity of developing cryoprotectant formulations specific for each variety remains an important shortcoming. For example, recent efforts to cryopreserve wheat embryos required extensive experimentation to identify successful cryoprotectant mixtures (Gnanapragasam and Vasil, 1992). As an alternative to cryoprotectant treatment, ABA pretreatment was tested as a method of hardening cultures and thus enhancing their overall cryostability.

Abscisic acid possessed several features that initially recommended its use as an agent to promote cryostability.

Table 3. The effect of carbon source and abscisic acid on the soluble sugar composition of cultured SWP9302 spring wheat embryos.[a]

carbon source	fructose	glucose	inositol	sucrose	maltose	total
	mg/gfw					
sucrose	3.73 ± 0.20	4.65 ± 0.58	0.12 ± 0.04	14.57 ± 4.51	0	23.07 ± 4.55
sucrose + ABA	3.49 ± 1.35	3.67 ± 2.31	0.80 ± 0.61	36.72 ± 13.90	0	44.68 ± 12.06
maltose	2.14 ± 0.14	5.40 ± 1.63	0.12 ± 0.02	12.16 ± 1.85	1.28 ± 1.14	20.50 ± 4.08
maltose + ABA	0.79 ± 0.05	2.03 ± 0.31	0.53 ± 0.07	27.47 ± 1.58	6.93 ± 0.67	37.75 ± 1.48
glucose	1.21 ± 0.14	2.05 ± 0.28	0.27 ± 0.14	7.63 ± 1.67	0	11.17 ± 1.71
glucose + ABA	1.52 ± 0.30	4.96 ± 1.46	0.69 ± 0.21	22.14 ± 11.13	0	29.32 ± 10.28
fructose	1.03 ± 0.10	0.43 ± 0.07	0.06 ± 0.00	1.51 ± 0.85	0	3.02 ± 0.98
fructose + ABA	2.26 ± 0.81	0.79 ± 0.15	0.53 ± 0.13	27.77 ± 5.21	0	31.35 ± 5.55

[a]Embryos were cultured for 15 days on MS medium containing 3% sucrose or maltose, 1.5% glucose or fructose and supplemented with 0.5 mg/L abscisic acid as required. Each value is the mean from at least two replications of three sub-samples.

Table 4. Fatty acid and sterol composition of cultured spring wheat embryos treated with abscisic acid.

a. Total fatty acid composition.[a]

Treatment	Palmitate	Stearate	Oleate	Linoleate	Linolenate	Total
	μmoles/gfw					
control 7d	8.24a	0.77a	2.30a	24.53a	4.67a	40.51a
ABA 7d	19.25b	2.10b	7.09b	56.10b	8.98b	93.52b
control 17d	4.65c	0.73a	0.93c	10.21c	2.61c	19.13c
ABA 17d	4.31c	0.31a	1.46a	11.24c	1.66c	18.98c

b. Sterol composition.[a]

Treatment	Campesterol	Stigmasterol	β-sitosterol	Total	Sterol/TFA
	μmoles/gfw				
control 7d	0.10a	0.04a	0.28a	0.42a	0.010
ABA 7d	0.52b	0.08a	1.21b	1.81b	0.019
control 17d	0.21c	0.08a	0.50c	0.79c	0.041
ABA 17d	0.16ac	0.08a	0.45ac	0.69ac	0.036

[a]Values in a column followed by the same letter are not significantly different at the 5% level according to the LSD test. Values represent the means of two replications of three sub-samples. Sterol/TFA = [Total sterol]/[Total fatty acid](μmole/μmole).

It has been shown by others that many cell lines harden when exposed to ABA (Reaney et al. 1988, Chen and Gusta 1983). In addition, this laboratory has demonstrated that ABA can enhance synchronization in embryo cultures and promote the development of embryogenic, regenerable cultures (Qureshi et al. 1989). These are desirable features in any strategy for the cryopreservation of valuable cell cultures or germplasm.

In reference to our earlier work, the ABA concentration chosen in this study (2M, 0.5mg/L) was that which induced optimum embryogenesis in SWP9302 zygotic embryos (Qureshi et al. 1989). At 1mg/mL a reduction in the embryogenic activity was noted, while at lower concentrations precocious germination occurred.

The factors contributing to freezing tolerance have not been unequivocally established. However, observations made at the whole plant level point to correlations between dry matter percent (Brule-Babel and Fowler 1989), carbohydrate concentration (Livingston et al. 1989, Anderson et al. 1984) or phospholipid content (Borochov et al. 1989; Kendall and McKersie 1989) and freezing tolerance in winter cereals. We questioned whether similar relationships might exist in zygotic embryos and in embryo-derived callus cultures of the relatively tender spring cereal crops.

The ABA treatment increased both the total proportion of dry matter and the sucrose concentration in freshly cultured spring wheat embryos. Part of the sucrose accumulation could be accounted for by enhanced uptake. However, sucrose was also accumulated when embryos were cultured on media containing glucose, fructose or maltose as the carbon source (Table 3). This latter observation suggests that ABA influences not only the uptake but also the intracellular deposition of sucrose.

The increase in sucrose concentration, which took place in the embryos before extensive proliferation of the scutella (callus formation), corresponded temporally with the development of tolerance to cryopreservation (Fig 1b). There would appear to be several advantages in accumulating sucrose prior to cryopreservation. First, it is relatively non-toxic and can be readily utilized during a subsequent growth phase. Second, it will colligatively depress the freezing point of the cytoplasm and reduce the amount of freezable water and thus the likelihood of intracellular ice formation. Third, sucrose (Koster and Leopold 1988, Crowe et al. 1984) has been shown to stabilize proteins and membrane bilayers exposed to extreme desiccation. Extreme desiccation can occur during storage at -196 °C.

The ABA treatment induced an accumulation of polar diacyl lipids (Table 4). The relative abundance of the fatty acid species and their degree of unsaturation were independent of ABA treatment and are similar to those reported previously for established callus (Kendall et al. 1990). These data also agree with earlier findings that cold hardening is accompanied by membrane proliferation and lipid accumulation (Kendall and McKersie 1989; Johnson-Flanagan and Singh 1986). The reduction in the non-esterified fatty acid pool size (Table 4) may reflect a state where degradative activity is reduced to a greater extent than is synthetic activity. If so, this could account, at least in part, for the accumulation of polar diacyl-lipid.

Our data demonstrate that, ABA can induce a high measure of tolerance to cryopreservation in spring wheat. Thus, this technique is an attractive alternative to conventional approaches for cryopreserving specimens that are known to be responsive to ABA.

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