

Preculture Conditions Influence Cold Hardiness and Regrowth of *Pyrus cordata* Shoot Tips After Cryopreservation

Yongjian Chang

Department of Horticulture, Oregon State University, Corvallis, OR 97331

Barbara M. Reed¹

United States Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, 33447 Peoria Road, Corvallis, OR 97333

Additional index words. ABA, cryopreservation, freezing tolerance, pear, shoot tip, sucrose

Abstract. Cold hardiness and cryogenic survival of micropropagated pear (*Pyrus cordata* Desv.) shoots were evaluated after pretreatments with ABA and sucrose. Shoot cold hardiness increased by 3 °C, and cryopreserved shoot tip growth increased by 17% after a 4-week 150 μM ABA pretreatment. Low temperature (LT) pretreatments improved the recovery of cryopreserved *P. cordata* shoot tips. Six to 10 weeks of LT were required for reaching high cryopreservation recovery. ABA and LT treatments produced significant synergistic effects on both cold hardiness and cryopreservation recovery. ABA shortened the LT requirement for high cryopreservation growth from 10 to 2 weeks. The optimal treatment for recovery of cryopreserved shoot tips was a 3 week culture on 50 μM ABA followed by 2 weeks of LT, while the maximum cold hardiness (–22.5 °C) was obtained with 150 μM ABA and 2-week LT. A 4 week culture on 150 μM ABA at 25 °C induced dormancy in 74% of shoot tips, but had little effect on cryopreservation growth unless combined with LT. Control and ABA-treated shoot tips, lateral buds, and leaves had similar cold hardiness (–10 to –12 °C), but LT and LT+ABA-treated shoot tips survived the lowest temperatures (–17 to –23 °C), lateral buds next (–15 to –20 °C), and finally leaves (–14 to –18 °C). An increase in the preculture-medium sucrose concentration from 2% to 7% combined with 2-week LT significantly increased cryopreserved shoot tip growth (0% to 75%) and decreased the LT₅₀ from –7.8 to –12.4 °C. The optimal shoot pretreatment for successful recovery of cryopreserved *P. cordata* shoot tips was a 3 week culture on either 50 μM ABA or 5% to 7% sucrose medium followed by 2 weeks of LT, and increased shoot tip growth from zero to >70%. Chemical name used: abscisic acid (ABA).

Cryopreservation for germplasm storage is increasingly important for national germplasm collections (Withers, 1991). Clonally propagated crops such as fruit, nut, and many root and tuber vegetables cannot be stored as seed and are especially suited for cryopreserved storage. Pear germplasm is now stored in the cryopreserved form as base storage for the active field collections of the U.S. National Plant Germplasm System (Reed et al., 1998b). Cryopreservation techniques that are effective for a wide range of genotypes are important for both genebanks and breeder's collections.

Many techniques are used to prepare plant materials for cryopreserved storage, including osmotic and temperature conditioning and chemical additives at various stages of the cryopreservation process (Reed and Chang, 1997). Osmotic conditioning with sugars is often used for cryopreservation. Natural

sucrose, an important chemical in dehydration and freezing tolerance, is a commonly used cryoprotectant (Dumet et al., 1993; Niino et al., 1992). Sucrose cryoprotectant solutions are highly concentrated (10% to 50%) and applied for short duration (16 h to several days). High sucrose concentration in the medium of in vitro-grown apples and blackberries significantly increases tissue freeze tolerance (Caswell et al., 1986; Palonen and Junttila, 1999).

ABA is an important stress hormone produced during cold acclimation. Chen et al. (1983) suggest that increased ABA concentrations in cells trigger cold acclimation and expression of low temperature-responsive genes. ABA increases the cold hardiness of cell cultures and whole plants of many species (Chen and Gusta, 1983; Lang et al., 1989; Tanino et al., 1990a; Veisz et al., 1996). Exposing tissues to ABA at room temperature substitutes for low temperature (LT) treatments for cold acclimating embryogenic wheat callus and the resulting lethal temperature at which 50% of cultures die (LT₅₀) is similar for callus treated with ABA and LT acclimation. ABA has a much weaker effect on intact wheat than on wheat callus (Dallaire

et al., 1994). ABA in the preculture medium improves the recovery of cryopreserved shoot tips of several plant species (Pence, 1998; Reed, 1993; Ryyanen, 1998; Vandebussche and De Proft, 1998).

LT pretreatments increase cold acclimation and improve the recovery of cryopreserved in vitro-grown shoot tips. However, some plant species require long LT treatments, some genotypes do not respond to LT, and others do not tolerate LT (Chang and Reed, 1997). Cold acclimation is essential for shoot tip cryopreservation of most pear genotypes (Reed et al., 1998a). The relationship of ABA, cold hardiness, and cryopreservation of in vitro-grown pear has not been studied.

Our objectives were to determine the effects of pretreatments of LT, ABA, and sucrose on the cold hardiness of pear shoots and on cryopreserved shoot tip recovery, and to examine the interactions between ABA and LT for inducing cold acclimation and improving recovery from cryopreservation.

Materials and Methods

Plant materials. *Pyrus cordata* (accession PYR 750.001) was selected because of its poor response to standard cryopreservation protocols in earlier studies (Chang and Reed, 2000). Micropropagated shoots of *P. cordata* were cultured in Magenta GA7 boxes (Magenta Corp., Chicago) on 40 mL of Cheng medium (Cheng, 1979), with 4.9 μM N⁶-benzyladenine (BA), 3% sucrose, 0.35% agar (Bitek agar, Difco, Detroit) and 0.145% Gelrite (Kelco, San Diego, Calif.). The pH was adjusted to 5.2 before autoclaving. Growth room conditions were 25 °C with a 16-h photoperiod (25 μmol·m⁻²·s⁻¹) and 40% relative humidity (RH). Shoots were transferred at 3 week intervals. Shoots were LT acclimated in a growth chamber at 22 °C with 8-h light (10 μmol·m⁻²·s⁻¹) and –1 °C 16-h darkness (Reed, 1990).

ABA treatments. Shoots were cultured for 3 weeks on the medium described above with 0, 10, 25, 50, 75, 100, and 150 μM (±) cis-trans-abscisic acid (Sigma Chemical Co., St. Louis). After 3 weeks, the shoots were placed in the LT chamber for 0, 1, or 2 weeks without transfer to new medium. Cold hardiness and the cryogenic recovery of the shoots were tested at the end of the LT treatment. To determine the effect of ABA on growth, we dissected apical shoot tips (1.0 mm) from shoots grown for 3 weeks on each ABA concentration and transferred them to regular medium. Growth data were recorded at 4 weeks. If the shoot tips remained green but did not grow new shoots or leaves by 4 weeks, they were considered dormant.

Sucrose and BA treatments. Shoots of *P. cordata* were cultured on medium with 2%, 3%, 5%, or 7% sucrose and 0.89, 2.22, or 4.44 μM BA for 3 weeks and then placed in the LT chamber for 0, 1, or 2 weeks without transfer to new medium.

Freezing procedure. After ABA, sucrose, BA, or LT pretreatment, 25 shoot tips (0.8–

Received for publication 12 Oct. 2000. Accepted for publication 16 Mar. 2001. Use of trade names in this publication does not imply endorsement of the U.S. Dept. of Agriculture (USDA) or Oregon State Univ. ¹To whom requests for reprints should be addressed. E-mail address: reedbm@bcc.orst.edu

1.0 mm) of each treatment were dissected and precultured on medium with 0.35% agar, 0.18% Gelrite, and 5% dimethyl sulfoxide (DMSO) for 48 h under the same temperature conditions as the parent shoots. Samples were subjected to slow freezing (Reed, 1990). Shoot tips were transferred to 0.25 mL liquid medium in 1.2 mL plastic cryo-vials (Cryovial, Beloeil, Quebec, Canada) on ice. The cryoprotectant PGD (Finkle and Ulrich, 1979), a mixture of 10% (w/w) each of polyethylene glycol (MW 8000), glucose, and DMSO in liquid medium was added dropwise up to 1.2 mL over 30 min. After 30 min equilibration on ice, the shoot tips were frozen to -40°C at $0.1^{\circ}\text{C}/\text{min}$ in a programmable freezer (Cryomed, Forma Scientific, Mt. Clemens, Mich.) with nucleation at -9°C and immersed in liquid nitrogen for at least 1 h. Vials were thawed in 45°C water for 1 min, then in 23°C water for 2 min. The cryoprotectant was removed and replaced with liquid Cheng medium. Shoot tips were plated in 24-cell plates with 2 mL Cheng medium per cell (Costar, Cambridge, Mass.) for recovery. Growth data were taken 4 weeks after thawing. Each experiment comprised 20 shoot tips in one vial for each treatment and five shoot tips for unfrozen controls, with at least three replications per experiment.

Cold hardiness test. Ten shoots were enclosed in a piece (11.4×11.5 cm) of moist sterile tissue paper (Kimwipes, Kimberly-Clark Corp., Roswell, Ga.) in each test tube (15×100 mm) and closed with a plastic cap sealed with parafilm. Tubes were cooled at $0.1^{\circ}\text{C}/\text{min}$ in a programmable freezer with automatic ice nucleation at -2°C . Five tubes per treatment were removed at each 2.5°C interval. Shoots were thawed at 4°C for 3 h and recultured on Cheng medium. Leaves that retained green color for 2 weeks were considered alive. Only shoot tips and buds that developed into plantlets were considered to have survived. Viability was expressed as LT_{50} , the temperature at which 50% of the shoots were killed.

Fresh weight/dry weight. Shoot water content was determined as the difference between dry weight (DW) and fresh weight (FW). DWs were determined after heating the samples in an oven at 95°C for 24 h. Percentage of water was calculated as: $[(\text{FW} - \text{DW})/\text{FW}] \times 100$.

Osmotic potential measurement. The water potential of the medium and the osmotic potential of *P. cordata* shoots at different culture or LT durations were measured on expressed sap using a vapor pressure osmometer (5100C; Wescor, Logan, Utah). The medium was frozen at -20°C for 16 h, then thawed for 5 min at room temperature (22°C). Aliquots of the medium (0.5 mL) were centrifuged in 1.5-mL microfuge tubes (Fisher Scientific, Pittsburgh) at 3000 rpm for 5 min. Shoots were frozen in the microfuge tubes overnight, thawed for 3 min at room temperature, then pressed with a glass bar to extract the sap. Sap was centrifuged at 3000 rpm for 5 min to obtain a clear supernatant (Guak, 1998). Osmolalities were converted to MPa by multiplying by 2.48 (According to Van't

Hoff's equation $\Psi = -RT\Sigma C_j$, where R is the gas constant, T was the temperature in degrees Kelvin and C_j was the summation of the concentrations of all solutes in the solution). There were at least three replications for each treatment.

Statistical analysis. The results were analyzed by analysis of variance (ANOVA) and means separated with Duncan's multiple range test ($P \leq 0.05$) using Statgraphics Plus (Statistical Graphics Corp. and STSC Rockville, Md.).

Results and Discussion

ABA and LT effects on the cold hardiness of in vitro-grown pear shoots. Increasing concentrations of ABA produced increasingly lower LT_{50} s either with or without LT treatments (Fig. 1). Shoots grown at 25°C were the hardest on $150\ \mu\text{M}$ ABA with an LT_{50} of -12.5°C . This was 2.5°C lower than the non-ABA controls. LT treatments produced greater increases in cold hardiness than the ABA treatments. Non-ABA control shoot LT_{50} s were 4°C lower with 1-week LT and 7°C lower with 2-week LT compared to the non-LT, non-ABA controls. ABA and LT interacted to improve plant cold hardiness. The addition of $150\ \mu\text{M}$ ABA to the LT treatments reduced the LT_{50} to -17.5°C after 1 week and to -22.5°C after 2 weeks.

Effect of ABA and LT on cold hardiness of pear tissues. Lateral buds, leaves, and shoot tips of control shoots had LT_{50} s near -10°C (Table 1). ABA ($75\ \mu\text{M}$) treatment significantly increased tissue cold hardiness, but by only 1 to 2°C , and there were no significant differences among the three tissues ($P \leq 0.05$). LT treatments significantly increased shoot cold hardiness and the effect was significantly stronger than ABA treatment for all three tissues. Shoot tips had the greatest re-

sponse to LT and were significantly harder than leaves and lateral buds ($P \leq 0.05$). The combined ABA and LT treatment increased the hardness of all three tissues with shoot tips and lateral buds both significantly harder than leaves.

ABA (75 – $150\ \mu\text{M}$) in the preculture medium increased the cold hardiness of *P. cordata* shoots by only a few degrees (Fig. 1) and only slightly improved cryopreservation recovery. LT induced much deeper cold hardiness than ABA. ABA did not substitute for LT for inducing cold hardiness. These responses indicate that ABA and LT induce cold hardiness by different pathways. A similar conclusion was reached in studies on wheat (Dallaire et al., 1994), where ABA ($50\ \mu\text{M}$) application increased the cold hardiness of intact wheat plants by 3°C , while LT induced much deeper cold hardiness. For five *Rubus* genotypes, although cold hardiness was not directly tested, LT combined with ABA ($50\ \mu\text{M}$) treatment of stock cultures improved the growth of cryopreserved shoot tips, while ABA alone had little effect (Reed, 1993). LT treatments improved cryopreservation recovery of *P. cordata* shoots from no growth of controls (no LT) to $>80\%$ after 12 weeks of LT (Chang and Reed, 1997). Long periods of LT acclimation (2 to 12 weeks), are required to achieve maximum cold hardiness in many pear genotypes including *P. calleryana* Decne, *P. koehnei* Schneider, *P. pashia* Buch.-Ham. ex D. Don, and *P. communis* L. cvs. Beurre d'Amanlis Panachee, Bosc-OP-5, Monchallard, and Good Christian (Chang and Reed, 1997). In the current study, *P. cordata* shoot cold hardiness significantly improved when ABA and LT were applied together and less exposure to LT was required with the ABA treatment. This is similar to *Rubus* hybrid cv. Hillemeier that has improved cryopreser-

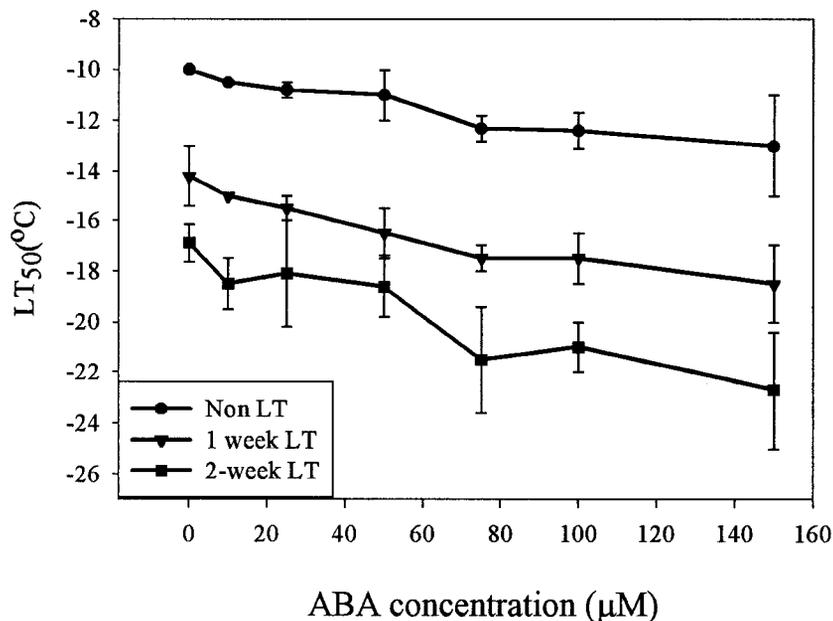


Fig. 1. Cold hardiness of in vitro-grown *P. cordata* shoots determined by LT_{50} after 3-week growth on Cheng medium with various ABA treatments followed by 0, 1, or 2-week low temperature (LT) treatment (22°C with 8-h light ($10\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and -1°C 16-h darkness). Five replicates of 10 shoots each ($N = 50$).

vation growth with LT pretreatment (from 17% to 21%), but growth more than doubles (52%) with combined LT and ABA pretreatments (Reed, 1993). In *P. cordata*, pretreatment with ABA and 2 weeks of LT produced cold hardness similar to that produced with 12 weeks of LT pretreatment (data not shown). Shoot tips appear to be best for cryopreservation not only because of their genetic stability, but also because they attain the deepest cold hardness and withstand freezing.

Dormancy induction by ABA. ABA induced apical dormancy in shoot tips of *P. cordata* and the rate increased with increasing ABA concentrations. After 3 weeks of growth, 30% of shoot tips on 50–80 μM , 50% on 100 μM , and 75% on medium with 150 μM ABA formed dormant apical buds. ABA concentrations below 25 μM did not affect shoot tip growth or induce dormancy.

Effects of ABA and LT pretreatments on the growth of cryopreserved pear shoot tips. ABA pretreatments alone slightly improved shoot tip growth (Fig. 2). Without LT, growth of shoot tips pretreated on 150 μM ABA was 17% compared to zero for the control (no ABA or LT). LT treatments for 2 weeks (without ABA) produced $\approx 20\%$ shoot tip growth while control (no ABA or LT) and 1 week of LT shoot tips did not survive. ABA and LT treatments interacted to improve growth of cryopreserved shoot tips (Table 2). Shoots treated with 50 μM ABA alone had <10% shoot tip growth after thawing, but growth increased to 38% with only 1 week of LT and to over 70% with 2 weeks of LT. Increasing ABA to 75 μM or 150 μM slightly improved non-LT treated shoot tip growth (from 8% to 20%), but shoots with 1 or 2 weeks of LT produced 40% to 50% shoot tip growth. Without ABA, extended LT treatments (10 weeks) are required for >80% growth of cryopreserved *P. cordata* shoot tips and 6 to 8 weeks of LT for >50% growth (Chang and Reed, 1997).

Dehydration tolerance of *Medicago sativa* L. and *Brassica napus* L. is increased by adding ABA to the culture medium (Anandarajah and McKersie, 1990; Senaratna et al., 1991). Dehydration and freezing tolerance are well correlated with improved growth of shoot tips following cryopreservation (Chang and Reed, 2000). In this study, ABA treatment slightly increased the shoot tip growth (Fig. 2) and decreased shoot water content (Fig. 3A). There are two suggested mechanisms by which ABA could confer freezing tolerance in plants: maintenance of a favorable water balance (Rikin et al., 1981; Tanino et al., 1990b) or induction of specific sets of genes that produce freeze-protective products (Lee et al., 1991). Without further analysis, it is difficult to say which mechanism is applicable to pear tissues.

Sucrose effects on shoot cold hardness. Shoot cold hardness increased significantly with increasing pretreatment-medium sucrose concentrations (Table 3). The LT_{50} of shoots grown on 7% sucrose with no-LT treatment was significantly lower ($P \leq 0.05$) than those

Table 1. Cold hardness of in vitro-grown *P. cordata* shoot tissues determined by LT_{50} following a 3-week growth on 75 μM ABA, a 2-week LT pretreatment, or both.

Type of tissues	LT_{50} ($^{\circ}\text{C}$)			
	Control ^z	+ABA ^y	+LT ^x	+ABA + LT ^w
Shoot tips	-10.8 aD ^y	-12.4 aC	-16.9 aB	-22.5 aA
Lateral buds	-11.1 aD	-12.5 aC	-15.2 bB	-20.1 bA
Leaves	-10.1 aD	-12.1 aC	-14.1 bB	-17.9 cA
Means	-10.7 D	-12.3 C	-15.4 B	-20.2 A

^zControl: 3 weeks of growth at 25 $^{\circ}\text{C}$ with no ABA.

^y+ABA: 3 weeks of growth at 25 $^{\circ}\text{C}$ with 75 μM ABA.

^x+LT: 2 week LT at 22 $^{\circ}\text{C}$ with 8 h light (10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and -1 $^{\circ}\text{C}$ 16 h darkness.

^w+ABA + LT: Growth at 25 $^{\circ}\text{C}$ for 3 weeks with 75 μM ABA then a 2-week LT treatment.

^yMeans in a row with different capital letters (A–D) are significantly different at $P \leq 0.05$; Means in a column with different lower case letters (a–c) are significantly different at $P \leq 0.05$.

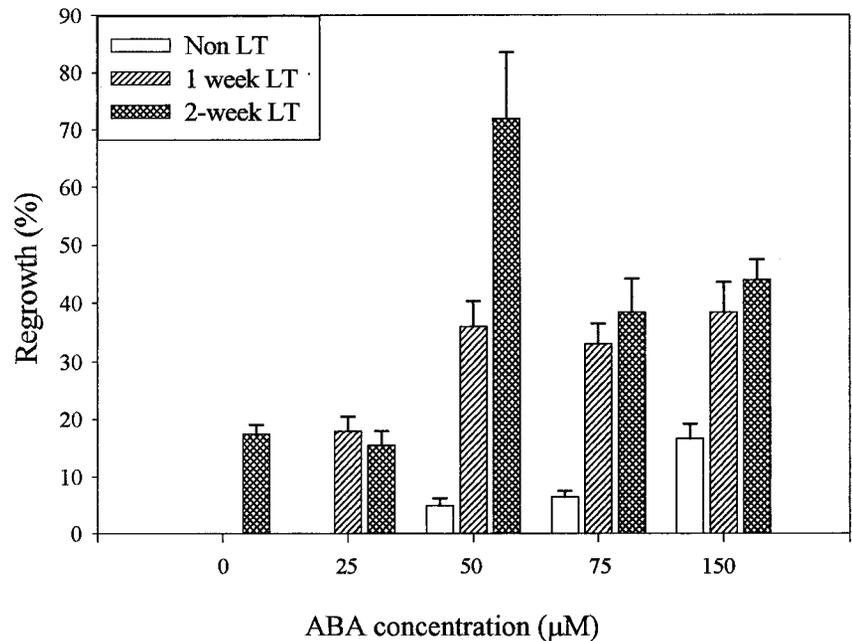


Fig. 2. Growth of in vitro-grown *P. cordata* shoot tips after a 3-week culture with various ABA treatments followed by 0, 1, or 2 week low temperature (LT) treatment (22 $^{\circ}\text{C}$ with 8 h light (10 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and -1 $^{\circ}\text{C}$ 16 h darkness) on the same medium. N = 60; means + sd.

Table 2. Analysis of variance for frequency of recovery of in vitro-grown *P. cordata* shoot tips after 3 weeks of growth with 0.75 μM ABA followed by 0, 1, or 2 weeks of LT treatment and cryopreservation in liquid nitrogen.

Source of variation	Sum of squares	df	Mean square
Cold treatment	369.8	2	184.9*
ABA concentration	1067.6	4	266.9**
Cold treatment \times ABA concentration	2599.2	6	433.2**
Residual	1785.6	32	55.8
Total	5822.2	44	

*. **Significant F test at $P \leq 0.05$ and $P \leq 0.01$.

on 2% or 3%. LT treatments significantly increased the cold hardness of the pear shoots grown on all sucrose concentrations. There was an interaction between medium sucrose concentration and LT treatment. Shoots grown on 7% sucrose had significantly lower LT_{50} s after 2 weeks of LT than with 0 or 1 week of LT, but the LT_{50} s were not significantly different from the 3% and 5% sucrose plus 2 weeks of LT treatments.

High sugar concentrations in plant tissues are strongly correlated with freezing tolerance and cold hardness (Gusta et al., 1996; Dumet et al., 1993). We increased the concentration of sucrose in the pear pretreatment

medium and significantly increased cold hardness of non-acclimated shoots. High sucrose concentrations also enhanced the effect of LT treatments on cold hardness (Table 3) and decreased shoot water content (Fig. 3A). Generally, moisture content correlates negatively with cold hardness in plants (Caswell et al., 1986; Tanino et al., 1990b; Yelenosky and Guy, 1989). Palonen and Junttila (1999) demonstrated that sucrose in the culture medium increased the sugar content of raspberry shoots and is readily taken up by shoots without prior hydrolysis.

Water content and osmotic potential of sucrose and ABA-cultured shoots. Shoot mois-

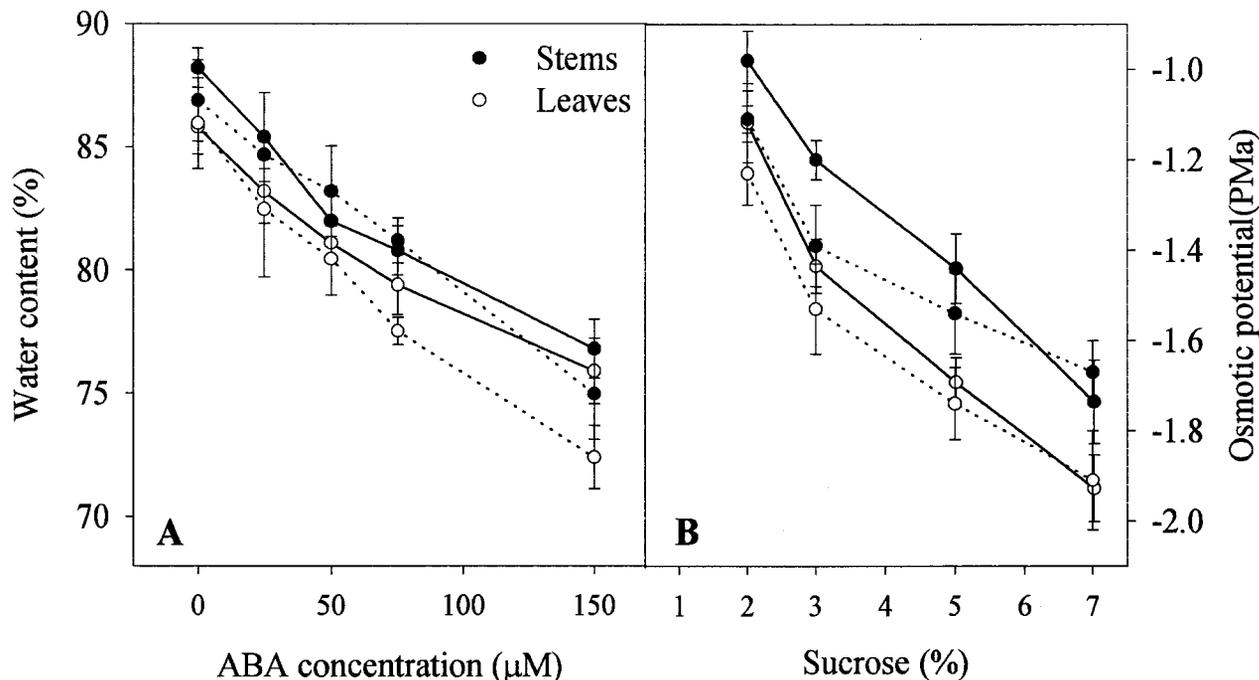


Fig. 3. Water content and osmotic potential of stems and leaves of *P. cordata* shoots following 3-week culture on Cheng medium with (A) five ABA concentrations or (B) four sucrose concentrations. Shoots were grown at 25 °C with a 16-h photoperiod. (dotted line) osmotic potential; (solid line) water content. N = 60; means \pm SD.

ture content decreased from 86% to 71% for leaves and 89% to 75% for stems when the medium sucrose concentration increased from 2% to 7% (Fig. 3B). The osmotic potential of the shoots became more negative with the increased sucrose concentrations and the decline was similar to that for water content. Shoot water content decreased significantly, from 88% to 77% for stems, and 86% to 76% for leaves, after 3 weeks of ABA (150 μ M) treatment (Fig. 3A). The osmotic potential of ABA-treated tissues decreased with the increase in ABA concentration.

Effects of sucrose and BA on cryopreservation. Sucrose and BA in the preculture medium affected the growth of pear shoot tips following cryopreservation (Table 4). Shoots grown on 2% sucrose had poor recovery at all BA concentrations. Standard growth medium concentrations of BA (4.44 μ M) and sucrose (3%) resulted in significantly less shoot tip growth than lower BA or higher sucrose concentrations. Growth of shoot tips following cryopreservation is influenced by the physiological condition of the parent shoots (Reed and Chang, 1997; Wu et al., 1999). Lower BA concentrations in the culture medium improved pear shoot growth (data not shown) and increased the growth of shoot tips following cryopreservation. High sucrose (5% to 7%) and BA (2.22–4.44 μ M) concentrations combined also produced good growth of cryopreserved shoot tips (Table 4).

LT treatments were the most effective pretreatments for increasing shoot cold hardness and growth of cryopreserved pear shoot tips. Without LT treatment, ABA and sucrose in the culture medium decreased water content and osmotic potentials, but only slightly increased cold hardness and tolerance to liquid nitrogen. ABA and sucrose effects

Table 3. Cold hardness of in vitro-grown *P. cordata* shoots cultured 3 weeks on medium with 2%, 3%, 5%, or 7% sucrose followed by 0, 1, or 2 weeks of LT pretreatment as determined by LT_{50} .

Sucrose (%) ^z	LT_{50} (°C) ^y			Means
	LT duration (wk)			
	0	1	2	
2	-7.8 cB	-12.6 cA	-13.3 bA	-11.2 c
3	-10.6 bC	-15.5 bB	-18.6 aA	-14.9 b
5	-11.5 abC	-16.7 abB	-20.4 aA	-16.0 ab
7	-12.4 aC	-17.5 aB	-20.3 aA	-16.7 a
Means	-10.5 C	-15.6 B	-18.2 A	

^zShoots grown on 2%, 3%, 5%, or 7% sucrose medium at 25 °C for 3 weeks and then treated with 0, 1, or 2 weeks. LT treatments at 22 °C with 8 h light (10 μ mol·m⁻²·s⁻¹) and -1 °C 16 h darkness.

^yMeans in a row with different capital letters (A–C) are significantly different at $P \leq 0.05$; Means in a column with different lower case letters (a–c) are significantly different at $P \leq 0.05$.

Table 4. Percentage of recovery of cryopreserved *P. cordata* shoot tips excised from shoots grown for 3 weeks on preculture medium with sucrose and BA and 2 weeks in combination with LT treatments.

BA concentration (µM)	Sucrose concentration (%) ^z			
	2	3	5	7
0.89	35 \pm 11.4 b	60 \pm 15.8 a	70 \pm 7.8 a	58 \pm 12.3 a
2.22	0 c	69 \pm 10.7 a	68 \pm 9.5 a	72 \pm 6.1 a
4.44	0 c	25 \pm 8.9 b	67 \pm 6.7 a	75 \pm 5.0 a

^zShoots were precultured on Cheng medium with sucrose and BA for 3 weeks followed by LT treatment for 2 weeks [22 °C with 8-h light (10 μ mol·m⁻²·s⁻¹) and -1 °C 16-h darkness] (n = 60). Means followed by different letters are significantly different at $P \leq 0.05$

were greatly enhanced when combined with LT treatment. Shoot tips and lateral buds were the most cold hardy (-22.5 and -20.1) with the combined LT-ABA treatment. The optimal shoot pretreatment for successful recovery of cryopreserved *P. cordata* shoot tips was a 3-week culture on either 50 μ M ABA or 5% to 7% sucrose medium followed by 2 weeks of LT, and increased shoot tip growth from 0 to >70%. This new procedure will increase the

number of pear genotypes that can be successfully stored in liquid nitrogen.

Literature Cited

- Anandarajah, K. and B.D. McKersie. 1990. Manipulating the desiccation tolerance and vigor of dry somatic embryos of *Medicago sativa* L. with sucrose, heat shock and abscisic acid. Plant Cell Rpt. 9:451–455.
- Caswell, K.L., N.J. Tyler, and C. Stushnoff. 1986.

- Cold hardening of in vitro apple and Saskatoon shoot cultures. *HortScience* 21:1207–1209.
- Chang, Y. and B.M. Reed. 1997. The effects of in vitro-growth condition on the cryopreservation of *Pyrus* meristems. *In Vitro Cell. Dev. Biol.* 33:50A.
- Chang, Y. and B.M. Reed. 2000. Extended alternating-temperature cold acclimation and culture duration improve pear shoot cryopreservation. *Cryobiology* 40: 311–322.
- Chen, H.H., P.H. Li, and M.I. Brenner. 1983. Involvement of abscisic acid in potato cold acclimation. *Plant Physiol.* 73:71–75.
- Chen, T.H.H. and L.V. Gusta. 1983. Abscisic acid-induced freezing resistance in cultured plant cells. *Plant Physiol.* 73:71–75.
- Cheng, T.Y. 1979. Micropropagation of clonal fruit tree rootstocks. *Compact Fruit Tree* 12:127–137.
- Dallaire, S., M. Houde, Y. Gagne, H.S. Saini, S. Boileau, N. Chevrier, and F. Sarhan. 1994. ABA and low temperature induce freezing tolerance via distinct regulatory pathways in wheat. *Plant Cell Physiol.* 35:1–9.
- Dumet, D., F. Engelmann, N. Chabrilange, Y. Duval, and J. Dereuddre. 1993. Importance of sucrose for the acquisition of tolerance to desiccation and cryopreservation of oil palm somatic embryos. *Cryo-Letters* 14:243–250.
- Finkle, B.J. and J.M. Ulrich. 1979. Effects of cryoprotectants in combination on the survival of frozen sugarcane cells. *Plant Physiol.* 63:598–604.
- Guak, S. 1998. Water relations, stomatal conductance, and abscisic acid content of container-grown apple (*Malus domestica*) plants in response to sorbitol-induced osmotic stress. Ph.D. Dissertation, Oregon State Univ., Corvallis.
- Gusta, L.V., R.W. Wilen, and P. Fu. 1996. Low-temperature stress tolerance: The role of abscisic acid, sugars, and heat-stable proteins. *HortScience* 31:39–45.
- Lang, V., P. Heino, and E.T. Palva. 1989. Low temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. *Theor. Appl. Genet.* 77:729–734.
- Lee, S.P., T.H.H. Chen, and L.H. Fuchigami. 1991. Changes in the translatable RNA population during abscisic acid induced freezing tolerance in bromegrass suspension culture. *Plant Cell Physiol.* 32:45–56.
- Niino, T., A. Sakai, H. Yakuwa, and K. Nojiri. 1992. Cryopreservation of in vitro-grown shoot tips of apple and pear by vitrification. *Plant Cell Tissue Organ Cult.* 28:261–266.
- Palonen, P. and O. Junttila. 1999. Cold hardening of raspberry plants in vitro is enhanced by increasing sucrose in the culture medium. *Physiol. Plant.* 106:386–392.
- Pence, V.C. 1998. Cryopreservation of bryophytes: The effects of abscisic acid and encapsulation dehydration. *Bryologist* 101:278–281.
- Reed, B.M. 1990. Survival of in vitro-grown apical meristems of *Pyrus* following cryopreservation. *HortScience* 25:111–113.
- Reed, B.M. 1993. Responses to ABA and cold acclimation are genotype dependent for cryopreserved blackberry and raspberry meristems. *Cryobiology* 30:179–184.
- Reed, B.M. and Y. Chang. 1997. Medium- and long-term storage of in vitro cultures of temperate fruit and nut crops, p. 67–105. In: M.K. Razdan and E.C. Cocking (eds.). Conservation of plant genetic resources in vitro, Vol. 1. Science Publishers, Inc., Enfield, NH.
- Reed, B.M., J. DeNoma, J. Luo, Y. Chang, and L. Towill. 1998a. Cryopreservation and long-term storage of pear germplasm. *In Vitro Cell. Dev. Biol.*—*Plant* 34:256–260.
- Reed, B.M., C.L. Paynter, J. DeNoma, and Y. Chang. 1998b. Techniques for medium- and long-term storage of (*Pyrus L.*) genetic resources. *Plant Gen. Res. Newsletter* 115:1–4.
- Rikin, A., C. Gitler, and C. Atsmon. 1981. Chilling injury in cotton: Light requirement for the reduction of injury and for the protective effect of abscisic acid. *Plant Cell Physiol.* 22:453–460.
- Ryynanen, L. 1998. Effect of abscisic acid, cold hardening, and photoperiod on recovery of cryopreserved in vitro shoot tips of silver birch. *Cryobiology* 36:32–39.
- Senaratna, T., L. Kou, W.D. Beversdorf, and B.D. McKersie. 1991. Desiccation of microspore derived embryos of oilseed rape (*Brassica napus L.*). *Plant Cell Rpt.* 10:342–344.
- Tanino, K.K., T.H.H. Chen, L.H. Fuchigami, and C.J. Weiser. 1990a. Metabolic alterations associated with abscisic acid-induced frost hardiness in bromegrass suspension culture cells. *Plant Cell Physiol.* 31:505–511.
- Tanino, K., C.J. Weiser, L.H. Fuchigami, and T.H.H. Chen. 1990b. Water content during abscisic acid induced freezing tolerance in bromegrass cells. *Plant Physiol.* 93:460–464.
- Vandenbussche, B. and M.P. De Proft. 1998. Cryopreservation of in vitro sugar beet shoot tips using the encapsulation-dehydration technique: Influence of abscisic acid and cold acclimation. *Plant Cell Rpt.* 17:791–793.
- Veisz, O., G. Galiba, and J. Sutka. 1996. Effect of abscisic acid on the cold hardiness of wheat. *J. Plant Physiol.* 149:439–443.
- Withers, L.A. 1991. In-vitro conservation. *Bio. J. Linnean Soc.* 43:31–42.
- Wu, Y., F. Engelmann, Y. Zhao, M. Zhou, and S. Chen. 1999. Cryopreservation of apple shoot tips: Importance of cryopreservation technique and of conditioning of donor plants. *Cryo-Letters* 20:121–130.
- Yelenosky, G. and C. Guy. 1989. Freezing tolerance of citrus, spinach, and petunia leaf tissue. *Plant Physiol.* 89:444–451.