for the tropics: I. Interspecific hybrids between *B. campestris* and *B. oleracea* through in vitro embryo culture. Jon. Soc. Hort. Sci. Autumn Mtg. p. 228-229. (Abstr.)
Maas, J.L. 1977. Pollen ultrastructure of strawberry and other small fruit crops. J. Amer. Sot. Hort. Sci. 102:560-571.
Namai, H., M. Sarashima, and T. Hosoda. 1980. Interspecific and intergeneric hybridization breeding in Japan, p. 191–201. In: S. Tsunoda, K. Hinata, and C. Gómez-Campo (eds.). *Brassica* crops and wild allies. Japan Scientific Societics Press, Tokyo.
U, N. 1935. Genome-analysis in *Brassica* with special reference to the experimcntal formation of B. *napus* and peculiar mode of fertilization. Jpn. J. Bet. 7:389-452.

HortScience 25(1): 111-113. 1990.

Survival of in Vitro-grown Apical Meristems of Pyrus Following Cryopreservation

Barbara M. Reed
Department of Horticulture, Oregon State University and National Clonal Germplasm Repository 33447 Peoria Road, Corvallis, OR 97333

Additional index words. conditioning, tissue culture, pear, cold-hardening, acclimation

Abstract. **Apical meristems of four pears *(Pyrus communis L.* cv. Beurre Hardy, *P. koehnei* Schneider, *P. cossonii* Coss. and Dur., and a hybrid, *P. dimorphophylla* Makino × *P. fauriei* Schneider) were tested for their ability to survive immersion in liquid nitrogen. Plantlets were grown in vitro at 25C or cold-hardened for 1 week at – 1C before cooling at rates of 0.1, 0.3, 0.5, and 0.8 C/rein to –40C, followed by plunging the vials into liquid nitrogen. Vials were thawed for 1 min at 40C. A cryoprotectant mixture of polyethylene glycol, glucose, and dimethylsulfoxide (DMSO) was used. Regrowth of meristems ranged from 0% to 61% for plants grown at 25C and from 5% to 95% for cold-hardened plants. Cold-hardening significantly improved the recovery rates of all species tested. Survival rates increased as cooling rates decreased. Survival rates were not linked to the geographic origin of the species tested.**

The development of methods to successfully store apical meristems in liquid N_2 is needed to aid in the conservation of genetic resources. Long-term storage methods for *Pyrus* spp. are needed to provide a backup collection of the 26 species and numerous cultivars present at the National Clonal Germplasm Repository, Corvallis, Ore. Cryopreservation may be a useful method for long-term storage using a minimum of space and maintenance.

In freezing actively growing apical meristems of *Pyrus* species, variable survival over a range of conditions for dehydration could be expected. The rate of cooling and the prefreezing.. temperature must be taken into account (15). Relatively slow cooling rates may have the net effect of greater dehydration at a given temperature than faster rates, while the same level of dehydration would be reached at a lower temperature at faster cooling rates.

Enhanced survival has been found in callus of several species that were cold-hardened before cryopreservation (8, 10, 12). One week of cold-hardening was found to increase the survival of cryopreserved apical meristems from tissue-cultured *Rubus* plants (6). Dormant apple buds survived best in liquid N_2 when the tissue had ≤20% liquid water at the precooking temperature and the cell solute concentration had increased from about 2 mmol to 5 mmol with dehydration, leading to higher survival rates (14).

Field-grown pear species and cultivars vary in hardiness to freezing damage of cambial cells from temperatures of – 16C to – 40C (5). *Pyrus communis* cultivars alone vary in hardiness from – 20 to – 35C (J.M. Montano, unpublished results). Other species exhibit somewhat greater or lesser hardiness that often is well-correlated with the geographic origin of the plant (7). Sakai (9) found that, in the cryopreservation of woody twigs, the greater the hardiness, the higher the precooking temperature that is required for survival. Cryopreservation of *Pyrus* spp. has been limited to the cooling of dormant buds during midwinter (4, 8) and to protoplasts and suspension-cultured cells (1, 16).

Both cold-hardening and dehydration due to pretreatment and cooling rate play important roles in conditioning meristems to survive immersion in liquid N_2 (13, 14). This study compares the survival of apices of four species of the genus Pyrus that were frozen at four rates following growth under cold-hardening or growth room conditions.

Tissue culture conditions. Tissue-cultured plants of *P. communis, P. koehnei,* and *P. cossonii* and of a hybrid, *P. dimorphophylla* Makino × *P. fauriei,* were grown on the medium of Cheng (2) with benzyladenine at 2 mg·liter^{-1} and 0.6% agar at pH 5.2. Growth conditions included a 16-hr photoperiod at 25C. Cold-hardening of in vitro plants involved 22C days (8 hr) and –1C nights (16 hr) for 1 week before excision of meristems (6). Dissected meristems (0.8 mm) of both treatments were grown for 48 hr on medium with 0.8% agar and 5% DMSO added. Meristems were grown under the same conditions as the parent plants.

Addition of cryoprotectant. Following cold-hardening, meristems were transferred to 0.25 ml of liquid medium in 1.2-ml plastic cryotubes on ice. The cryoprotectant PGD (3), a mixture of 10% each of polyethylene glycol (M_r 8000), glucose, and DMSO in water, was added dropwise up to 1.2 ml over 30

Table 1. Influence of cold-hardening or growth at 25C (non-hardened) on the survival of meristems of *Pyrus* spp. after cryopreservation in liquid N_2 (all cooling rates combined).

	No. meristems tested					
	Cold-hardened			Non-hardened		
Species	Living	Dead	Total	Living	Dead	Total
P. communis	73	57	130	9	99	108
P. hybrid	35	107	142	6	113	119
P. koehnei	31	110	141	1	114	115
P. cossonii	42	108	150	7	85	92
Total	181	382	563	23	411	434

	df	G^z
P. communis	1	66.34***
P. hybrid	1	20.83***
P. koehnei	1	32.89***
P. cossonii	1	16.45***
Species totals	3	40.15***
Total	7	176.67***

[z]G values calculated as in Sokal and Rohlf (11).
***Significant at $P < 0.001$.

Received for publication 18 Nov. 1988. Contribution of the Oregon Agricultural Experiment Station in cooperation with the ARS/USDA Technical paper no. 8706 of the Ore. Agr. Expt. Sta. 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.

Table 2. Survival of apical meristems of *Pyrus* spp. after four controlled freezing rates and immersion in liquid N_2 for 1 hr following cold-hardening or growth at 25C (non-hardened).

Cooling rate[z]	Survival (% ± SEM)[y]			
	P. communis	*P.* hybrid	*P. koehnei*	*P. cossonii*
		Non-hardened		
0.1	23 ± 12	8 ± 5	0	26 ± 0
0.3	51 ± 21	10 ± 3	0	3 ± 3
0.5	3 ± 2	10 ± 7	3 ± 2	13 ± 5
0.8	0	0	0	5 ± 3
		Cold-hardened		
0.1	95 ± 1	55 ± 15	75 ± 0	95 ± 0
0.3	82 ± 2	56 ± 15	31 ± 7	33 ± 2
0.5	27 ± 7	7 ± 3	5 ± 2	12 ± 3
0.8	10 ± 1	7 ± 1	5 ± 0	5 ± 0

[z]Degrees C/rein to – 40C.
[y]Tips showing normal or callus growth; 100% of control meristems survived.

Table 3. Shoot production in surviving apical meristems of *Pyrus* spp. following freezing at four controlled rates to – 40C and immersion in liquid N_2 for 1 hr.

Cooling rate[z]	Shoot production (% ± SEM)[y,x]			
	P. communis	*P.* hybrid	*P. koehnei*	*P. cossonii*
		Non-hardened		
0.1	100 ± 0	100 ± 0	---	100 ± 0
0.3	88 ± 9	75 ± 3	---	100 ± 0
0.5	100 ± 0	100 ± 0	100 ± 0	100 ± 0
0.8	---	---	---	100 ± 0
		Cold-hardened		
0.1	81 ± 13	100 ± 0	100 ± 0	100 ± 0
0.3	91 ± 6	63 ± 6	100 ± 0	100 ± 0
0.5	96 ± 3	100 ± 0	100 ± 0	100 ± 0
0.8	100 ± 0	7 ± 0	50 ± 35	100 ± 0

[z]Degrees C/rein to – 40C.
[y]Shoot tips showing normal shoot growth (not callus).
[x]Dashes = no survival.

min. A 30-min equilibration period at – 1C was followed by removal of excess cryoprotectant.

Cooling. Samples were frozen in 1 ml of the cryoprotectant at 0.1, 0.3, 0.5, or 0.8C/min to – 40C in a programmable controlled-temperature cooling chamber (Cryomed, Leona, Mich.) and then immersed in liquid N_2 for 1 hr.

Thawing. Vials were thawed in 40C water for 1 rein, then cooled in 23C water. The cryoprotectant was drained from the vials and replaced with liquid medium. Meristems were drained on sterile filter paper and plated on medium for "regrowth. Twenty treated meristems and five controls (not frozen) were tested for each of four replicates for each species.

Growth following cooling. Meristems were plated on 2 ml medium in sterile welled containers (Costar, Cambridge, Mass.) (24 wells per plate). Regrowth was under standard conditions described previously. Recovering meristems were observed for 30 days. Survival was measured as the formation of shoots or callus.

Results were analyzed using the G statistic as calculated in Sokal and Rohlf (11). Large G values indicate a significant interaction between the characteristics tested.

Cold-hardening effects on survival. Cold hardening for 1 week as compared to incubation at 25C had a significant effect on all species at all cooling rates tested (Table 1).

Of non-hardened plants, only *P. cossonii* survived at the fastest cooling rate (0.8C/rein) (Table 2). At slower cooling rates, survival improved for all species except *P. koehnei;* however, the survival rates of all species were lower than those of cold-hardened meristems cooled at the same rate. Decreasing the cooling rate for meristems that had been incubated at 25C had the greatest effect on *P. communis* and little or no effect on *P. koehnei.*

All four species had low survival rates at the fastest cooling rate following cold-hardening, and survival increased with decreasing cooling rate. *Pyrus koehnei,* which did not respond to a slow cooling rate when grown at 25C, survived better (by 70 percentage points) following cold-hardening (Table 2).

Both warm-grown and cold-hardened frozen meristems produced a high proportion of normal (non-callused) shoots after thawing (Table 3), which indicates that large portions of the shoot apex have survived intact rather than just a few cells that normally produce only callus. The favorable response may also be indicative of a recovery medium with optimal hormone concentrations that supported organized recovery (15). Surviving shoots were subculture and produced normal in vitro plants. The few meristems that produced callus did not redifferentiate into shoots.

The greatest differences in survival from cold-hardening occurred with *P. koehnei.* Plants conditioned at 25C survived at only one cooling rate, while cold-hardened plants had a high survival rate at the two slower cooling rates and some survival at the faster cooling rates. This response to cold hardening was not entirely predictable. The pear species in this study were chosen to correspond with varied environments to compare species that were cold-hardy and could be expected to harden with those from subtropical areas that would be less cold-hardy and perhaps unable to harden.

These results differ from studies of low-temperature exotherms in field-grown *Pyrus* spp., where Rajashekar et al. (5) found that, based on differential thermal analysis (DTA), *P. communis* was relatively hardy, as was *P. dimorphophylla,* with DTAs of – 29C and – 30C, while *P. koehnei* at – 18C was very tender. Pyrus *cossonii* was reported to have exotherms ranging from as low as – 18 to – 28C. DTA analysis (J.M. Montano, unpublished results) on the same plants used in this study indicated – 29C for *P. communis, – 21C* for *P. cossonii,* – 10C for *P. koehnei,* and – 23C for *P. dimorphophylla* × *P. fauriei.* The current study indicates that the ability to cold-harden in the field does not necessarily relate to the ability of apical meristems of in vitro plants to cold-harden and survive freezing in liquid N_2.

With the *Pyrus* spp. tested in this study, the degree of cold hardiness in itself may not be as important as the ability to cold-harden to any extent. The level of cold-hardening and the associated physiological changes produced may be causing reactions similar to those reported by Tyler et al. (14), with cells with greater osmolarity having a higher survival rate. These levels will need to be tested to determine if this is the case with *Pyrus* meristems or if other factors are involved.

Cooling rate. The effect of cooling rate on survival was highly significant for all four cold-hardened pears ($P < 0.001$), with the slower cooling rates producing the highest survival rates in all species tested. Survival of non-hardened pears (grown at 25C) also was affected by cooling rate, although less than that for hardened plants (Table 4). Overall, cooling rate was found to be highly significant in its interaction with conditioning (G = 123.42).

The response of individual species to cooling rate varied, but, in general, the slower the rate the higher the level of survival (Table 2). This indicates that dehydration may be important in cryopreservation of this genus. Preliminary testing indicated that – 40C is superior to – 35C as a prefreezing temperature. Perhaps an even lower temperature combined with one of the slower cooling rates would produce higher survival in the two species with < 95% survival.

Species differences. Sakai (9) predicted that there should be differential survival rates between hardy and less-hardy species. If this is the case, then we would expect that the highest rate of survival would occur in the Pyrus hybrid and *P. communis* and lowest in *P. cossonii* and *P. koehnei.* Greater survival of *P. koehnei* would be expected with

Table 4. Effect of freezing rate on the survival of *Pyrus* meristems frozen to – 40C and immersed in liquid N_2 for 1 hr following cold-hardening treatments or growth at 25C (non-hardened).

Cooling rate[z]	No. meristems tested					
	Cold-hardened			Non-hardened		
	Living	Dead	Total	Living	Dead	Total
0.1	62	18	80	9	106	115
0.3	100	87	187	11	138	149
0.5	9	141	150	3	108	111
0.8	10	136	146	0	59	59
Totals	177	382	559	23	411	434

	df	G^y
Conditioning	1	123.42***
Effect of freezing rate on hardened plants	3	222.47***
Effect of freezing rate on non-hardened plants	3	10.67*
Total	7	356.56***

[z]Degrees C/rein to —40C.
[y]G values calculated as in Sokal and Rohlf (11).
*,*** significant at $P < 0.05$ or 0.001, respectively.

slower cooling rates or lower prefreezing temperatures.

Species differences are apparent in the rate of survival following freezing. Survival at all cooling rates was *P. communis* > *P. cossorrii* > *P. koehnei* > the hybrid pear (Table 2). This sequence does not conform with the suggestion of Sakai (9), since both *P. dimorphoplsylla* and *P. fauriei* are temperate species and the hybrid of the two does riot respond as well as P. *cossonii or P. koehnei,* which are subtropical.

Several factors likely are involved in survival from cryopreservation in the genus *Pyrus*; one to be considered in the case of in vitro-grown plants is their vigor in culture. The *Pyrus* hybrid is less vigorous in culture and may require improved media for vigorous growth. Lack of vigor may contribute to lower tolerance to dehydration or cooling, as well as decreased growth on the culture medium after thawing. Preliminary studies of *Pyrus* (data not shown), as well as studies of other species (15), have demonstrated that survival rates are linked to medium composition as well as actual cooling conditions.

Cold-hardening as a method for improving the survival of cryopreserved Pyrus was successful with the three species and one hybrid tested. The results of this study indicate that many factors are involved in the successful cryopreservation of apical meristems. Significant increases in survival rate can be obtained by decreasing the cooling rate and cold-hardening parent plants for 1 week before cryopresetvation, and indicate that further increases may be possible.

The range of species used in this test was intended to provide initial data on which to base further studies of the genus Pyrus. It appears that those species that are not considered cold-hardy are capable of conditioning to enable them to withstand liquid N_2 temperatures.

Literature Cited

1. Bartelo, M. E., S.D. Wanner, and R.E. Ketchum. 1987. Comparison of cooling tolerance in cultured plant cells and their respective protoplasts. Cryobiology 24:53-57.
2. Cheng, T.Y. 1979. Micropropagation of clonal fruit tree rootstock. Compact Fruit Tree 12:127-137.
3. 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.
4. Mariguchi, T., T.A. Kihama, and I. Kozaki. 1985. Freeze preservation of dormant pear shoot apices. Jpn. J. Breed. 35:196-199.
5. Rajashekar, C., M.N. Westwood, and M.J. Burke. 1982. Deep supercooling and cold hardiness in genus Pyrus. J. Amer. Soc. Hort. Sci. 107:968-972.
6. Reed, B. 1988. Cold acclimation as a method to improve survival of cryopreserved *Rubus* meristems. CryoLetters 9:166-171.
7. Sakai, A. and Y. Sugawara. 1973. Survival of poplar callus at super-low temperatures after cold acclimation. Plant & Cell Physiol. 14:1201-1204.
8. Sakai, A. and Y. Nishijama. 1978. Cryopreservation of winter vegetative buds of hardy fruit trees in liquid nitrogen. HortScience 13:225-227.
9. Sakai, A. 1965. Survival of plant tissue at super-low temperatures: III. Relation between effective precooking temperatures and the degree of frost hardiness. Plant Physiol. 40:882-887.
0. Steponkus, P. and L. Bonnier. 1971. Cold acclimation of plant tissue cultures. Cryobiology 8:386–387.
1. Sokal, R.R. and F.J. Rohlf. 1969. Biometry. Freeman, San Francisco.
12. Tumanov, I., R. Butenko, and I. Ogolevets. 1968. Application of isolated tissue techniques for studying hardening processes in plant cells. Soviet Plant Physiol. 15:625-630.
13. Tyler, N. and C. Stushnoff. 1988. The effects of precooking and controlled dehydration on cryopreservation of dormant vegetative apple buds. Can. J. Plant Sci. 68:1163–1167.
14. Tyler, N., C. Stushnoff, and L. Gusta. 1988. Cooling of water in dormant vegetative buds in relation to cryopreservation. Plant Physiol. 87:201–205.
15. Withers, L. A., E. Benson, and M. Martin. 1988. Cooling rate/culture medium interactions in the survival and structural stability of cryopreserved shoot-tips of *Brassica napus.* CryoLetters 9:114-119.
16. Wu, M.-T. and S.J. Wahrer. 1985. Effect of temperature on cooling and heat stress tolerance of cultured plant cells. Cryobiology 22:191–195.