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CRYOPRESERVATION AND LONG-TERM STORAGE OF PEAR GERMPLASM[1]

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SUMMARY

Germplasm collections of vegetatively propagated crops are usually maintained as plants in fields or potted in greenhouses or screened enclosures. Safety duplication of these collections, as duplicate plants or separate collections, is costly and requires large amounts of space. Cryopreservation techniques which were recently developed for long-term storage of pear germplasm may offer an efficient alternative to conventional germplasm collection maintenance. Pear (*Pyrus* L.) germplasm may now be stored as seeds (species), dormant buds or pollen from field-grown trees, or shoot tips from *in vitro*-grown plants (cultivars). Pear germplasm may now be cryopreserved and stored for long periods (> 100 yr) utilizing slow-freezing or vitrification of *in vitro*-grown shoot-tips. Dormant bud freezing, pollen, and seed cryopreservation of other lines are being developed to complete the base collection for *Pyrus*. This cryopreserved collection provides base (long-term) storage for the field-grown pear germplasm collection at the National Clonal Germplasm Repository, Corvallis, Oregon.

Key words: cryopreservation; genebank; liquid nitrogen storage; *Pyrus*; slow freezing; vitrification.

INTRODUCTION

Cryopreservation of plant germplasm is researched in many laboratories throughout the world. Storage methods for plants kept as seeds, pollen, dormant buds and shoot tips are actively being developed (Withers, 1991; Bajaj, 1995). Although storage of genetic resources has been practiced for many years with seed-propagated crops, organized conservation of clonally propagated plants has developed only in the last 20 yr. Many nations have made the development of efficient methods for germplasm preservation a high priority. Germplasm conservation is becoming increasingly important due to the loss of diversity in agricultural systems as formerly isolated farming communities begin replacing old land race varieties with new cultivars (Tanksley and McCouch, 1997). The genetic diversity in wild relatives is very important for future crop improvement. Thus, all unique accessions need to be collected and preserved (Engelmann, 1991).

Active field or *in vitro* collections are those available for evaluation of important characteristics and distribution to plant breeders for crop improvement. Base collections are those stored and used only if the active collections are lost or in need of rejuvenation (Stanwood, 1985). Base collections of most seeds often show considerable longevity when stored at −20° C. Clonally propagated crops or large-seeded plants must be maintained as vegetative plant material under normal growing conditions because the plants are highly heterozygous, sterile, or have seeds that are short lived and temperature or desiccation sensitive. The backup collections of clonally propagated crops are grown as field collections at a second site, maintained in greenhouses, or held as *in vitro* collections.

Cryopreservation is the newest addition to the germplasm storage system. It is a goal of many national germplasm systems to store base collections of clonal material in liquid nitrogen (LN). Advantages include greater security for the collection, small space requirements, little input or cost for maintenance, and reduction in the number of duplicates needed in the active collection.

Pears are an example of an economically important, clonally propagated crop. The USDA-ARS National Clonal Germplasm Repository (NCGR) at Corvallis, OR has more than 1500 pear genotypes in its field collection. About 160 genotypes are also stored as *in vitro* cultures under low light at 4° C and regenerated at 2–3-yr intervals. Pollen of 30 genotypes is stored in LN. Storage in LN provides a stable, low maintenance, low risk, long-term base collection for clonal pear germplasm and seeds of pear species. This base collection has now been initiated at NCGR and the National Seed Storage Laboratory (NSSL) in Fort Collins, CO.

CRYOPRESERVATION RESEARCH

Research on cryopreservation of Pyrus *seed, pollen, dormant buds, and* in vitro*-grown shoot tips.* The following is a summary of research on the cryopreservation of *Pyrus* genetic resources. Details of ongoing cryopreserved storage of *Pyrus* germplasm are also included. These research reports indicate that cryopreservation techniques are in place and available for use in preserving world germplasm collections. Although techniques for storing some plant materials are more developed than others, cryopreservation should be applied to *Pyrus* genetic resources to insure future availability.

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Seed. There are no reports of *Pyrus* seed cryopreservation. Stanwood (1985) suggests that most desiccation-tolerant LN-tolerant seeds have high survival with moisture contents between 5 and 10% and a cooling rate of 1 to 30° C/min, and if a test sample is not damaged, the whole sample could be safely stored. Studies are needed to determine the optimal seed moisture content and freezing and thawing rates for seeds of the many *Pyrus* species available in germplasm collections.

Pollen. The many studies of pear pollen show that it is long lived. Dry pear pollen may survive (with low germination) for up to 9 yr under dry room-temperature storage (Akihama and Omura, 1986). Visser (1955) did extensive studies on the longevity of pear pollen at various temperatures and relative humidities and found that germination capacity was maintained for 2 to 3 yr at −196° C. Akihama et al. (1978) successfully cryopreserved freeze-dried pear pollen and found that rehydration of dried stored pollen before pollination was very important. Pear pollen stored for 3 yr in LN and rehydrated at 5° C for 6 h to 90% relative humidity successfully pollinated pear cultivars. Craddock (1987) cryopreserved pollen from many cultivars and species of pear. He found some variability in pollen tube germination after thawing, but had generally good results. Craddock collected unopened "popcorn stage" blossoms and separated the anthers from the flower using sieve screens; the undehisced anthers dried and dehisced overnight on the lab bench. Initial moisture content (MC) of air-dried pollen was 25 to 35% with 85 to 95% germination. Pollen (binucleate) MC between 5 and 30% resulted in the greatest viability following LN exposure, but moderate germination occurred with MC as high as 90%. Pollen/anther mixtures could be cryopreserved after 24 h of air drying or stored in a desiccator at 5° C until cryopreserved. Chen et al. (1993) found that pear pollen remained viable and successfully pollinated flowers even after several freeze/thaw cycles if the moisture content remained low (10 to 20%) each time the pollen was frozen. *In vitro* germination may also be used for viability testing (Towill, 1985).

Dormant buds. Sakai and Nishiyama (1978) first demonstrated that hardy *Pyrus* dormant shoots can survive in LN after prefreezing to −40 or −50° C. Four of five cultivars tested had high survival (78 to 100%) and the buds developed, although the xylem was severely injured in all cultivars. Several laboratories in Japan have studied cryopreservation of shoot apices excised from dormant buds (Moriguchi et al., 1985; Oka et al., 1991; Mi and Sanada, 1992, 1994). Moriguchi et al. (1985) and Moriguchi (1995) reported that pretreatment with dimethylsulfoxide (DMSO) and prefreezing to −40 to −70° C was required to successfully cryopreserve shoot tips from dormant buds of Japanese pear [*P. serotina* Rehd. var. *culta* cv. Kosui; synonym of *P. pyrifolia* (Burm. f.) Nakai]. Additional cryoprotectants were unnecessary and about 80% of shoot tips survived regardless of the rewarming procedure applied. Oka et al. (1991) recovered a few whole plants from cryopreserved 'Senryo' winter buds, but the overall frequency of shoot formation following grafting was low (0 to 8%). Suzuki et al. (1997) dehydrated, cryopreserved, and micrografted winter buds of pear (*Pyrus communis* L. cv. Beurre d'Amanlis and 12 other pear genotypes) onto seedling rootstocks with good regrowth.

In vitro-*grown shoot tips.* The first *in vitro* pear shoot-tip cryopreservation systems were developed simultaneously by Reed (1990) and Dereuddre et al. (1990a, 1990b). Reed (1990) developed a slow-freezing technique. Cold acclimatization (CA) and slow cooling resulted in a high rate of shoot regrowth (55% to 95%) for cryopreserved shoot tips of four *Pyrus* species. Species differences were apparent in the regrowth percentage after thawing: 95% for *P. communis* cv. Beurre Hardy and *P. cossonii* Rheder, 75% for *P. koehnei* C. Schneider and 55% for the hybrid *P. dimorphophylla* Makino × *P. fauriei* C. Schneider, hybrid. Pear plantlets were cold acclimatized for 1 wk (22° C, 8 h light; −1° C, 16 h dark). Shoot tips were pretreated on medium with 5% DMSO then cooled in PGD cryoprotectant [10% each polyethylene glycol (MW 8000), glucose and DMSO in liquid medium] at 0.1, 0.3, 0.5 or 0.8° C/min to −40° C before LN exposure and thawed for 1 min in a 40° C water bath. The response to different cooling rates varied among species, but in general the slower the cooling rate the higher the level of regrowth. Cold acclimatization was essential for high regrowth with all genotypes tested.

Dereuddre et al. (1990a, 1990b) developed the encapsulation-dehydration technique with 'Beurre Hardy' shoot tips. Shoot tips from plantlets CA for 2 mo. were encased in alginate-gel beads, precultured in a liquid medium with 0.75 *M* sucrose for 18 h, and dehydrated in an air-flow cabinet for 2 to 6 h. The beads were cooled rapidly by direct immersion in LN and rewarmed slowly in air at room temperature. High survival (80%) resulted from dehydration for 3 h; 40% of the surviving shoot tips produced new plantlets. In a later study the best results (80% shoot recovery) were obtained with a 0.75 *M* sucrose preculture and 4 h of dehydration, resulting in 20% residual water (Scottez et al., 1992).

Niino and Sakai (1992) obtained about 70% shoot formation for three pear cultivars (*P. pyrifolia* and *P. communis*) with a modified encapsulation-dehydration method. Plantlets were CA for 3 wk; then shoot tips were removed and precultured with successively higher concentrations of sucrose in the medium, captured in alginate beads, and precultured in 1 *M* sucrose for 16 h at 5° C, then dehydrated over silica gel to 33% moisture and immersed in LN.

Niino et al. (1992) successfully applied the vitrification method to eight pear cultivars (*P. pyrifolia* and *P. communis*) with 40% to 72.5% shoot formation. Shoot tips (1.5–2 mm) were removed from *in vitro*-grown plantlets CA for 3 wk at 5° C with an 8 h photoperiod. Excised shoot tips were pretreated for 1 d on 0.7 *M* sucrose medium at 5° C, followed by 20 min in plant vitrification solution 2 (PVS2) in cryotubes. Two changes of PVS2 over an additional 70 min preceded a plunge into LN. Shoot tips were rewarmed in a 35° C water bath and regrown on MS medium (Murashige and Skoog, 1962) without NH_4NO_3.

Germplasm storage. Many *Pyrus* genotypes at NCGR-Corvallis were screened for cryopreservation potential with slow-freezing (Reed, 1990) and vitrification techniques (modified from Yamada et al., 1991). The goal was to establish a base collection of pear germplasm stored as shoot tips (0.8 mm) in LN. Accessions were randomly chosen from the *in vitro* collection and screened for response to cryopreservation techniques. *In vitro*-cultured plantlets received 1 wk of CA in an incubator with 22° C, 8-h d (3 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and −1° C 16-h nights prior to shoot tip excision for either technique.

Using the slow-freezing method developed by Reed (1990), we pretreated 0.8-mm shoot tips for 2 d in the CA incubator on base medium with 5% DMSO and 2.05 g Gelrite added, then transferred them to 0.25 ml liquid medium in 1.2-ml plastic cryotubes. Cryotubes were slowly filled with the cryoprotectant PGD [10% each polyethylene glycol (MW 8000), glucose and DMSO in liquid medium] over a half-hour period. Samples were allowed to equilibrate at 4° C for 30 min, after which the cryoprotectant was drawn down to 1 ml.

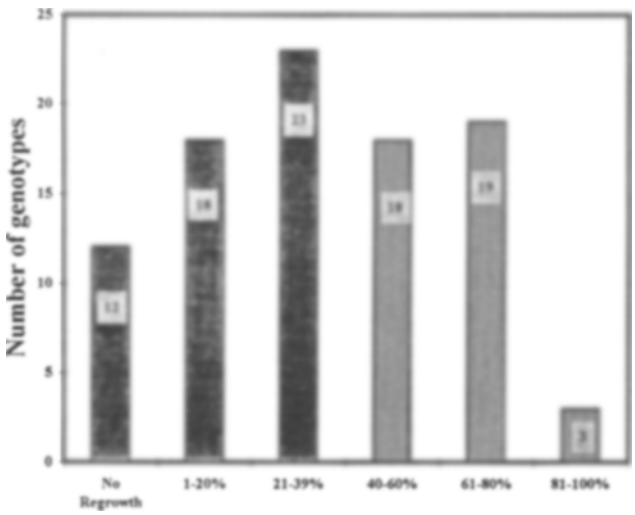

FIG. 1. Distribution of percent shoot regrowth of 93 pear genotypes cryopreserved by slow freezing. *Solid bars* = regrowth <40%, *striped bars* = ≥40% regrowth.

Samples were cooled in a controlled rate freezer (CryoMed, Forma Scientific, Marietta, Ohio) at 0.1° C/min to −9° C when an exotherm was induced; cooling continued at 0.1° C/min to −40° C, when samples were plunged into LN. Samples were warmed for 1 min in 45° C water then 1 min in 22° C water, rinsed in liquid medium, and plated on Cheng medium (Cheng, 1979).

Pear shoot tips were vitrified with a modification of the technique developed for white clover (Yamada et al., 1991). Shoot tips were pretreated as in the slow-freezing method, then transferred to 1.2-ml plastic cryotubes with PVS2 cryoprotectant (30% glycerol, 15% ethylene glycol, and 15% DMSO in liquid medium with 0.4 *M* sucrose) on ice, and stirred. After 20 min, cryotubes were submerged in LN. Samples were rewarmed as in the slow-freezing method and then rinsed twice in liquid MS medium with 1.2 *M* sucrose for 2 min.

Comparison of cryopreservation methods for germplasm storage. A comparison of 28 *Pyrus* genotypes showed that 61% had good shoot regrowth (>50% regrowth) after slow freezing (0.1° C/min), whereas only 43% of the genotypes responded well to the vitrification technique (Luo et al., 1995). We chose the slow-freezing technique for long-term storage over the vitrification technique for several reasons. First, it was easier to process a large number of samples with slow freezing; the timing of addition and removal of the cryoprotectant PGD was not highly critical in slow freezing, so survival was less likely to be affected by interruptions during handling. Second, more genotypes survived the slow-freezing treatment than survived vitrification. Both methods were suitable for pear cryopreservation and the choice of one over the other would depend on individual laboratory situations. Genotypes with greater than 40% shoot formation with the slow freezing method were cryopreserved and shipped to the National Seed Storage Laboratory, Ft. Collins, CO for long-term storage (Reed et al., 1997).

Genotype response to cryopreservation methods. Ninety-three genotypes were screened with the slow-freezing technique originally developed for four genotypes (Reed, 1990). Forty genotypes had regrowth greater than 40%; of these, 22 genotypes had regrowth greater than 60% and 18 had regrowth between 40 and 60% (Fig. 1). We arbitrarily set 40% regrowth as the lowest acceptable regrowth rate; however, with clonal materials only one shoot tip is needed to maintain the genotype. Methods are usually developed using a few genotypes at a time; however, it is very important to know how diverse genotypes will respond if the goal is long-term storage of a highly variable germplasm collection. Some *Pyrus* genotypes did not regrow following cooling and these were identified as having cultural problems which required further study. The accessions with low recovery are being tested with modified pretreatment procedures (Chang and Reed, 1997).

We believe that each of the available cryopreservation methods has distinct advantages and disadvantages (Reed and Yu, 1995). Slow freezing is effective for many taxa and material handling is easy, but it usually requires expensive equipment. This would limit its use in many laboratories. Vitrification is quick and easy for small samples and no special equipment is needed. However, the cryoprotectant solutions tend to be very toxic to the shoot tips and careful timing is often necessary for successful recovery, thus limiting the number of cryotubes that can be handled at one time. Encapsulation and dehydration of cells and shoot tips in alginate beads is also successfully used for pear shoot-tip cryopreservation (Dereuddre et al., 1990a, 1990b; Niino and Sakai, 1992; Scottez et al., 1992). The encapsulation method requires individual handling of shoot tips several times during the process, which makes it more time consuming and tedious than the other methods. It appears to be the best method to use with fragile materials and those that do not respond to other methods.

Shipping and storage of pear germplasm. For storage, 150 shoot tips were cryopreserved (25/cryotube) and placed in a shipping dewar (Minnesota Valley Engineering, New Prague, MN). Five unfrozen controls were plated immediately. One cryotube (25 shoot tips) was removed from the dewar before shipping and was thawed; the shoot tips were then regrown. The remaining 125 shoot tips were sent to the National Seed Storage Laboratory in Fort Collins, CO. The shipping dewar was prepared in advance by cooling with LN until the absorbent material was saturated and LN remained in the well. The shipping dewar retained LN temperatures for more than 1 wk, so it would be suitable for international shipments. Cryotubes on canes were transferred to the shipping dewar and sent by express mail with the required hazardous materials shipping documents (air freight, Federal Express). Canes were removed from the travel dewar and placed in long-term storage in LN. After a short period of storage (1 wk to 3 mo.) one cryotube was thawed as a control for travel and storage, and regrown as noted above. The remaining 100 shoot tips are in long-term storage in LN to form the base pear collection at NSSL. Some variability was seen in the regrowth of cryopreserved shoot tips between the two locations and between the test freeze and the shipping freeze (data not shown). This variation may be due to plant condition, differences in individual cryotubes, the particular thawing procedure for an individual cryotube, growth room differences at the two locations, or other handling anomalies which are difficult to quantify.

Effect of plant condition on regrowth. Healthy plants are required for the best survival and regrowth of cryopreserved shoot tips. Initially all *Pyrus* cultures were screened regardless of vigor in culture. Future screening will exclude cultures which are growing poorly. Contraindications include hyperhydricity, slow growth, and shoot tip necrosis. Factors such as medium composition may impact survival following cryopreservation (Withers et al., 1988). *In vitro* cultures exhibiting poor growth characteristics, including some genotypes

TABLE 1

EFFECT OF 1 WK OF COLD ACCLIMATIZATION ON REGROWTH OF CRYOPRESERVED PEAR (*PYRUS* L.) SHOOT TIPS AFTER SLOW FREEZING, IMMERSION IN LIQUID NITROGEN, AND THAWING

Name	NCGR accession no.	Cold acclimatized regrowth %	Non-acclimatized regrowth %
Pyrus communis L. cultivars[a]			
Doyenne du Comice-Regal Red	2150.001	75	10
Itala Pirovano	2898.001	80	30
Notaire Lepin	418.001	75	15
Nouveau Poiteau	420.001	50	30
Species			
P. betulifolia Bunge	1310.001	50	25
P. calleryana Decne.	675.001	50	10
P. koehnei C. Schneider	812.001	80	0
P. pashia Buch.-Ham. ex D. Don	876.001	65	45

[a]Based on single tests of 20 cryopreserved shoot tips and 5 nonfrozen controls for each treatment.

TABLE 2

COMPARISON OF 1 AND 3 WK COLD ACCLIMATIZATION (CA) ON REGROWTH OF PEAR (*PYRUS* L.) SHOOT TIPS FOLLOWING SLOW FREEZING AND IMMERSION IN LIQUID NITROGEN

		% Recovery following cryopreservation[a]	
Name	NCGR accession no.	1 wk CA	3 wk CA
Pyrus communis L. cultivars			
Fondante de Charneu	230.001	5 ± 0.3	42.5 ± 24.8
Admiral Gervais	11.001	0	19.5 ± 6.36
Species			
P. pashia Buch.-Ham. ex D. Don	1394.001	14.9 ± 14.3	73.2 ± 2.6

[a]Two replicates, n = 40.

tested, required changes in culture medium or growth room conditions before reaching high recovery percentages following cryopreservation (data not shown). Harding et al. (1991) found that long-term culture of potato significantly decreased recovery of plants following cryopreservation. Recovery of pear shoot tips following cryopreservation does not appear to be affected by long-term maintenance in tissue culture (Reed, unpublished).

Cold acclimatization of the *in vitro* plants prior to shoot tip excision is crucial to survival and regrowth of *Pyrus* shoot tips following cryopreservation (Reed, 1990; Niino et al., 1992). Differences in regrowth were apparent for eight pear genotypes cryopreserved with and without CA (Table 1). These results are similar to those found earlier in replicated trials with four pear genotypes for which regrowth ranged from 0 to 26% for non-CA shoot tips and 55 to 95% for CA shoot tips (Reed, 1990). Standard CA treatment of 1 wk was successful for many of the pear genotypes tested in this study; however, some required longer CA for high regrowth. Three wk of CA improved recovery of some genotypes (Table 2).

CONCLUSIONS

Depletion of germplasm resources throughout the world due to loss of natural areas, plant diseases, and changes in agricultural practices mandates a move toward conserving as much genetic material as possible. Present methods of germplasm storage such as field, greenhouse and *in vitro* gene banks are important as active (working) collections. Cryopreservation provides a secure, low maintenance, reliable means to back up these active collections.

The research on pollen, dormant buds, and *in vitro*-grown shoot tip cryopreservation described here illustrates the feasibility of cryopreserving *Pyrus* germplasm in several forms and confirms the reliability of shipping cryopreserved materials to remote locations. The storage of *Pyrus* dormant buds, shoot tips, and pollen provides a backup (base) collection of *Pyrus* germplasm to insure against loss due to disease, insect damage, or natural disaster. The form of base storage in LN will vary with the plant material and the method available. Some cold-hardy accessions may be stored as dormant buds, some *Pyrus* species as seeds or pollen, and other accessions as shoot tips of *in vitro*-grown plants. The initiation of this and other base collections of clonal germplasm in LN is an important step in developing safe, efficient long-term storage.

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