Reprint

Conservation of Plant Genetic Resources *In Vitro*

Volume 1: General Aspects

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Medium- and Long-term Storage of *In Vitro* Cultures of Temperate Fruit and Nut Crops

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INTRODUCTION

Slow-growth and cryopreservation strategies are used in a number of institutions throughout the world for the preservation and distribution of fruit and nut germplasm (Brettencourt and Konopka, 1989). Techniques for some crops require additional research, but methods for others are well defined. Slow-growth conditions provide a secondary storage method for clonal field collections, a storage mode for experimental material, or a reserve of germplasm for plant distribution. Cryopreservation provides a low-input method for storing a base collection (long-term backup) of clonal materials. Both *in vitro* and cryopreserved collections provide insurance against the loss of valuable genetic resources and may provide alternative distribution methods.

The term "medium-term storage" encompasses slow-growth strategies ranging from temperature reduction or environmental manipulation to chemical additions in the growth media. The variety of storage techniques which have been developed so far provides options to match techniques with a facility's needs and resources.

The number of cryopreserved species is rapidly increasing because of new techniques and improvements in cryopreservation research. Liquid nitrogen storage is now available for plants as suspension or callus

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cultures, dormant buds, *in vitro*-grown apical meristems, isolated embryonic axes, somatic embryos and pollen. A few cryopreserved collections of temperate plants of economic importance have been established. Only time and financial resources remain as barriers to the storage of most temperate horticultural crops as base collections in liquid nitrogen.

MEDIUM-TERM STORAGE OF *IN VITRO* CULTURES OF TEMPERATE SMALL FRUITS

A limited number of small (soft) fruit genera have been stored *in vitro* for nearly 20 years. A number of laboratories throughout the world routinely use *in vitro* storage techniques, but little has been published on the subject (Table 1). Reduced temperatures (–1°C to 8°C) are most commonly used to slow growth but some investigators have altered medium composition for room temperature (25°C to 28°C) storage. Storage occurs either in darkness or with 12- to 16-h photoperiods. Published *in vitro* storage methods are available for *Actinidia, Fragaria, Ribes, Rubus,* and *Vitis* and are summarized below. In addition, we present some unpublished data from the U.S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository in Corvallis, OR (NCGR-Corvallis), and Changli institute of Pomology, Changli, Hebei Province, PRC.

Actinidia: Slow-growth storage studies for *Actinidia deliciosa* Liang and Ferguson cv. Hayward (formerly *A. chinensis* Planch.) found that shoot-tip cultures could be stored in dark for one year at 8°C, while those stored at 4°C began declining after six months (Monette, 1986, 1987). The quality of surviving shoot tips was also better at 8°C than at 4°C. Storage medium was composed of one-fourth strength Murashige and Skoog (1962) (MS) salts and 4 mgl^{-1} 6-benzylaminopurine (BAP). Transient physiological abnormalities were observed in a few cultures but no field evaluations were conducted to determine genetic stability (Monette, 1995). Cultures of *Actinidia chinensis* Planch. derived from seedlings showed chilling sensitivity at 4°C but storage at 25°C on medium with activated charcoal was successful for nine months (Wilkins et al., 1988).

Fragaria: In vitro storage of *Fragaria* germplasm began in the mid-1970s. Mother plants were stored on basal medium and weak illumination at 1 to 3°C for 12 to 18 months for the production of virus-free strawberry planting stock (Boxus, 1976). Multiple genotypes of commercial strawberries were stored for up to six years on filter-paper

Table 1. Medium-term storage of *in vitro* cultures of temperate small fruits

Species or Cultivars		Results	References
(1)	(2)	(3)	(4)
Actinidia deliciosa (A. chev.) C.F. Liang et A.R. Ferguson cv. Hayward	8°C or 4°C, dark	1 year, 100% viability at 8°C 60% viability at 4°C	Monette, 1986, 1987
Actinidia chinensis Planch.	25°C, 16-h photoperiod, charcoal in medium	9 months storage with 40% viability	Wilkins et al., 1988
Fragaria species and cultivars (over 50)	4°C, dark, add liquid medium at 3-month intervals	Viable and healthy for up to 6-year storage	Mullin and Schlegel, 1976
Fragaria cultivars (21)	2°C, dark	9–27 months storage, 10–100% survival	Damiano, 1979
Fragaria species and cultivars (96)	4°C, dark, in tissue-culture bags	15 month mean storage time, range 9–24 months, no contamination	Reed, 1991, 1992a; Reed and Hummer, 1995
Fragaria species and cultivars (9)	4°C, 12-h photoperiod or dark, 4 BA concentrations, CH or not	More than 12 months, best with CH, photoperiod, and low BA	Reed, 1995
Ribes nigrum L. (14 cvs.); *R. nigrum* × *R. ussuriense* Jancz. hybrid	6°C, light	3 months, 95–100% survival	Brennan et al., 1990
Rubus species and cultivars (250)	4°C, 12-h photoperiod 4°C, dark in bags 25°C, reduced nitrogen for cold sensitive genotypes	12 months storage, 4°C, light, 40–100% survival 9 months, 60–90% survival with 25% nitrogen	Reed, 1993a
Vitis	Room temperature, low light	8–10 months, 100%	Cao et al., 1988
Vitis	Growth inhibitors CCC, TIBA and ABA at 28°C	Good with CCC at 10–20 ppm at 18 months	Li et al., 1992
Vitis species and cultivars (12)	26°C, 15-h light/20°C, 9-h dark 9.5°C, dark	9–12 months, mixed results, 0–70% recovery	Barlass and Skene, 1983
Vitis vinifera L. cv. Chardonnay and *V. rupestris* cv. du Lot	12°C, 12 $\mu E \cdot m^{-2} \cdot s^{-1}$ 0.3% or 1.5% sucrose in medium	11 months storage, Chardonnay best at 1.5%, du Lot best at 0.3%	Galzy and Compan, 1988
Vitis vinifera L. cv. Rizamat, *V. thumbergii* Sieb, & Zucc., *V. labrusca* L. cv. Delaware	28°C, 16 h light, reduced ammonium nitrate	9 months, 70–80% survival with 6% or 25% ammonium nitrate	Moriguchi and Yamaki, 1989

bridges in liquid medium with growth regulators at 4°C in dark with additional medium added at three-month intervals (Mullin and Schlegel, 1976). Strawberry cultures were stored for many years on phytohormone-free medium at low temperatures with annual transfers (Jungnickel, 1988). Strawberry germplasm of over 350 *Fragaria* genotypes stored in gas-permeable tissue-culture bags in dark at 4°C were healthy and contamination free for 12 to 24 months with a mean storage time of 1.5 years (Table 2) (Reed and Hummer, 1995). The addition of a 12-h photoperiod increased the length of storage in tissue-culture bags for most genotypes. Plantlets cold hardened for one week (22°C, 8 h light/–1°C, 16 h dark) before storage and those stored on medium with N^6 benzyladenine (BA) survived better than those without hardening or BA (Reed, 1995).

Table 2. Medium-term storage of *in vitro* cultures at the U.S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository-Corvallis. Storage periods include studies from 1989 through 1995 and were at 4°C in dark unless otherwise noted

Mean length of storage without repropagation for each genus[a].			
Genus	Number of accessions	Mean storage (years)	Range (years)
Corylus	33	1.26 ± 0.5	0.7 – 2.7
Fragaria	278 (96 sampled)	1.50 ± 0.5	0.7 – 2.0
Humulus	56	1.20 ± 0.4	0.7 – 2.7
Mentha	306 (116 sampled)	3.40 ± 0.9	0.7 – 5.7
Pyrus	175 (82 sampled)	2.68 ± 1.1	0.7 – 4.7
Ribes	101 (80 sampled)	1.40 ± 0.8	0.7 – 3.0
Ribes[b]	40 sampled	2.76 ± 0.7	
Rubus[c]	258 (101 sampled)	2.21 ± 1.3	0.3 – 5.0
Vaccinium	94 (81 sampled)	2.10 ± 1.1	0.3 – 4.0

[a]Large differences exist between individual genotypes. The length of storage may also be affected by mechanical problems or technical skills of personnel. Each genus is inventoried at 4-month intervals and repropagated as needed.
[b]–1°C, dark storage
[c]12-h photoperiod for 1993–95

Ribes: Few reports are published on the storage of *Ribes* germplasm. Some *Ribes* cultivars and species stored well for one year at –1°C in dark, while others declined much earlier, and most at stored at 5°C declined in a few months (Gunning and Lagerstedt, 1985). Preliminary investigations on the storage of three *Ribes* cultivars at 6°C for three months found survival and morphogenic potential remained constant (Brennan et al., 1990).

Ribes cold-storage studies at NCGR-Corvallis—In vitro cultures of over 100 *Ribes* genotypes have been preserved in the collection at

NCGR-Corvallis. These cultures were typically kept at 4°C in dark and had a mean storage duration of 1.5 years (Table 2). We compared *Ribes* cultures stored in 4°C dark with those in a –1°C dark refrigerator. A second experiment compared cultures in a 12-h photoperiod at 4°C with those in –1°C dark conditions. Growth room conditions provided were a 16-h photoperiod at 25°C with 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance provided by cool white fluorescent bulbs; the cold-hardening incubator had 8 h light at 22°C and 16 h dark at –1°C. Plantlets from the propagation medium were transferred to two five-chamber tissue culture bags (Agristar, Conroe, Texas), placed for one week in the growth room and then one week in the cold-hardening incubator before storage.

Shoots were multiplied on NCGR-RIB medium with (per liter): 0.1 mg BA, 0.2 mg gibberellic acid (GA), 3.5 g agar (Bitek, Difco, Detroit, Mich.), and 1.45 g Gelrite (Schwizerhall, South Plainfield, N.J.) at pH 5.7. Growth regulators were obtained from Sigma Chemical Co., St. Louis, Mo. This medium has one-third of the nitrogen level of normal MS medium. Plantlets were grown in Magenta GA7 culture vessels (Magenta Corp., Chicago, Ill.).

Ten plantlets of each accession were stored with 10 ml medium in an individual section (15 × 150 mm) of a five-section bag. Storage bags contained a firmer medium (3.5 g agar and 1.75 g Gelrite per liter) compared to the multiplication medium. We stored one bag of each genotype per treatment. Plants were inventoried at 4-month intervals and rated from 0 to 5 for their condition. Those with a rating of ≤ 2 were removed for repropagation (Reed, 1992a). The first experiment divided the bags between 4°C dark and –1°C dark and continued from 1990 to 1993. The second experiment was with 4°C, 12-h photoperiod (3 $\mu E \cdot m^{-2} \cdot s^{-1}$), and –1°C dark storage from 1993–1995. Not all genotypes were used in both experiments.

After 15 to 24 months in dark storage the mean storage duration of plantlets at –1°C was 2.76 ± 0.7, while at 4°C the mean was 1.40 ± 0.8 (Table 2). *Ribes* accessions stored at 4°C with a 12-h photoperiod for 11 to 15 months had means of 2.4 ± 0.9 and those at –1°C in dark had 2.2 ± 0.6. Means of plantlets stored at –1°C dark were similar to those at 4°C with a 12-h photoperiod (Table 3). Storage lengths varied among genotypes, ranging from four months to three years for those stored at 4°C in dark. Differences in storage time may arise due to the condition of the plant when stored, size of propagule, preparation of medium, or placement of the plant in the medium. Storage consistency depends on the technical skills of lab workers. *Ribes* are particularly sensitive to size variation and small plantlets store for shorter periods than do intermediate or large plantlets. Genotypes which have a bushy

Table 3. Condition ratings for *Ribes* genotypes stored under various conditions for 11 to 25 months. Treatments were inventoried at 4-month intervals and rated for condition (0 = dead, 5 = excellent).

Months stored	Genotype	Storage Rating			
		Experiment 1		Experiment 2	
11	Captivator (*R. grossularia* L.)	–	–	4	2
11	Portal Ruby (*R. rubrum* L.)	–	–	3	2
11	*R.* hybrid	–	–	4	2
15	Consort (*R. nigrum* L.)	1	2		
15	Kerry (*R. nigrum*)	2	3	–	–
15	Raby Castle (*R. rubrum*)	1	4	3	2
18	Boskoop Giant (*R. nigrum*)	–	–	2	2
18	*R. ciliatum* Humb. and Bonpl.			0	3
18	Viking (*R. rubrum*)	–	–	3	2
19	Pruterberry (*R. odoratum* Wendl. f.)	1	2		
19	Weisse Aus Juteborg (*R. petraeum* Wulf)	1	3		
20	Kosmiczeskaja (*R. nigrum*)	1	3	3	2
20	*R. curvatum* Small	2	3	–	–
20	*R. hudsonianum v. petiolare* Dougl.	1	3	2	3
20	*R. viscocsissium* Pursh	2	4	–	
20	Rosa Hollandische (*R. rubrum*)	1	2	3	2
23	Invigo (*R. nigrum*)	3	3		
25	Heros (*R. rubrum*)	1	3		
25	*R. echinellum* (Cov.) Rehd. B74B	1	3		
25	Wilder (*R. rubrum*)	1	3		

growth habit exhibit a shorter storage duration than those with more elongated plants (data not shown).

Rubus: Preliminary storage experiments with *Rubus* germplasm indicated that broad genetic variation exists in relation to cold tolerance of different genotypes for *in vitro* cold storage (Gunning and Lagerstedt, 1985). Most of the 250 *Rubus* species and cultivars at NCGR-Corvallis could be stored on medium with no growth regulators for one to five years at 4°C with a 12-h photoperiod ($3\,\mu E \cdot m^{-2} \cdot s^{-1}$) (Reed, 1993a). Mean storage duration was 2.2 years with a range of four months to five years (Table 2). Accessions such as tropical species, which declined quickly under cold conditions, could be stored for nine months in the growth room without transfer (25°C, 16-h photoperiod) with 25% MS nitrogen concentrations in the growth medium (Reed, 1993a).

Vaccinium: *Vaccinium* genotypes held at 5°C in darkness for one year were etiolated, depleted the medium, and many were contaminated. New basal shoots developed during storage when the apex died (Gunning and Lagerstedt, 1985). The mean storage time for 81 genotypes of *Vaccinium*, held at 4°C in darkness in the tissue-culture collection at NCGR-Corvallis, was 2.1 years with a range of four months to four years (Table 2). Cultures are currently being evaluated at 4°C with a 12-h photoperiod ($3\,\mu E \cdot m^{-2} \cdot s^{-1}$).

Vitis: Barlass and Skeene (1983) stored cultures of 12 *Vitis* species and hybrids for up to 12 months at 9.5°C with a 15-h photoperiod ($15–30\ \mu E \cdot m^{-2}.s^{-1}$). Cao et al., (1988) successfully stored grape germplasm at room temperature for 10 years with transfer at 8- to 10-month intervals; some genotype variation was noted. Two species of *Vitis* stored at 12°C for 11 months with light ($12\ \mu E \cdot m^{-2} \cdot s^{-1}$) showed considerable variation among genotypes (Galzy and Compan, 1988). *V. rupestris* cv. du Lot stored longer on low sucrose concentrations (0.3%) while *V. vinifera* cv. Chardonnay stored best on medium with 15% sucrose. Room temperature storage for eight to nine months on medium with reduced ammonium nitrate was more successful than 5°C or 10°C storage for three grape species (Moriguchi and Yamaki, 1989). Li et al. (1992) stored ten grape genotypes at room temperature for up to 18 months with 10 to 20 ppm of CCC ([2-Chloroethyl] trimethylammonium chloride) in B5 growth medium but other CCC concentrations, TIBA (2,3,5-Triiodobenzoic acid), and abscisic acid (ABA) were not effective. Chang (unpublished) used a modified B5 medium with 2 ppm pacbutrazol (PP333) to store eight grape genotypes at room temperature for up to 15 months with 100% survival. Cultures of more than 130 grape genotypes are stored at Changli Institute of Pomology at 8°C to 10°C with light ($10\ \mu E \cdot m^{-2} \cdot s^{-1}$, 15-h photoperiod) and yearly transfers.

MEDIUM-TERM STORAGE OF *IN VITRO* CULTURES OF TEMPERATE FRUIT AND NUT TREES

Genetic conservation of fruit trees in field genebanks is risky because of losses from weather, pests, and diseases. The global exchange of field germplasm is restricted by quarantine laws designed to prevent the spread of diseases or insects. *In vitro* storage systems are valuable as a backup to field genebanks and in some countries satisfy quarantine restrictions to insure disease and insect-free germplasm exchange. *In vitro* culture systems are available for most temperate fruit and nut

crops but information on medium-term storage is limited for many genera. Published research is available for *Malus, Morus, Prunus, Punica,* and *Pyrus*; however, most studies are restricted to a few genotypes and storage conditions (Table 4). There are no published reports of *in vitro* storage systems for temperate nut trees. The following review includes available literature and some previously unpublished data on germplasm collections at NCGR-Corvallis and Changli Institute of Pomology.

Table 4. Medium-term storage of *in vitro* cultures of temperate tree fruit

Species or Cultivars	Storage Methods	Results	References
(1)	(2)	(3)	(4)
Malus domestica L. cv. Golden Delicious	25°C 1°C or 4°C, dark	12 months, 70–100% 3–12 months, 60–100%	Lundergan and Janick, 1979
M. domestica cv. Golden Delicious, rootstocks M26 M27, M111	2°C, dark, half-submerged in agar medium	100% for 1.5 to 3.5 years depending on genotype	Druart, 1985
M. domestica Borkh. (5 cvs.), *M. baccata* (L.) Borkh., *M. prunifolia* (Willd.) Borkh.	4°C, 16-h photoperiod	12 to 28 months, 13–100%	Wilkins et al., 1988
M. domestica	2°C–4°C, diffused light 0.04 μM, BAP, 1.5% sucrose	1.5 years storage	Eckhard, 1989
M. domestica rootstock cvs. M9 and P2	4°C, dark Three media Three explant types	9 months, 80 to 100% on proliferation or reduced BAP medium, best explant type varied with genotype	Orlikowska, 1991, 1992
Morus alba L.	4°C, dark multiplication medium	80% at 6 months 7% at 12 months	Sharma and Thorpe, 1990
M. nigra L.	4°C, 16-h photoperiod 6–9 months, 25°C	100% at 6 months 5% at 9 months 42% at 9 months	Wilkins et al., 1988
Prunus domestica L. cv. D1869, *P. persica* (L.) Batsch. × *P. amygdalus* Batsch. cv. GF677, *P. cerasus* L. cv. CAB 11E (all rootstocks)	Dark or 16-h photoperiod, –3°C, 4°C or 8°C	10 months, dark, low temperature, culture for 7–14 days before storage	Marino et al., 1985

(Contd)

Table 4. ***(Contd)***

(1)	(2)	(3)	(4)
Prunus 12 species	2°C, dark, defoliated, half-submerged in agar medium	1.5 to 4 years, varied with genotype	Druart, 1985
P. avium L. × *P. pseudocerasus* Lindl. cv. Colt, *P. insitita* L. cv. Pixy, *P. domestica* cv. Jefferson's Gage, *P. padus* L., *P. cerasifera* Ehrh. cv. Pissardii	4°C, 16-h photoperiod	9–18 months, 62–100%	Wilkins et al., 1988
Punica granatum L.	4°C or 10°C, 16-h photoperiod	Cold sensitive at 4°C, 18 months at 10°C	Wilkins et al., 1988
Pyrus communis L. cv. Caucasica	4°C, 8°C, 12°C, 16-h photoperiod	56–94% at 18 months 4°C high sucrose/mannitol not effective	Wanas et al., 1986
P. communis cvs. La France, Bartlett, and Bartlett x La France hybrid, *P. pyrifolia* Nakai cvs. (6)	5°C, 10°C, 15°C, 16-h photoperiod 1°C, dark	60–100% at 1°C for 12–20 months, lower for other treatments	Moriguchi et al., 1990
P. pashia D. Don.	4°C, 16-h photoperiod 10°C, 16-h photoperiod	100% at 12 months 100% at 12 months	Wilkins et al., 1988

Corylus: At NCGR-Corvallis, 33 genotypes of hazelnut (*Corylus* sp.) *in vitro* cultures are stored at 4°C in dark. The mean storage duration is 1.26 years and the range is eight months to 2.5 years (Table 2).

Malus: The first papers on *in vitro* storage of *Malus domestica* Borkh. reported successful storage of "Golden Delicious" for 12 months at 1°C or 4°C. The authors were the first to suggest the feasibility of an *in vitro* apple germplasm repository (Lundergan and Janick, 1979). Additional studies indicate similar success with other *Malus* species and cultivars, with storage ranging from nine months to 3.5 years (Druart, 1985; Orlikowska, 1991, 1992; Wilkins et al., 1988) (Table 4). Wilkins et al. (1988) studied cultures of five *Malus domestica* cultivars, as well as *M. prunifolia* (Willd.) Borkh. and *M. baccata* (L.) Borkh., under several storage regimes. Apple cultivars were stored successfully for 12 to 28 months at 4°C with a 16 h photoperiod on agar-based multiplication medium and some rootstock cultivars were successful in

liquid medium. The addition of charocal to the medium improved storage time but eliminated proliferation. Orlikowska (1991, 1992) found that two apple rootstock cultivars kept at 4°C in dark stored better on medium with 6-benzylaminopurine (BAP) than on medium lacking the growth regulator. Explants stored as tufts of shoots were more productive following storage than were single-shoot cultures. Storing plants immediately after subculture was important for the survival of shoot tufts, but shoot tips and middle segments survived whether stored immediately or at 10 or 20 days after subculture. Eckhard (1989) stored apple-shoot cultures at 2–4°C under low light for 1.5 years on a reduced sucrose, low BAP medium. Topped, partially submerged shoots of three *Malus* rootstocks and "Golden Delicious" were stored on hormone-free medium in dark, at 2°C for 1.5 to 3.5 years with 100% survival (Druart, 1985). More than 150 apple species and cultivars are stored at Changli Institute of Pomology on modified MS medium at 2 to 4°C in light (10 $\mu E \cdot m^{-2} \cdot s^{-1}$, 15-h photoperiod) with yearly transfer (Chang, unpublished).

Morus: Single shoots of *Morus nigra* L. stored on multiplication medium at 4°C with a 16-h photoperiod survived for only six months. This survival could be increased to 42% at nine months by storing them at 25°C with activated charcoal (Wilkins et al., 1988). Sharma and Thorpe (1990) stored 15 genotypes of *Morus alba* L. at 4°C in dark on shoot proliferation medium with high viability (80%) for six months. All shoots rooted and surviving shoots retained their multiplication potential.

Prunus: Shoot cultures of three *Prunus* genotypes (two peach, one cherry) were stored at 8°C, 4°C or –3°C for up to 10 months on multiplication medium (Marino et al., 1985). A 16-h photoperiod was important for successful 4°C and 8°C storage for 90 days. Ten-month, dark storage at –3°C was better than under light, particularly for some genotypes. Established cultures (Stored 14 days after subculture) survived better than those stored immediately after subculture and lower temperatures resulted in longer storage times. Druart (1985) tested 12 *Prunus* species and cultivars on basal medium at 2°C in dark. Some genotypes stored successfully for up to four years; adding dimethyl sulfoxide or glycerol to the medium proved toxic to the cultures. Shoots were topped and partially submerged in the medium prior to storage; survival was genotype dependent (Druart, 1985). Five *Prunus* genotypes stored on multiplication medium at 4°C with a 16-h photoperiod showed high survival after 9 to 18 months (Wilkins et al., 1988).

Punica: Pomegranate, *Punica granatum* L., shoot cultures were sensitive to 4°C but could be stored for 18 months at 10°C with a 16-h photoperiod (Wilkins et al., 1988).

Pyrus: Shoot tips of *Pyrus communis* L. cv. Caucasica were stored on basal medium at 4°C, 8°C, and 12°C with a 16-h photoperiod. Each temperature tested depressed growth for 12 to 18 months, but survival was highest at 4°C. Adding mannitol or increasing sucrose in the medium did not produce acceptable survival at 4°C or 28°C (Wanas et al., 1986). *Pyrus pashia* D. Don stored on multiplication medium at 4°C or 10°C with a 16-h photoperiod had 100% survival after 12 months (Wilkins et al., 1988). *P. communis* cultivars La France, Bartlett, and La France × Bartlett had high survival at 5°C a 16-h photoperiod and at 1°C in dark after 20 months, but survived poorly at 10°C and 15°C with light. The Japanese pears (*P. pyrifoila* (Burm.) Nakai) "Shinsui", "Nijisseiki", "Shinchu", "Kosui" "Hosui", and "Hakataao" did not survive storage at 5°C, 10°C, or 15°C with a 16-h photoperiod, but had 100% survival when stored at 1°C in dark for 12 months. Adding ABA to the storage medium did not improve their survival (Moriguchi et al., 1990).

Pyrus cold-storage studies at NCGR-Corvallis—Pears have been stored *in vitro* at NCGR-Corvallis since 1984. Initially they were stored in 20 × 100 mm tubes at 4°C in dark but tubes were replaced with tissue-culture bags in 1989. *Pyrus* storage in tissue-culture bags in dark at 4°C has ranged from eight months to 4.7 years with mean storage time of 2.7 years (Table 2).

A cold-storage experiment was initiated with 46 pear genotypes from the NCGR-Corvallis *in vitro* collection to test three storage conditions. Shoots were multiplied on Cheng's medium (Cheng, 1979) with (per liter): 1 mg N^6-benzyladenine, 3 g agar (Bitek, Difco, Detroit, Mich.) and 1.25 g Gelrite (Schwizerhall, South Plainfield, N.J.) at pH 5.2. Cultures were grown in Magenta GA7 culture vessels (Magenta Corp., Chicago, Ill.) at 25°C under a 16-h photoperiod with 25 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance provided by cool white fluorescent bulbs.

Shoots (2 to 3 cm) from *in vitro* cultures were stored in dark using three treatments: 1) upright, base submerged, 4°C; 2) defoliated, topped and 3/4 submerged, 4°C; and 3) upright, base submerged, 1°C. Five plantlets of each accession were stored per treatment, each in an individual section (15 × 150 mm) of a five-section bag (Agristar, Inc., Conroe, TX) with 10 ml of medium per section. Bags contained a firmer medium (3 g agar and 1.5 g Gelrite per liter), compared to that in boxes and tubes, to compensate for the low amount of water loss through the bag walls (Reed, 1991). Every four months the plants were inventoried and rated on a scale of 1 to 5, with a rating of 5 for excellent

condition (Reed, 1992a). Approximately three months into the experiment the 4°C cold room malfunctioned and warmed to 32°C for several hours.

Pyrus in vitro shoots can be stored at 1°C or 4°C for 1 to 5 years depending on the genotype. A range of species and cultivars were tested in this study (Table 5). In most cases genotypes could be stored from one to three years. "Admiral Gervais", "Clapp Favorite", "Good Christian", *P. calleryana* Decne., "Poirier Fleurissant Tard", and "Rosee de Juillet" were stored for four or five years in all three treatments.

Table 5. Storage life of *in vitro* cultures of pear genotypes held at the U.S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository—Corvallis. Cultures (one bag with five plants for each treatment) were stored in total darkness, inventoried at 4-month intervals, and stored upright or 3/4 submerged in the medium

Accession No.	Genotype	Years Stored		
		Treatments		
		Upright 4°C	Submerged 4°C	Upright 1°C
(1)	(2)	(3)	(4)	(5)
Pyrus communis L. cultivars				
11	Admiral Gervais	5.0	5.0	5.0
14	Alliance Franco-Russe	2.5	2.0	2.5
478	Bartlett—Red Max	1.25	1.0	1.25
493	Bartlett—Rosired	2.0	2.0	2.0
549	Bartlett—Striped	2.5	2.5	2.5
228	Belle Lucrative	4.0	3.5	4.0
1066	Bergamote Tardive de Ganzel	2.25	2.0	2.25
61	Beurre d'Amalis Panachee	3.75	4.0	4.0
81	Beurre Hardy	3.0	3.0	3.0
115	Butirra Precoce Morettini	3.0	4.0	4.0
1725	Cascade	1.0	1.5	1.0
1078	Citron D'Ete	3.5	3.5	5.0
140	Clapp Favorite	5.0	5.0	5.0
142	Colonel Wilder	3.5	3.5	5.0
144	Columbia	1.25	1.25	1.25
161	Count A.W. Moltke	3.5	3.5	5.0
181	Doyenne d'Alecon	3.5	3.5	5.0
2143	F.12-173 (P-2366)	1.25	1.25	1.25
230	Fondante de Charneau	2.25	2.25	4.5

(Contd)

Table 5. *(Contd)*

(1)	(2)	(3)	(4)	(5)
2146	G.28-119 (P-2462)	3.5		3.0
248	Gliva Ukrainskaya/Wilenska Plenna	1.25		1.25
313	Kalle	3.0	3.0	2.5
380	Marchland	3.5	4.0	4.0
1419	MM-38 (rootstock)	2.5	2.5	2.5
397	Monchallard	3.75	2.0	2.25
415	Nijisseiki[a] × OH decline susceptible	3.5	3.5	4.0
418	Notaire Lepin	3.0	3.0	4.0
1375	Oregon Pear Rootstock-001	2.5	2.5	2.5
434	Orient	2.5	2.5	2.5
1518	Poirier Fleurissant Tard	4.0	4.0	4.0
491	Rosee de Juillet	4.0	4.0	4.0
596	Vineland 29018	2.5	2.5	2.5
657	*P. betulifolia* Bunge	3.5	3.5	5.0
650	*P. calleryana* x *P. fauriei* C., Schneider	3.5	2.5	3.0
1276	*P. calleryana* Decne.	4.0	4.0	5.0
1589	*P. cordata* Desv.	3.5	3.0	5.0
828	*P. cossonii* Rheder	3.5	3.5	5.0
764	*P. dimorphophylla* Makino x *P.* sp.	2.5	2.5	5.0
390	*P. elagrifolia* Pallas x *P. communis* L.	.25	1.25	1.25
631	*P. hondoensis* Kikuchi and Nakai	2.25	2.0	2.0
818	*P. koehnei* C. Schneider	1.25	1.25	1.25
808	*P. koehnei*	3.0	3.0	3.5
528	*P. pyrifolia* cv. Shinseiki	2.0	2.0	1.25
250	Good Christian[b]	4.0	4.0	4.0
795	*P. regelii* Rheder	2.0	2.0	2.25
1121	*P. ussuriensis* Maxim, cv. Mien Suan Li	1.0	2.0	3.5

[a] *P. pyrifolia* (Berm. f.)
[b] *P. pyrifolia* cv. Nakai × *P. communis*
The 4°C cold-room malfunctioned approximately three months after the experiment began and heated the room to over 32°C for several hours.

After one year most of the 46 genotypes remained in storage with ratings greater than two (Fig. 1). At two years only 15 to 29 accessions were rated greater than two. A rating of two indicated declining condition and need for repropagation. Little change occurred from two to three years for plantlets at 4°C, while those at 1°C declined slightly. Only a few accessions remained in storage at four and five years. An analysis of variance of the mean ratings of the accessions indicated no significant differences among the three treatments during the first year ($P \geq 0.05$), but by the second year's inventory the mean rating of the submerged treatment was significantly lower than the 1°C treatment. At 2.3 years and longer, the 1°C treatment rating means were significantly better than those of either 4°C treatment ($P \geq 0.01$) and more accession remained in culture. Submerged *Pyrus* cultures did not show a survival advantage over regular cultures. The higher mean

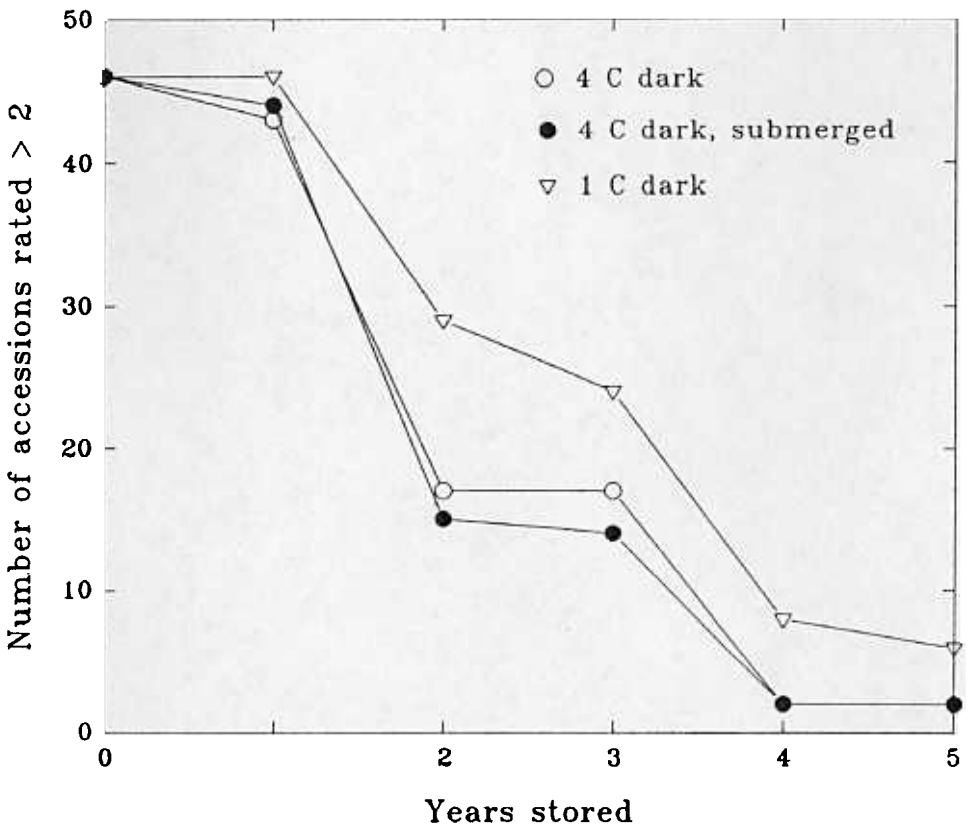

Fig. 1. Storage of *Pyrus* accessions in three conditions. 46 accessions were stored in heat-sealed plastic tissue-culture bags and inventoried at 4-month intervals for five years.

ratings for the 1°C treatment may have resulted from the heat shock received by the 4°C treatments early in the experiment. Genotype differences were evident for the length of storage but for many there was little or no difference between treatments (Table 5).

DISCUSSION OF *IN VITRO* STORAGE TECHNIQUES

In vitro collections play an increasingly important role in storing and distributing germplasm throughout the world. Many certification programs have incorporated *in vitro* culture as a standard technique for producing virus-negative plants from stock collections.

A number of factors are involved in the successful *in vitro* storage of temperate small fruit, fruit trees and nuts. Temperature is the most studied parameter and other factors mentioned in individual studies need more investigation. The size and type of propagule stored is very important. Barlass and Skene (1983) noted that the response of single-rooted shoots differed from that of proliferating cultures of *Vitis*. Single shoot tips, nodal segments, and cultures with multiple shoots also responded differently to various storage conditions (Orlikowska, 1991).

Light quality and intensity, both before and during storage, needs further investigation. In most cases experimentation is limited by growth-room availability. Culture conditions before storage and duration of growth after subculture have important affect on the length of storage. The season of transplanting was significant for *Vitis* cultures stored at room temperature; cultures stored longer when transferred during high summer temperatures (Cao et al., 1988). Proliferation of cultures following cold storage varied with genotype and in some genotypes was better than nonstored plants (Orlikowska, 1992). Proliferation was often dependent on the prestorage culture period (Marino et al., 1985).

Culture and storage media vary greatly for the many genera studied and may influence the survival of cultures. The value of growth regulators in storage media appears to be genotype dependent and may improve or decrease storage time (Orlikowska, 1992; Reed, 1995). Further research needs to address growth regulators in storage medium and genetic stability of stored cultures (Wilkins et al., 1988).

Contaminants often become evident after a period of storage (Gunning and Lagerstedt, 1985). Latent infection may contribute to the death of stored cultures (Wanas et al., 1986). An important but often ignored step in germplasm storage is indexing the cultures for latent bacterial and fungal infections (Reed and Tanprasert, 1995). Indexing procedures using bacteriological media to detect contaminants are more

effective than visual examination of cultures (Reed et al., 1995). Improved detection of bacterial contaminants will provide healthier cultures, longer storage times and safer materials for distribution. Fungal, bacterial, and insect contamination in storage can be minimized by using heat-sealed tissue-culture bags for germplasm storage (Reed, 1992a).

Analysis of genetic stability is lacking for most *in vitro*-grown and stored plants. Available studies indicate that genetic instability is genotype dependent and generalizations are not warranted (Moore, 1991). Field and molecular analyses are needed to determine whether genetic instability is problematic (Harding, 1994; Kumar, 1995). Adventitious shoot production is one of the main causes of genotype variation during *in vitro* storage. Improvements in multiplication and storage media should reduce the likelihood of adventitious shoot production.

The possibility of genetic variation complicates the storage of large germplasm collections. Additional research to develop standard techniques, combined with regular evaluations of stored cultures, will provide safe medium-term storage for *in vitro* cultures for the range of diversity of temperate fruit and nut crops.

LONG-TERM STORAGE (CRYOPRESERVATION) OF TEMPERATE FRUIT AND NUT CROPS

Retention of clonal genotypes of temperate fruit and nut trees requires vegetative propagation. For this germplasm, cold or room temperature storage of *in vitro* cultures provides only medium-term storage (nine to 36 months). Cryopreservation (storage in liquid nitrogen at –196°C) is considered an ideal method for long-term germplasm storage (more than ten years). At cryogenic temperatures cell division and metabolic processes are stopped, minimizing genetic change. Minimal storage space and maintenance are needed for cryopreserved samples. During the last two decades cryopreservation methods have been developed for many species of temperate fruit and nut crops (Tables 6, 7 and 13).

Cryopreservation of Small Fruits

Actinidia: Jain and Sun (1989) studied cryopreservation of Chinese gooseberry (*Actinidia chinensis* Planch.) stem segments. Surface-sterilized winter shoots were cut into four segments and cryoprotected with combinations of cryoprotectants (10% DMSO + 0.5 M sorbitol or 5% glycerol + 5% sucrose). Shoot segments were cooled at 1.0 °C/min to –40°C and held at –40°C for 2 h before immersion in liquid nitrogen

Table 6. Small fruit cryopreservation research

Species or Cultivars	Explant Used	Freezing Method	Results	References
(1)	(2)	(3)	(4)	(5)
Actinidia chinensis Planch. cv. No. 36		1°C/min, PF: –40°C	More than 75% survival with callus regrowth	Jian and Sun, 1989
Fragaria x *ananassa* Duch. cv. Hokowase	Runner apex	PF: –20 to –30°C, cryoprotectant: 16% DMSO cooling rapidly in LN	60–80% survival	Sakai et al., 1978
Fragaria x *ananassa* cv. Redcoat	*In vitro*-grown meristems	0.5–1.0°C/min, 5% DMSO PF: –40°C	Maximum 95% survival	Kartha et al., 1980
Fragaria x *ananassa* cv. Indoka	*In vitro*-grown rosette buds	CH at 3–5°C, 7–10 days, 2°C/min to –20°C, 0.5°C/min to –43°C	14.3% survival	Shi and Cao, 1992
Fragaria x *ananassa* cvs. and *F.* species (56 genotypes)	*In vitro*-grown meristems	CH 1 week, cryoprotectant: PGD, 0.8°C/min, PF: –40°C	25 genotypes with greater than 50% survival	Reed and Hummer, 1995
Ribes hirtellum x *R. grossularia* cv. Oregon Champion, *R. rubrum* L. London Market	Dormant buds	5°C/day, PF: –40°C	Cuttings, 100% survival	Sakai and Nishiyama, 1978
R. aureum Pursh. cv. Bronze, *R. odoratum* H.L. Wendl., *R. aureum* Pursh., *R. diacantha* Pall., *R. quercetorium* Greene, *R. nigrum* L. cv. Ben Nevis, *R. sativum* Rchbch. cv. Cherry	*In vitro*-grown meristems	0.3 or 0.5°C/min, vitrification (PVS2) and encapsulation-dehydration	Varied with genotype and method	Reed, 1992b; Reed and Yu, 1995 Benson, et al., 1996
R. aureum Pursh *R. ciliatum* Homb + Bonpl	*in vitro*-grown meristems	Soakin bovinie serum albumen and vitrification in PVS2	68% regrowth	Luo and Reed, 1997

(Contd)

Table 6 (Contd.)

(1)	(2)	(3)	(4)	(5)
Rubus idaeus L. cv. Latham	Dormant buds	5°C/day, PF: –40°C	Cutting,100% survival	Sakai and Nishiyama, 1878
R. idaeus var. *strigosus*, *R. occidentalis* L., *R. neglectus* Peck, *R. allegheniensis* Porter.	Dormant buds	PF: –25°C	Survival to 60% if acclimated	Wu et al., 1981
R. ideaus cv. Heritage, *R. leucodermis* Torr. & Gray, *R. spectabilis* Pursh. *R.* spp. cv. Logan Thornless and Merton Thornless	*In vitro*-grown meristems	PGD, 0.8°C/min, PF: –40°C	Maximum 63% survival	Reed and Lagerstedt, 1987
R. idaeus cv. Heritage, *R.* spp. cv. Logan Thornless and Merton Thornless, *R. spectabilis*	CH 25°C day/–1°C night, meristems	1 week, 0.5°C/min, PF: –40°C	CH improved survival	Reed, 1988
R. fruticosus L.	Suspension cells	Preculture for 24 h with 1% BSA, cryoprotectant; 15% DMSO, PF: –40°C	60% survival at 8 days after thawing	Lett and Schmitt, 1992
R. cissoides Cunn., *R. parvifolius* L., *R. parviflorus* Nutt., *Rubus* hybrid cvs. Hillemeyer and Silvan	*In vitro*-grown meristems	CH and 5 µM ABA pretreatment, slow freezing	Best results with CH, ABA + CH good for one genotype	Reed, 1993b
Vaccinium corymbosum L., *V. ovatum* Pursh., *V. uliginosum* L.	*In vitro*-grown meristems	3–8 weeks CH, 0.1°C/min, PF: –40°C	About 50% shoot formation	Reed, 1989
Vitis vinifera L. cv. Chardonnay	*In vitro*-grown shoot tips	Encapsulation-dehydration slow thawing	24% shoot formation	Plessis et al., 1991
V. vinifera cv. Chardonnay	*In vitro*-grown shoot tips	Encapsulation-dehydration and 0.1°C/min, PF: –100°C	40–60% shoot survival	Plessis et al., 1993
V. vinifera cv. Chasselas x *V. belandiri* rootstock cv 41 B, *V. vinifera* cv. Chardonnay	Embryogenic suspension cultures	Preculture: 0.25 M maltose and 5% DMSO at 0°C for 1 h, 0.5°C/min to –40°C	60% survival with special recovery medium	Dussert et al., 1991, 1992

Table 7. Temperate fruit tree cryopreservation research

Species or Cultivars		Freezing Method	Results	References
(1)		(3)	(4)	(5)
Malus domestica Borkh. cvs. Red Gold, Jonathan, Starking Delicious, Golden Delicious, Fuji, Akane	Dormant buds	5°C/day, PF: −30°C Slow thawing	Buds and cortex survived; xylem killed	Sakai and Nishiyama, 1978
M. domestica cv. Fuji and Golden Delicious, *M. paradisiaca* Shneid. rootstock cvs. M.9 and M.26	Dormant buds	2.5°C/5min, PF: −10°C	Regrown *in vitro*	Katano et al., 1983
M. domestica	Callus	CH, PF: −30°C	16% survival	Kuroda and Nishiyama 1983
M. domestica cv. Jonathan	*In vitro* shoot tips	CH at 4°C for 6 weeks, cryoprotectant, SF, PF: −40°C	Callus formed, no shoot regrown	Kuo and Lineberger, 1985
M. domestica cultivars (16)	Dormant buds	Drying at −30°C or −40°C for 24 h to 20–30% water content	80–100% survival	Tyler and Stushnoff 1988b
M. domestica several cvs.	Dormant buds	Encapsulation and vitrification	All tissues remain uninjured	Seufferheld et al., 1991
M. domestica cvs. Golden Delicious and Fuji	*In vitro* shoot tips	CH at 2°C, 5 weeks, PF: −40°C, 0.1°C/min, rapid thawing	90% shoot formation	Chang et al., 1992; Chen et al., 1994
M. domestica cv. Fuji, Golden Delicious, hybrid 423-1, *M. paradisiaca, M. prunifolia* Borkh	*In vitro* shoot tips	CH at 5°C for 3 wks, vitrification (PVS2) or encapsulation-dehydration	80% shoot formation	Niino and Sakai, 1992 Niino et al., 1992c
Malus sp. and cvs. 500 accessions	Dormant buds	Drying to 30% moisture, cool 1°C/h to −30°C, hold 24 h, store in vapor phase	Successful recovery after 5 years in LN	Forsline et al., 1993

(Contd)

Table 7. *(Contd)*

(1)	(2)	(3)	(4)	(5)
M. domestica cv. Golden Delicious, Huifeng Orin, New Jonagold, Republican Fugi and Gala, *M. micromalus* Borkh.	*In vitro* shoot tips	Vitrification (PVS3), 80-min exposure	50–80% survival *in vitro*	Zhao et al., 1995
Morus bombycis Koidz. cv. Kenmochi	Dormant buds	10°C/day, PF: –20 or –30°C	About 60% shoot formation	Yakuwa and Oka, 1988
M. bombycis cv. Kenmochi	*In vitro* shoot tips	Preculture, encapsulation-dehydration	65% shoot formation	Niino and Sakai, 1992
M. bombycis cv. Kenmochi, Yukishinogi, and 6 other species	Dormant buds	5°C/day, PF: –20°C	80% shoot regeneration	Niino et al., 1992b
M. multicaulis Loud. cv. Lusang	Dormant buds	PF: –3°C for 10 days, –5°C for 3 days, –10°C for 1 day, –20°C for 1 day	55–90% survival *in vitro*	Wang et al., 1988
M. bombycis cvs. Kenmochi and Yukishinogi, *M. alba* L. *M. australis* Poir cvs. Shimaguwa M-28 and Sulawesi, *M. multicaulis* L., *M. nigra* L., *M. rotundiloba* Koidz cvs. Poo, Som and Dapang	*In vitro* shoot tips	CH at 5°C, 3 weeks, encapsulation-dehydration or vitrification (PVS2)	40–81.3% shoot formation	Niino et al., 1992a, b
Prunus persica L. cv. GF 305	*In vitro* shoot tips	Preculture: 5% DMSO + 5% proline, 1°C/min to –40°C	70–80% survival	Paulus et al., 1993
P. pennsylvanica L., *P. fruticosa* Pall. and 8 other species	*In vitro* shoot tips	Preculture: 14% sucrose at –4°C, PF: –30°C for 24 h	75% recovery	Stushnoff, 1985
P. interspecific cross rootstocks: Fereley-Jaspi and Ferlenain-Plumina	*In vitro* shoot tips	Preculture: 5% DMSO + 2% proline, slow freezing	69% and 74% survival	Brison et al., 1995
Pyrus communis L. cvs. Flemish Beauty, Bartlett, *P. serotina* Rehd. cvs. Mishirazu and Hokusei	Dormant buds	5°C/day, PF: –40 or –50°C	78–100% regrowth for most genotypes	Sakai and Nishiyama, 1978

(Contd)

Table 7. *(Contd)*

(1)		(3)	(4)	(5)
P. serotina cv. Kosui	Dormant buds	PF: –40°C to –70°C no cryoprotectant	More than 80% survival	Moriguchi et al., 1985
P. serotina cv. Senryo	Dormant shoot tips	5°C/day, PF: –40°C	Maximum 67% survival 8% shoot formation	Oka et al., 1991
P. communis cv. Beurre Hardy, *P. cossonii* Coss. and Dur., *P. koehnei* Schneider, *P. dimorphophylla* Makino x *P. fauriei* Schneider hybrid	*In vitro* shoot tips	CH one week, preculture 5% DMSO 2 days cryoprotectant: PGD, 0.1°C/min, PF: –40°C	55–95% shoot formation	Reed, 1990
P. communis L. cv. Beurre Hardy	*In vitro* shoot tips	CH at 0°C, 3–8 weeks, preculture; 0.75M sucrose and 15% DMSO, 0.5°C/min PF: –30 or–40°C	60% shoot formation	Dereuddre et al., 1990a
P. communis cv. Beurre Hardy	*In vitro* shoot tips	CH two months, encapsulation-dehydration	80% survival 40% shoot formation	Dereuddre et al., 1990b
P. pyrifolia Nakai. cvs. Hokkaiwase, Yoshino and Senryo *P. communis* 5 cvs.	*In vitro* shoot tips	CH, preculture: 0.7 M sucrose at 5°C, 1 day, vitrification (PVS2)	40–72.5% shoot formation	Niino et al., 1992c
P. communis cvs. Beurre d'Amanlis and Winter Nelis, *P. pyrifolia* cv. Senryo	*In vitro* shoot tips	CH, preculture with high sucrose concentration, encapsulation-dehydration	67.5–70% shoot formation	Niino and Sakai, 1992
P. communis L. cv. Beurre Hardy	*In vitro* shoot tips	Encapsulation-dehydration, CH at 0°C, 8–12 wks, preculture: beads with 0.75 M sucrose, dehydration (20% residual water)	80% shoot formation	Scottez et al., 1992
P. pyrifolia Nakai. cv. Choujuurau	*In vitro* shoot tips	CH 35 days; Cryoprotectant; 0.5°C/min to –40°C	Maximum 100% survival and 25% shoot formation	Mi and Sanada, 1994

(LN). Following fast thawing, 75% of segments produced callus and numerous adventitious shoots were produced from the callus.

Fragaria: Sakai et al., (1978) first reported that apices of field-grown strawberry runners frozen to –196°C developed normal shoots *in vitro* after thawing. Survival ranged from 60% to 80% for apices prefrozen to –20 to –30°C in the presence of 12 to 16% DMSO and cooled rapidly. *In vitro*-grown meristems precultured on medium supplemented with 5% DMSO and cooled at 0.84°C/min to –40°C before LN, had 95% survival (Kartha et al., 1980). Fifty-six *Fragaria* accessions were tested for possible cryopreservation at NCGR-Corvallis (Reed and Hummer, 1995). *In vitro* plantlets were cold hardened for one week, 0.8 mm meristems were cut and pretreated on 5% DMSO for two days then frozen at 0.8°C/min to –40°C in the cryoprotectant PGD (a combination of 10% polyethylene glycol, 10% glucose, and 10% DMSO) and plunged in LN. Survival was dependent upon genotype; 25 genotypes had greater than 50% regrowth. A slower cooling rate (0.3°C/min) partially improved survival. No off-types were found during field evaluations of 15 cultivars regrown from cryopreserved meristems.

Ribes: Sakai and Nishiyama (1978) demonstrated that winter-dormant *Ribes* buds could be frozen at 5°C/day to –40°C, plunged in LN, and slowly thawed with 100% survival. Modifications to slow freezing, vitrification and encapsulation-dehydration techniques were successful for cryopreserving apical meristems from *in vitro*-grown *Ribes* plants (Reed and Yu, 1995). *R. diacantha* Pall. meristems had moderate to high regrowth with each of the seven modifications tested and most methods resulted in regrowth for some *R. aureum* Pursh meristems. *R. rubrum* L. cv. Cherry meristems had moderate (60%) regrowth with encapsultion-dehydration (3 h dehydration) and very poor results with the other techniques. PVS2 vitrification and encapsulation-dehydration techniques were effective for *Ribes nigrum* cvs. Ben Tron and Ben More (Benson et al., 1996). A 2 h pretreatment in bovine sorum albumen (BSA) significantly improved survival of *R. aureum* and *R. ciliatum* following vitrification in PVS2 solutions (Luo and Reed 1997).

Rubus: Sakai and Nishiyama (1978) demonstrated that winter dormant buds of raspberry could be frozen at 5°C/day to –40°C, immersed in LN, and slowly thawed with 100% survival; some injury to the cortex and xylem was reported. *In vitro*-grown *Rubus* meristems were successfully cryopreserved using slow-freezing techniques (Reed, 1988, 1993b; Reed and Lagerstedt, 1987). PGD (Finkle and Ulrich, 1979) produced good results as a cryoprotectant with *in vitro*-grown *Rubus* meristems (Reed and Lagerstedt, 1987). Cold acclimation (22°C

day/–1°C night) improved survival of cryopreserved *Rubus* meristems. Nonacclimated meristems had regrowth rates between 18% and 41% while cold-acclimated plants had 51% to 67% regrowth following slow freezing (Reed, 1988). Cold acclimation was effective for most genotypes tested but the combination of ABA and cold acclimation improved survival for one *Rubus* genotype (Reed, 1993b). Lett and Schmitt (1992) reported that a 1% bovine serum albumin (BSA) pretreatment for 24 h in Heller's medium was effective for suspension-cultured *Rubus* cells when used with 15% DMSO as a cryoprotectant.

Vaccinium: Reed (1989) cryopreserved *in vitro*-grown *Vaccinium* meristems by slow freezing with the cryoprotectant PGD. *V. corymbosum* L. survival increased from 6% to 58% following three or more weeks of cold hardening (CH) (22°C day/–1°C night). Cooling meristems at 0.1°C/min produced the highest survival. Cold acclimation requirements and optimum cooling rates varied among species.

Vitis: Plessis et al. (1991, 1993) successfully cryopreserved *in vitro*-grown *Vitis* shoot tips. Axillary shoot tips encapsulated in alginate beads, precultured in sucrose-enriched medium, dehydrated at room temperature, and slowly frozen from 20°C to –80°C, then immersed in LN, had 20% survival. Higher survival rates, between 40% and 60% with 30% shoot recovery, were noted using lower prefreezing temperatures (–100°C) and slower cooling rates (0.1°C/min). Dussert et al. (1991) obtained 60% survival of embryogenic grape cell suspensions with 30% packed cell volume after 1 h pretreatment at 0°C with 0.25 M maltose and 5% DMSO, freezing at 0.5°C/min to –40°C, and immersion in LN. Six weeks after thawing the growth rate of cryopreserved cells was equivalent to that of the unfrozen control. The best recovery was obtained on a medium with 1 mgl^{-1} napthoxyacetic acid. A minimum retention of six days on a semisolid medium was necessary to obtain regrowth from cryopreserved cells before placing them in a liquid medium (Dussert et al., 1992).

Cryopreservation of Temperate Fruit Trees

Many reports on the cryopreservation of temperate fruit trees have been written in the last two decades (Table 7). Improved cryopreservation techniques have been successfully applied to apple, pear, peach, plum, and cherry; and now some entirely new techniques are available.

Malus: Sakai and Nishiyama (1978) first demonstrated that apple and pear winter buds retained their viability after immersion in liquid

nitrogen if provided with an initial exposure to subzero temperatures at slow freezing rates. Apple buds taken from the shoots stored in LN grew after grafting onto rootstocks in the greenhouse (77% regrowth). Eighty to 100% viability was obtained from cryopreserved dormant vegetative buds from *Malus* using newly developed techniques (Stushnoff, 1987; Tyler and Stushnoff, 1988a, b; Tyler et al., 1988). The moisture content of the winter-dormant buds ranged from 48% to 60% and desiccation to 20% to 30% was required for survival; a few very tolerant species could be desiccated below 10%. Buds at maximum hardiness were most tolerant of desiccation (Stushnoff, 1987, 1991; Tyler and Stushnoff, 1988a, b). Drying of dormant buds before freezing is applicable to genotypes which naturally tolerate desiccation and freezing to –30°C or colder at maximum hardiness. Cryopreserved dormant buds are thawed either slowly (room temperature air) or rapidly (40°C water) (Sakai and Nishiyama, 1978; Tyler and Stushnoff, 1988a; Tyler et al., 1988).

Seufferheld et al. (1991) demonstrated that pretreating dormant apple buds with sugars and other cryoprotectants enhanced survival of taxa that are less cold hardy or do not sufficiently acclimate. The buds could be grafted directly onto rootstocks in the greenhouse after rehydration in moist peat moss, or the shoot tips excised for tissue culture. Shoot tips excised from winter buds of apple survived *in vitro* after slow freezing below –10°C and immersion in LN without a cryoprotectant (Katano et al., 1983).

Dormant buds of 500 apple genotypes were stored in the vapor phase of LN at the National Seed Storage Laboratory (NSSL) in Ft. Collins, Colorado (Forsline et al., 1993). Single-bud sections were prepared from cold-hardened, dormant apple shoots, dried to 30% moisture, cooled at 1°C/h to –30°C, held for 24 h, and then stored in the vapor phase of LN.

In vitro-grown shoot tips are considered ideal materials for germplasm storage and international exchange. Kuo and Lineberger (1985) first tested cryopreservation on *in vitro*-grown shoot tips but recovered only callus. Niino and Sakai (1992) cryopreserved *in vitro*-grown apple shoot tips with the encapsulation-dehydration method. Shoot tips cold hardened at 5°C for three weeks were progressively precultured on MS media with increasing sucrose, then encapsulated in alginate beads. The beads were dried to about 33% water content before exposure to LN. Shoot regrowth after thawing was about 80%. Eighty percent survival was obtained when shoot tips of *in vitro*-grown apples were precultured with 0.7 M sucrose at 5°C for one day and then cryoprotected with plant vitrification solution number two (PVS2) for 80 min before LN exposure (Niino et al., 1992c).

Malus cryopreservation studies at Changli Institute of Pomology—Several aspects of *in vitro* apple shoot-tip cryopreservation studies at Changli Institute of Pomology, Hebei Academy of Agricultural and Forestry Sciences are described here (Chang et al., 1992). *Malus domestica* Borkh. cvs. Golden Delicious, Fuji, Shengli and Jincui plants were cultured on MS medium containing 1.0 mgl^{-1} BA, 4% sucrose, 200 mgl^{-1} casein enzymatic hydrolysate, and 0.5% agar at pH 5.8. Plantlets were grown on 25 ml medium in 100 ml glass flasks with cotton plugs under white fluorescent light (16-h photoperiod) at 25°C. Following cold hardening (CH) at 2 ± 1°C in darkness, 1.5 to 2 mm shoot tips were tested using slow freezing, vitrification, and encapsulation-dehydration.

Plants cultured for 70 days without transfer before CH had more shoot formation following cryopreservation than those cultured for 35 days (Chang et al., 1992) (Table 8). Plantlets cultured without transfer for 35 days were faster growing and had higher meristem moisture contents than those cultured for 70 days. Seventy-day plantlets had almost stopped growing and their water content was reduced because of gradual drying of the medium. After 70 days, more than 70% of the original moisture in the medium was lost and the osmotic potential doubled. As the shoot tips acclimated to the medium's increasing osmotic potential, shoot tip tolerance to dehydration improved. These results are similar to those of Caswell et al. (1986) who demonstrated that a high sucrose concentration in the medium improved the hardiness of apple shoots. During a long culture period, the medium concentration gradually increases and plantlets acclimate better than those placed directly onto a highly osmotic medium.

Table 8. Effects of culture duration on shoot formation in four *Malus domestica* cultivar following cryopreservation

Days in Cultur	Shoot Formation (%			
	Fuji	Golden Delicious	Shengli	Jincui
35	45.6	43.1	27.1	46.
70	68.5	86.9	75.0	89.

[a]14 days CH at 2 ± 1°C in addition to days in culture. 5% DMSO + 5% glycerol use as cryoprotectant. Slow freezing (0.2°C/min to −40°C). n = 60 (Chang et al., 1992). (n refers to number of replicates in a sample).

In vitro-grown apple shoot tips require CH for cryopreservation bu the response is genotype dependent. Following one week of CH a 2 ± 1°C, "Golden Delicious" that had grown 70 days without transfe reached 80% shoot formation while 35-day-old "Fuji" required nearl

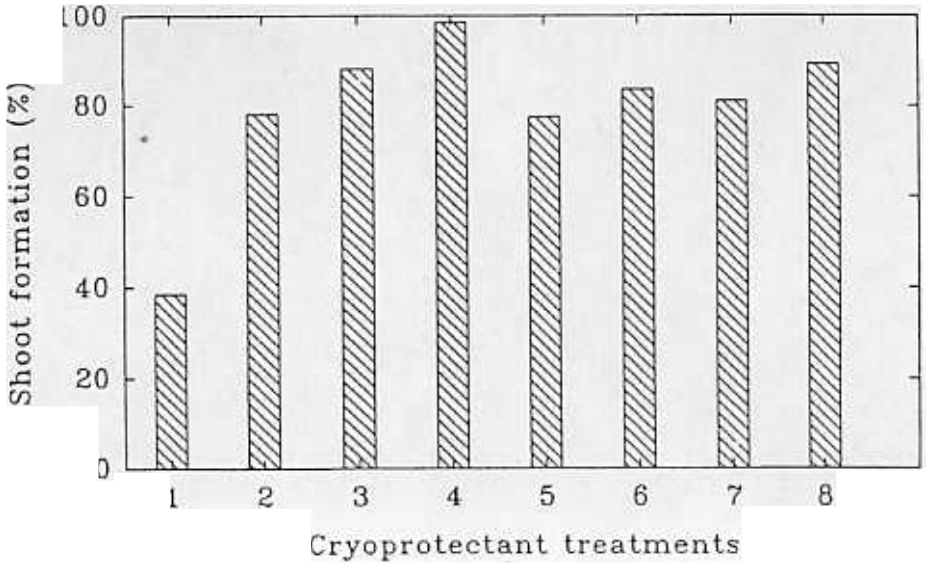

Fig. 2. Effect of cryoprotectants on shoot formation following cryopreservation of *in vitro*-grown shoot tips of *Malus domestica* cv. Golden Delicious. 70-day-old shoots at 40 days cold hardening; slow freezing (0.2°C/min to –40°C) (Chang et al., 1992). 1. MS liquid medium. 2. 5% DMSO. 3. 10% DMSO. 4. 5% DMSO + 5% glycerol. 5. 5% glycerol. 6. 10% glycerol. 7. 5% DMSO + 5% PEG + 5% glycerol. 8. 10% PEG.

Table 9. Effects of cold hardening on cryopreservation of *in vitro*-grown apple shoots[a]

Cultivars	Days of Cold Hardening					
	0	7	14	21	38	50
	Shoot Formation (%)					
Golden Delicious	38.9	80.0	86.9	90.7	94.5	**98.2**
Fuji	15.0	29.1	45.6	69.2	81.2	–

[a]Golden Delicious cultured 70 days; Fuji cultured 35 days before CH at 2 ± 1°C (dark). Cryoprotectant: 5% DMSO + 5% glycerol; cooling rate: 0.2°C/min to –40°C rapid thawing; n = 80 (Chang et al., 1992).

6 weeks of CH to produce similar recovery rates (Chang et al., 1992) (Table 9).

The cryoprotectant used altered the rate of shoot formation for 70-day-cultured "Golden Delicious" shoot tips cryopreserved by slow freezing (Chang et al., 1992) (Fig. 2). Seven combinations of cryoprotectants were tested; all provided sufficient protection for high levels of shoot formation following cryopreservation but 5% DMSO + 5% glycerol was the best. The highly desiccated nature of the plants was demonstrated

by shoot production in 38.5% of control meristems frozen in liquid MS medium without cryoprotectants.

Cooling rate determines the amount and rate of dehydration and is a key factor for success using the slow-freezing method. *In vitro*-grown apple shoot tips have a high water content and require slow cooling to complete dehydration before being exposed to LN. Chang et al. (1992) found 0.1–0.2°C/min suitable for *in vitro*-grown apple shoot tips (Table 10). This is similar to the 0.1°C/min rate required for pear meristems (Reed, 1990).

Table 10. Effect of cooling rate on shoot formation of cryopreserved apple shoot tips[a]

Cooling Rate (°C/min)	Golden Delicious		Fuji	
	Number Tested	% Shoot Formation	Number Tested	% Shoot Formation
0.8	40	30.0	40	0
0.5	44	54.5	42	33.3
0.2	200	94.0	200	46.0
0.1	44	95.4	40	50.0

[a]'Golden Delicious' cultured 70 days; Fuji cultured 35 days and CH at 2 ± 1°C for 20 days; rapid thawing (Chang et al., 1992).

Vitrification with highly concentrated cryoprotectant solution allows cells to dehydrate quickly and liquids to form glasses at low temperatures. Sakai (1993) developed several vitrification solutions for plants. The vitrification solution and the duration of exposure are applied according to the size of plant material, the cryoprotectant toxicity, and the temperature. Niino et al. (1992c) dehydrated with PVS2 (30% glycerol, 15% ethylene glycol and 15% DMSO in MS medium containing 0.4 M sucrose) at 25°C for 80 min and obtained 80% shoot formation. Zhao et al. (1995) tested plant vitrification solutions PVS1 through PVS2 on three apple cultivars with several exposure lengths before vitrification. The best results were obtained using PVS3 (50% sucrose, 50% glycerol) for 80 min (Table 11).

Chang (unpublished) compared the three main cryopreservation methods, slow freezing, vitrification, and encapsulation-dehydration, on *in vitro*-grown apple shoot tips of "Gloden Delicious" and "Republican Fuji" (Table 12). Results were similar with each of the three methods if the materials came from plants cultured for 70 days and cold hardened for three weeks. Plants cultured for only 20 days did not survive with any of the methods tested, demonstrating the importance of a plant's physiological status on survival following cryopreservation. Shoot tips

Table 11. Effect of PVS3 exposure time on shoot formation of vitrified *in vitro* apple shoot tips[a]

Cultivars	Shoot Formation (%)			
	20 min	60 min	80 min	100 min
Golden Delicious	17	45	75	47
Huifeng Orin	8	50	70	55
New Jonagold	10	55	65	50

[a]CH 3 weeks at 5°C (8-h photoperiod); PVS3: 50% sucrose + 50% glycerol; rapid thawing n = 60 (Zhao et al., 1995).

Table 12. Shoot formation of *in vitro* shoot tips of two apple cultivars cryopreserved using three different techniques[a]

Cultivars	Shoot Formation (%)		
	Slow Freezing	Vitrification	Encapsulation-dehydration
Golden Delicious	85	77	80
Republican Fuji	83	75	85

[a]70-day cultured plants with 3 weeks CH; slow freezing at 0.2°C/min to –40°C; vitrification with a 0.7 M sucrose pretreatment for 2 days and PVS3 for 80 min. Encapsulation-dehydration with a sucrose series from 0.3–1.2 M for 3 days, then 3-h dehydration in airflow and rapid thawing; n = 40 (Chang et al., unpublished.)

which successfully recovered from cryopreservation resumed growth immediately after reculture; all parts of the shoot tip remained green in color and continued growing. The growth of frozen and control shoot tips was similar. The leaf primordia of some frozen shoot tips turned brown after reculture but new shoot tips emerged after one week. The thawed shoot tips grew rapidly and reached 1 cm in length by one month (Chang et al., 1992). More than 70 cultivars have been cryopreserved at Changli Institute of Pomology and stored for over three years. Samples were removed from LN after one month, one, two, and three years and recultured with no change in regrowth.

Morus: Yokoyama and Oka (1983) reported that the shoot tips of prefrozen winter buds of *Morus bombycis* Koidz. cv. Kenmochi survived immersion in liquid nitrogen but the same did not occur with grafts and cuttings. Wang et al. (1988) modified this method and succeeded in regenerating plants of *M. multicaulis* Loud. cv. Lusang through shoot-tip culture from frozen winter buds. Shoot segments were prefrozen at –3°C for 10 days, –5°C for three days, –10°C for one day, and –20°C

for one day before being immersed in LN. After slow thawing in air at 0 to 2°C, the buds were cultured on MS medium with a survival rate of 55% to 90%. Niino et al. (1992b) demonstrated that excised shoot tips from winter buds of *M. bombycis* cv. Kenmochi prefrozen to –20°C at 5°C/day produced more shoots than buds prefrozen at 10°C/day. Parital dehydration to about 38.5% moisture content at 25°C prior to prefreezing to –20°C also improved the recovery rates. The survival rate of the winter buds stored in LN from one month to 3.5 years did not change (Niino, 1995). Excised shoot tips (2 mm long) from winter buds could be directly dehydrated with silica gel at 25°C before LN exposure. Shoot formation increased with decreasing water content to a maximum of 80% survival at about 19% water content. Alginate-coated winter-hardened shoot tips of several *Morus* species had maximum shoot formation (81%) with 22 to 25% water content (Niino et al., 1992b). Thirteen cultivars of mulberry were tested for cryopreservation as *in vitro*-grown shoot tips (Niino, 1995). Slow freezing (0.5°C/min to –42°C), vitrification (PVS2, 90 min), and air-drying (24% water content) or encapsulation-dehydration (33% water content) were tested with survival ranging from 40 to 81.3%.

Prunus: Seventy to 80% of axillary apices excised from *Prunus persica* cv. GF 305 *in vitro*-cultured plants survived following vitrification when the preculture medium included 5% DMSO and 5% proline (Paulus et al., 1993). The same preculture medium (5% DMSO and 2% proline) was effective for two *Prunus* rootstocks with average shoot formation rates of 69% and 74% (Brison et al., 1995). Dormant buds of ten species of *Prunus* were successfully cryopreserved and high recovery (up to 100%) rates obtained by grafting onto rootstocks after thawing (Stushnoff, 1985). *In vitro*-hardened shoots could survive cryopreservation (75% regrowth) if held at –30°C for 24 h before transfer to LN. Culturing plants on a medium containing 14% sucrose and chilling them at –4°C enhanced low-temperature tolerance.

Pyrus: 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 (78–100%), although the xylem was severely injured. Cryopreservation of meristems excised from dormant buds has been studied by several laboratories (Mi and Sanada, 1992; Moriguchi et al., 1985; Oka et al., 1991). Moriguchi et al. (1985) reported that prefreezing to –40 to –70°C was required for successful cryopreservation of shoot tips from dormant buds of Japanese pear. Cryoprotectants were unnecessary and about 80% of shoot apices survived regardless of the rewarming procedure

applied. Oka et al. (1991) recovered whole plants from cryopreserved buds.

The first *in vitro* pear meristem cryopreservation was developed by Reed (1990). Cold hardening and slow cooling resulted in a high rate of regrowth (55% to 95%) of cryopreserved shoot tips of four *Pyrus* species, including 75% regrowth for *P. koehnei*, a subtropical species. Pear plantlets were CH for one week (22°C, 8 h light; –1°C, 16 h dark), the apical meristems cooled in PGD cryoprotectant at 0.1°C/min to –40°C before LN exposure, then thawed for 1 min in a 40°C water bath.

Dereuddre et al. (1990a, b) developed the encapsulation-dehydration technique with pear axillary-shoot tips. Shoot tips from plantlets, cold hardened for two months, were encased in alginate-gel beads, precultured in a liquid medium with 0.75 M sucrose for 18 h, and dehydrated in an airflow cabinet for two to six h. The beads were cooled rapidly by direct immersion in LN and rewarmed slowly in air at room temperature. High survival (80%) was obtained after dehydration for three h and 40% of the surviving shoot tips produced new plantlets. The best results (80% shoot recovery) were obtained with a 0.75 M sucrose preculture and four-h dehydration (20% residual water) (Scottez et al., 1992.) Niino and Sakai (1992) obtained about 70% shoot formation for three pear cultivars with a modified encapsulation-dehydration method. They also successfully applied the vitrification method to pears with 40 to 72.5% shoot formation rates (Niino et al., 1992c).

Many *Pyrus* genotypes at NCGR-Corvallis have been screened by slow-freezing (Reed, 1990) and vitrification techniques (Yamada et al., 1991). A comparison of 28 *Pyrus* genotypes found that 61% had good survival (> 50% regrowth) following slow freezing (0.1°C/min) while only 43% of the genotypes responded well to the vitrification technique (Luo et al., 1995). Genotypes with greater than 50% shoot formation were cryopreserved and shipped to the National Seed Storage Laboratory, Ft. Collins, CO for long-term storage. Each genotype shipped required 150 meristems (25 meristems per vial); 25 meristems thawed before shipping in Corvallis, 25 thawed at NSSL after storage, and the remaining 100 meristems were stored in LN.

Cryopreservation of Temperate Nut Trees

Most temperate nut seeds are sensitive to dehydration or cold and may not survive storage for more than one year. Excised embryonic axes are the preferred plant material for cryopreservation of nuts (Table 13). Pence (1990) tested embryonic axes of *Carya*, *Castanea*, *Corylus*, and *Juglans* for survival following cryopreservation. Each genus tested

had some axis tissue survive LN treatment: *Juglans* and most *Carya* germinated, or showed partial germination, with callus production; *Corylus* had mostly callus production; and *Castanea* produced only callus (Pence, 1990). Desiccation of axes to 20% to 30% improved survival of chestnut (*Castanea*) axes following cryopreservation, and some shoot formation was obtained (Pence, 1992).

Corylus: Gonzalez-Benito and Perez (1994) reported *Corylus avellana* L. "Morell" produced maximum recovery (85%) of newly harvested hazelnut embryonic axes when frozen at 12% moisture content; "Butler" required 11% moisture to obtain 50% recovery. Whole *C. avellana* "Barcelona" hazelnut seeds did not survive LN exposure despite desiccation pretreatment, but embryonic axes could be excised from the thawed seed and grown in tissue culture (Normah et al., 1994). Stratifying (moist storage at near-freezing temperatures) stored "Barcelona" hazelnut seed improved shoot production from both control and cryopreserved isolated embryonic axes. Shoot growth of axes from seed stored for four months increased from 33% to 94% when seeds were stratified for two weeks before removing the axes. Axes from stored stratified seed were dried to 8% moisture under laminar flow and cryopreserved with resultant 85% viability and 70% shoot growth rates, while only 30% of the unstratified axes produced shoots (Reed et al., 1994). In 1995, 300 embryonic axes from seeds of both *Corylus colurna* L. and *C. americana* Marsh. were stored in LN using this technique at NCGR-Corvallis (Reed, unpublished). Frozen axis regrowth of controls was 80% for both species.

Juglans: De Boucaud et al. (1991) obtained 75% to 91% survival and regrowth of *Juglans* embryonic axes with a cryoprotectant composed of 5 M 1,2-propanediol and 20% sucrose. Cryoprotectants increased the dehydration of axes without significantly influencing survival or regrowth of control axes. *In vitro*-grown shoot tips of walnut were successfully cryopreserved by slow freezing (de Boucaud and Brison, 1995). Plantlets were CH at least eight days (23°C day/4°C night) and shoot tips were precultured on medium with 5% DMSO and 0.5% proline for 24 h. Slow freezing (0.5°C/min) of shoot tips in modified PVS2 cryoprotectant produced 34% survival (Brison et al., 1991). Isolated walnut somatic embryos, 1 to 2 mm in length, were successfully cryopreserved with both encapsulation-dehydration and slow-freezing methods. Thermal analysis performed on naked and encapsulated somatic embryos showed the necessary dehydration time (1.5 h or 3 h respectively) to obtain vitrification upon cooling and rewarming (de Boucaud et al., 1994).

Table 13. Temperate nut tree cryopreservation research

Species or Cultivars	Explant Used	Freezing Method	Results	References
Carya cordiformis (Wang.) K. Koch, *C. illinoensis* (Wang.) K. Koch, *C. laciniosa* (Michx.) Loud., *C. tomentosa* Nutt.		Rapid drying, rapid cooling	Germination or partial germination with callus	Pence, 1990
Castanea mollissima Bl.	Embryonic axes	Rapid drying, rapid cooling	Callus	Pence, 1990
C. sativa Mill.	Embryonic axes	Rapid drying, rapid cooliing	Callus, roots and some shoots	Pence, 1990, 1992
Corylus avellana L.	Embryonic axes	Rapid drying, rapid cooling	Callus and shoots	Pence, 1990
C. avellana cvs. Morell & Butler	Embryonic axes (fresh seed)	Rapid drying, to 11% or 12% moisture content, rapid cooling	Morell: 85% recovery Butler: 50% recovery	Gonzalez-Benito and Perez, 1994
C. avellana cv. Barcelona	Embryonic axes (stored seed)	Stratification 2 weeks before excision, drying to 8–10% moisture, rapid cooling	85% viability 70% shoot growth	Normah et al., 1994; Reed et al., 1994
Juglans regia L., *J. nigra* L., *J. cinera* L.	Embryonic axes (mature seeds)	Rapid cooling	Germination or partial germination with callus	Pence, 1990
J. regia cv. Franquette	Embryonic axes	Rapid cooling	75–91% regrowth rates	de Boucaud et al., 1991
J. regia	*In vitro* shoot tips	CH, preculture: 5% DMSO and 0.5% proline, cryoprotectant (PVS2), 0.5 or 1.0°C/min, prefreezing temperature (PF): –40°C	10% shoot tips regrown	Brison et al., 1991
J. regia x *J. nigra* hybrid	Somatic embryos from cotyledons	Preculture: 5% DMSO and 0.5% proline, encapsulation, plasmolysis in sucrose up to 1 M, dehydration in airflow, then 1°C/min, PF: –40°C	Regrowth and multiplication	de Boucaud et al., 1994

DISCUSSION OF CRYOPRESERVATION OF TEMPERATE FRUITS AND NUTS

Many cryopreservation techniques have been developed for cell suspensions, callus, shoot tips, somatic embryos, and embryonic axes; some of these techniques are now used for long-term storage of germplasm. Several major areas need to be addressed for future improvements in cryopreservation techniques. The choice of materials is an important consideration for any cryopreservation technique. Genotypes vary widely in their response to cryopreservation, as well as to cryopreservation techniques (Niino, 1995; Reed and Yu, 1995). Changes in the physiological status of plants of a single genotype produce different survival rates following cryopreservation (Chang et al., 1992; Reed, 1988). More emphasis needs to be placed on the plant's physiological condition prior to cryopreservation.

Cold-hardening pretreatment is necessary for most cryopreservation techniques and is now included in most procedures. Standardized cold-hardening conditions are needed before comparisons can be made on the efficacy of cryopreservation techniques. The wide variation in temperature, light conditions, and duration of CH make comparisons of survival difficult.

Study of freezing rates and cryoprotectants is still needed. Slow freezing was the first technique developed and is still an important protocol with myriad modifications. For *in vitro*-grown shoot tips, a very slow freezing rate (0.1°C/min to –40°C) produces the best survival for many species, including *Vaccinium, Pyrus, Malus, Fragaria,* and *Vitis* (Chang et al., 1992; Reed, 1989, 1990; Reed and Hummer, 1995; Plessis et al., 1993). Cryoprotectants are necessary for high survival with most *in vitro* systems and highly concentrated solutions (PVS2) are now used for some slow-freezing methods (Brison et al., 1995).

Difficult genotypes may be successfully cryopreserved by combining methods. Encapsulated grape axillary-shoot tips were cooled at 0.1°C/min to –100°C before LN exposure; survival and shoot formation increased significantly over encapsulation alone (Plessis et al., 1993). Encapsulated apple dormant buds, dehydrated with a vitrification solution, were uninjured following LN exposure (Seufferheld et al., 1991). Further exploration of combined techniques may bring great advances in cryopreservation of difficult genotypes.

Several studies on genetic variation in cryopreserved plant material have been conducted. No observable changes from the original genotypes occurred due to cryopreservation (Chang and Reed, 1995; Harding and Benson, 1994; Reed and Hummer, 1995). More studies

are needed to evaluate the stability of plants held in LN but genetic abnormalities due to cryopreservation will likely be rare.

CONCLUSIONS

Research in slow-growth and cryopreservation techniques has greatly advanced in the last decade. These storage technologies are now available for many important temperate fruit and nut crops and are being developed for many more. *In vitro* and cryopreserved collections of important germplasm are now established in several countries and some genera are primarily held as *in vitro* plants. *In vitro* collections are increasingly used for virus elimination, storage, and distribution of clonal crops. Cryopreservation is now practical for base collections of clonal crops formerly preserved only as field collections. Future advances in cryopreservation will make the techniques applicable to more genera. Both cryopreservation and slow-growth storage can be implemented to provide added security in the preservation of important clonal genetic resources.

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