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Recovery and Longevity of Cryopreserved Dormant Apple Buds

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Abstract. **Clonally propagated crops, unlike seed-propagated crops, require intense and costly maintenance, generally in ex situ field gene banks. Consequently, large germplasm collections of tree species especially, are difficult to conserve in a well-replicated fashion and are vulnerable to damage from environmental stresses. Accordingly, long-term storage in liquid nitrogen presents a viable conservation alternative. To assess effectiveness of one approach to cryopreservation, dormant buds from 64 apple (*Malus ×domestica* Borkh. and other *Malus* spp.) accessions were collected and preserved in liquid nitrogen using a dormant-vegetative-bud method. Buds were retrieved from liquid nitrogen storage, rehydrated, and grafted onto rootstocks to determine survival. Mean recovery was 76% for 40 cold-hardy accessions, 66% for 20 moderately cold-hardy accessions, and 24% for four cold-tender accessions (range: 16% to 100%). Only four accessions had ≤25% recovery while 54 accessions had ≤50% recovery and 35 accessions had ≤75% recovery. No significant decline in recovery of these accessions by bud grafting occurred after 4 years of liquid nitrogen storage.**

To ensure security of plant germplasm held ex situ in genebanks, each accession in an active collection should be replicated by a duplicate sample in a base collection. While the active collection focuses on germplasm maintenance, characterization, documentation and distribution to the user community, the base collection provides long-term preservation and is distributed only when needed for repropagation at the active site. For seed-propagated crops in the USDA–ARS National Plant Germplasm System (NPGS), the base collection consists of seeds held at the National Seed Storage Laboratory (NSSL), Ft. Collins, Colo. For most clonally propagated crops, however, base collections either do not exist or consist of duplicate clones maintained in the field or greenhouse, often in close proximity to each other.

The national collection of apple (*Malus* spp.), managed by the Plant Genetic Resources Unit (PGRU), Geneva, N.Y., includes 2300 clonally propagated accessions representing cultivars, landraces, and breeding lines currently maintained as two or three plants in the field. Other accessions include wild species acquired and maintained as seed or grown as seedling trees for evaluation.

Field maintenance has been the only technique available to preserve germplasm and provide propagules for distribution (Stushnoff, 1991). Field maintenance of multiple plants per accession provides some security, though it exposes the often unadapted germplasm to damage from weather, pests, diseases, and other stresses. Furthermore, it is expensive, with costs approaching $75/accession/year, and, because all plants are on the same site, they are equally vulnerable. For example, despite considerable, regular protective measures, PGRU apple orchards are frequently heavily damaged by epidemics of *Erwinia amylovora* (fire blight), and susceptible genotypes can be killed. A particularly severe fire blight epidemic killed 450 trees in 1996, but duplicate plantings prevented loss of accessions.

Alternatives to current technologies that increase security and significantly decrease costs for maintenance of both active and base collections for clonally propagated crops are needed (Roos et al. 1996; Towill and Roos, 1989). The two general approaches to alternative preservation technologies are either short- or long-term storage. Short-term storage of in vitro plant tissues uses slow growth induced by low light and cool temperatures and has been successfully applied to preserve germplasm for active and base collections in sweet potato (*Ipomoea* spp.) (Jarret and Gawel, 1991), strawberry (*Fragaria* spp.) (Reed, 1992), and potato (*Solanum* spp., Mix 1982).

Programs for long-term preservation of plant tissues in liquid nitrogen (LN) are based on use of exogenously applied cryoprotectants for cold-tender plant tissues (Towill, 1995) or production of endogenous cryoprotectants in specific cold-acclimated tissues of cold-hardy species. Cryopreservation using exogenously applied cryoprotectants such as glycerol, ethylene glycol, or dimethylsulfoxide has been investigated in a range of species and tissues, including: protoplasts (Langis and Steponkus, 1990), cells (Sakai et al., 1990), somatic embryos (Mycock et al., 1995), zygotic embryos and whole seeds (Marin et al., 1990), and shoot tips (Niino et al., 1992; Plessis et al., 1991; Reed, 1995; Towill,

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1990; Towill and Jarret, 1992). Despite significant success in laboratory settings, application to a genebank setting must address obstacles such as variable responses to growth media, large investment of resources and time, juvenility in stored tissues, genotype-specific reactions to freezing and thawing protocol steps, removal of toxic cryoprotectants before regeneration, and potential genetic instability.

Cryopreservation of dormant vegetative buds for woody, cold-hardy plants was first described by Sakai (1960) and for apple by Sakai and Nishiyama (1978). Protocols based on endogenous production of cryoprotectants in dormant apple buds were refined by Stushnoff (1987), Tyler and Stushnoff (1988a, 1988b) and Tyler et al. (1988) with recovery by grafting. Dormant pear (*Pyrus communis* L.) buds have been successfully cryopreserved followed by recovery of meristems in vitro (Oka et al., 1991). Cryopreservation of dormant vegetative buds offers distinct advantages over other alternatives: a) clonal integrity is maintained; b) it is simple and space efficient; c) it has low maintenance requirements with LN as a coolant; d) long-term storage is theoretically possible; e) procedures can be optimized by varying desiccation, freezing and thawing rates; f) desiccated dormant buds can be shipped easily to other laboratories for recovery by grafting (Forsline et al., 1996b, Stushnoff, 1991); and g) grafted trees can be forced into early flowering.

We investigated whether dormant apple buds representing an array of genotypes from the USDA–ARS *Malus* germplasm collection could be preserved and recovered reliably and efficiently from LN storage (cryopreservability) and, if so, whether length of time in LN affected recovery success (longevity). The only other report of longevity of cryopreserved dormant buds has been in mulberry (*Morus bombycis* Koids.), which has been successfully stored for up to 5 years (Niino et al., 1993, 1994). We have designed this experiment to run over the next 50 years. Herein, we report on the cryopreservability and longevity of apple buds stored up to 4 years.

Materials and Methods

Test population. An array of 64 *Malus* accessions from the USDA–ARS collection was chosen to represent a broad range of variability for important traits including geographic origin or region of development, worldwide commercial importance, tree architecture, use as rootstock or scion, and chilling requirement. This array, our first studied, was selected to contain mostly cold-hardy material, since this trait has been associated with ease of cryopreservability (Tyler and Stushnoff, 1988a). Selection of cold-hardy genotypes was based on published records (Strang and Stushnoff, 1975; Forsline, 1983) as well as geographic origin, passport records, characterization data, and curator's knowledge of the accession.

Scion collection. We collected dormant, 1-year-old scions with ≈15 buds per scion section on eight dates over four consecutive winters (two collections per year, one in December and one in either January or February) at the PGRU orchard. From 100 to 150 scion sections were collected from vigorous 3- to 6-year-old trees of each accession at the end of a 72-h period when orchard ambient temperatures remained ≤0 °C . Of the 64 accessions studied, 51 were collected on a single date, five were collected on two dates, and eight were collected on three dates, making a total of 85 entries. The eight scion collection dates and number of accessions collected (no.) were 12 Dec. 1988 (8); 7 Feb. 1989 (8); 12 Dec. 1989 (6); 22 Jan. 1990 (10); 14 Dec. 1990 (12); 15 Jan. 1991 (13); 18 Dec. 1991 (13); and 18 Jan. 1992 (15). All scion sections were double wrapped in plastic bags and shipped with ice packs via overnight express to Ft. Collins, where scions were placed in a walk-in freezer at –3 to –5 °C for 1 to 6 weeks to maintain a state of cold acclimation before desiccation treatments were applied.

Scion desiccation. To initiate desiccation treatments, scions were cut into sections seven to ten cm long with two buds each and having at least 1.5 cm of internode at each end. Sections were spread on cafeteria trays and held in a chest freezer at –4 °C. Scion section diameters ranged from 3 to 8 mm. Since previous work indicated desiccation occurred more quickly in smaller diameter scion wood (Tyler and Stushnoff, 1988a), scion sections within a test entry were handled in three groups based on stem diameter: small (3.0 to 4.4 mm), medium (4.5 to 5.9 mm), and large (6.0 to 8.0 mm). For each entry, two samples of 10 individual scion sections (three small, four medium, and three large) were weighed immediately. One of these two samples was oven dried for three to five d at 85 °C and reweighed for gravimetric determination of percent moisture. The other sample was returned to the –4 °C freezer and weighed daily to calculate percentage moisture for each diameter size class. As each class reached 28% to 32% moisture, the sections were double wrapped in plastic and held at –4 °C until the cooling treatment. Desiccation periods ranged from 4 to 6 weeks.

Scion section treatments. Immediately before cooling, 20 scion sections were placed into cellulosic tube containers (41 mm diameter × 100 mm length) and the container ends plugged with tight-fitting rubber covers (containers and covers by Sinclair Rush, Inc.). At least 15 tubes of each test entry were prepared for periodic sampling during the 50-year span of the experiment. One tube was held at –4 °C as a desiccated control (DC) treatment. The remaining tubes were set into stainless steel boxes (64 × 115 × 285 mm), which then were placed in a freezer (Revco) at –4 °C. The freezer was modified by incorporating a heat tape with a controller (Omega Corp.) set to pulse heat to reduce the temperature 1 °C per hour to –30 °C. After the 26-h period for cooling from –4 to –30 °C, all boxes were held at –30 °C for 24 h, then quickly transferred to an LN tank for storage in the vapor phase above LN at –165 °C. Tanks were resupplied weekly with LN. The four LN treatments in this study included desiccation followed by 1 month or 1, 2, or 4 years continuous storage over LN. Treatments held in LN for 1 month are termed LN0.1 (≈0.1 year) while the remaining treatments are termed LN1, LN2, and LN4.

Bud recovery. Two days before initiating rehydration of scion sections, apple seedling rootstocks (Columbia Basin Nursery) 5.0 mm to 9.0 mm in diameter were removed from 2 °C storage and potted in 64 × 255 mm 'Deepots' (McConkey Co.) containing a peat–vermiculite medium (Boodley and Sheldrake, 1973) and grown for 17 d at 24/18 °C day/night in PGRU greenhouses. Supplemental lighting was supplied by high pressure sodium lights for 15 h each day. One tube of each accession was removed from LN and transferred to a 4 °C freezer to thaw for 24 h. For this study, 10 of the 20 desiccated-scion sections in each container were shipped via overnight express in the cellulosic containers to the PGRU, where they were rehydrated in moist peatmoss for 15 d before grafting.

The chip-budding procedure described by Hartman and Kester (1964) was modified to maximize contact between healthy tissues of the rootstock and the rehydrated chip bud. The bud section was sliced thinly from the scion section to remove only phloem and bark tissue with the bud, because LN treatment damages xylem ray parenchyma. Choosing one bud from each of the 10 scion sections, 10 buds per treatment of each entry were grafted onto rootstocks matching the diameter of the scion. Two buds were grafted to opposite sides of each rootstock 150 to 200 mm above soil level

Table 1. Percent bud recovery of cryopreserved *Malus* buds for 64 accessions representing different levels of cold-hardiness classification. Recovery from liquid nitrogen (LN) is based on pooled results for 1 month, 1, 2, and 4 years of storage.

Accession		Cold hardiness	Bud recovery (%)		Buds grafted[y]
PI no.	Name	classification	DC[z]	LN storage	(no.)
588846	Battleford	High	100	100	40
589478	Novosibirski Sweet	High	100	100	40
588790	Bisbee (Starkrimson)	Moderate	90	98	40
588841	Idared	Moderate	90	98	40
588801	Duchess (Red)	High	100	98	40
588775	Osman	High	95	95	80
483259	*M. baccata* 'Genvina'	High	95	95	80
589450	Parkland	High	90	95	40
589463	Breakey	High	100	94*	80
589481	Norland	High	100	93	40
588787	Snowdrift	Moderate	100	93	40
589363	Wellington	Moderate	90	90	40
588814	Trusevitch V-5-38	High	100	90*	40
589447	Norcue	High	70	90	40
383503	Chulanka	High	80	90	40
589389	*M. prunifolia* 'Macrocarpa'	High	90	90	40
392302	*M. sylvestris*	Moderate	90	90	39
589469	Haralson	High	100	88*	40
589483	Altaiski Sweet	High	95	88	80
588881	Ottawa 3	High	90	88	40
589491	Korichnoe Polosatoje	High	90	85	40
588848	Cortland	Moderate	95	85	80
589546	Zelenovka Sotchnaya	High	80	85	40
143974	Sugar Crab	High	100	83**	40
483268	Butterball	High	90	83	40
588803	Chestnut Crab	High	90	83	40
255899	Akane	Moderate	80	83	40
589485	Norson	High	90	80	40
588802	Anaros	High	80	80	40
589454	Earl	High	80	80	40
588883	Demir	Moderate	90	80	40
589649	Exeter Cross	Moderate	60	78	40
589360	Rescue	High	70	78	40
588875	Dab 325	High	100	75***	40
589367	Mantet	High	100	75***	40
589338	Columbia	High	80	73	40
589480	Westland	High	70	73	40
588884	Lord Lambourne	Moderate	90	72	40
588788	Wealthy	High	70	70	90
588872	Northern Spy	Moderate	100	70***	40
588839	Beautiful Arcade	High	100	68***	40
589521	Noret	High	90	65*	40
589359	Bedford	High	67	65	40
383518	Solncedar	Moderate	90	63*	40
589509	Dab 97	High	70	60	40
589357	Ranger	High	70	60	40
589339	Trail	High	90	60*	40
588862	Heyer #12	High	90	58**	40
588825	*M. ×robusta* 'Robusta 5'	High	90	58**	40
588866	Kerr	High	90	55**	40
589358	Quinte	Moderate	50	55	40
437055	*M. baccata* 'Flexilis'	High	60	53	40
588819	Vista Bella	Moderate	70	51*	120
588943	Liberty	Moderate	100	50***	40
392301	*M. baccata*	High	90	47**	30
588941	Jonagold	Low	87	43***	120
588835	Burgundy	Moderate	100	43***	40
588842	Empire	Moderate	73	40***	120
588789	Antonovka Shafran	Moderate	100	30***	40
588817	McIntosh Summerland Red	Moderate	70	28***	120
588873	MM 106	Moderate	77	21***	120
280401	Ein Shemer	Low	100	18***	40
588853	Cox's Orange Pippin	Low	73	17***	120
590184	Golden Delicious	Low	87	16***	120
Total			85	66***	3359

[z]DC = desiccated control, with means based on recovery of 10, 20, or 30 buds for one, two, or three collection periods.
[y]For LN-stored buds only: n = 30 ± 40, one period of bud collection; n = 80, two periods of bud collection; n = 90 ± 120, three periods of bud collection.
*,**,***Significant LN storage from DC at $P \leq 0.05, 0.01, 0.001$, respectively, for each accession, according to the test for difference between two proportions.

and tied in place with 100 × 12 mm budding rubbers. Seventeen days after grafting, rootstock tops were cut 50 mm above the grafted top bud to reduce apical dominance. Emerging rootstock buds were removed periodically to encourage growth of the grafted buds. Recovery was defined as elongation of buds into shoots 35 d after grafting. Data were analyzed as percent bud recovery of 10 buds per treatment. As positioned on the rootstock, top and bottom bud recovery on each entry was recorded to determine the effect of position for double budding.

The LN0.1 treatments were grafted in April or May following scion collection, as were the DC treatments, which were only done in the years of scion collection. Grafting for LN1, LN2, and LN4 was performed at nearly the same date each year (5 to 13 Feb. in 1990 through 1996). All other conditions, including rootstock type and personnel grafting and tying buds remained consistent from year to year.

To determine the control success rate for the modified chip-bud method, untreated buds from eight accessions harvested on the first date (12 Dec. 1988) were chip budded along with the other treatments. This treatment was not repeated for the remainder of the experiment.

Statistical analysis. The SAS procedure PROC FREQ (SAS Institute, Cary, N.C.) was used to conduct a chi-square test of independence of bud recovery quartiles versus hardiness rankings of high, moderate and low determined by published reports (Forsline, 1983; Strang and Stushnoff, 1975) and other criteria including geographic origin. The test for difference between two proportions (Zar, 1984) was used for mean comparisons.

Results and Discussion

Recovery from cryopreservation. Buds from all 64 accessions across all storage treatments and collection dates were recovered successfully from cryopreservation, although accessions differed in percent recovery (Table 1). Mean recovery across entries and the four storage treatments was 66%, compared with 85% mean recovery across entries for the DC treatment (Table 1). Our modified chip bud technique, determined with the one time control on 12 Dec 1988, was extremely effective, with over 98% recovery on untreated buds. Across all accessions, significant variability existed for mean recovery across the LN0.1 to LN4 treatments, with recovery of individual accessions ranging from 16% to 100%.

A chi-square test of accession cold hardiness classification versus the quartile to which the accession was assigned based on bud recovery was highly significant ($P = 0.006$ by Fisher's exact test). Thirty-three accessions showed recovery rates from 76% to 100%, 20 accessions from 51% to 75%, seven accessions from 26% to 50%, and four accessions from 0% to 25%. Accessions classified as least cold hardy were disproportionately found in the lowest quartile (Table 1). 'Ein Shemer', 'Cox's Orange Pippin', and 'Golden Delicious', among the least cold hardy *M.* ×*domestica* types in the PGRU collection, ranked lowest in recovery. Accessions classified as moderately hardy were disproportionately found in the 26% to 50% recovery quartile. In the upper two quartiles, accessions with moderate or high levels of cold hardiness were evenly distributed. In general, our results tend to support the relationship between degree of cold hardiness and cryopreservability proposed by Stushnoff (1991), although exceptions occurred. 'Bisbee' and 'Idared' were classed as moderately cold hardy, yet they showed 98% recovery. Three accessions classified as highly cold hardy, PI 392301 *M. baccata,* PI 437055 *M. baccata* 'Flexilis', and 'Kerr', had ≤55% recovery. More elaborate studies are needed to elucidate the relationship between cold hardiness and cryopreservability and the physiological processes involved, similar to those of Vertucci and Stushnoff (1992) and Wesley-Smith et al. (1992), which demonstrated the relationship between tissue water status and tolerance to cryogenic temperatures. Previous work has determined that an extremely cold-hardy species such as *Amelanchier alnifolia* Nutt. cryopreserves more readily than the hardiest *Malus* spp. Additionally, the extremely cold-hardy species of *Prunus virginiana* L. cryopreserves much easier than less cold-hardy species *P. armeniaca* L., *P. avium* L. and *P. persica* (L.) Batsch. (Stushnoff, 1991). We have demonstrated success in cryopreserving the moderately cold-hardy *Prunus cerasus* (Towill and Forsline, 1996) with cultivar differences positively correlated to extent of cold hardiness.

Most of the variability for bud recovery could be due to the steps involved in exposure to and recovery from cryogenic temperatures. Regardless of cold-hardiness classification, nearly all accessions had high levels of recovery in the DC treatment (mean = 85%, range 50% to 100%). In most of the highly cold-hardy accessions, there was no significant difference between the means for DC and LN storage (Table 1). However, some of the moderately cold-hardy accessions and all those with low cold hardiness had significantly lower recovery for LN vs. DC treatments. Further studies are being conducted to improve recovery of accessions that had low recovery by modifying protocol steps, including: cold acclimation, desiccation, cooling and thawing rates prior to and after LN storage, rehydration, and grafting.

While some genotypes might require modified protocols to enhance cryopreservability, we believe most will show sufficient recovery levels to use the current protocol for developing a base collection. First, however, it is essential to establish viability standards. In seed propagated accessions, regeneration of the base sample is recommended when viability as measured by standard germination tests falls to 65% (Policy Guidelines, NSSL, July, 1993). Recovery percentage of cryopreserved buds is a viability measurement comparable to germination testing; however, the

Table 2. Mean percent recovery of apple buds after liquid nitrogen storage, pooled over treatments for six accessions collected different years and months during dormancy.

Accession		Date of collection from the field		
name	PI no.	12 Dec. 1988	7 Feb. 1989	22 Jan. 1990
McIntosh S.R.	588817	58 a[z]	42 ab	10 b
Wealthy	588788	70 a	68 a	100 a
Vista Bella	588819	60 a	63 a	45 a
Empire	588842	44 a	56 a	33 a
Cox's Orange Pippen	588853	26 a	20 ab	5 b
Jonagold	588941	72 a	46 ab	18 b

[z]Entries in a row that are followed by the same letter are not significantly different from one another at $P \leq 0.05$ by test for difference between two proportions.

Table 3. Percent bud recovery for 64 accessions pooled over accessions.

Treatment	Recovery (%)
DC[z]	85.3 a[y]
LN0.1 (1 month storage)	63.0 b
LN1 (1 year storage)	64.2 b
LN2 (2 years storage)	66.5 b
LN4 (four years storage)	68.6 b

[z]DC = desiccated control.
[y]Separation of grand means of 64 accessions at $P \leq 0.01$ by test for difference between two proportions (LSD = 7.2).

genetic integrity of a clonal accession can be preserved in only one viable propagule. While it would not be prudent to rely on recovering a single bud from a stored sample, even relatively low levels of bud recovery may be adequate for long term storage. If a 65% viability standard were adopted, this study shows that 43 of 64 accessions were successfully recovered after 4 years LN storage. However, we have concluded that a viability standard of 40% provides an acceptable probability level that a sample can be recovered. For example, each accession in the base collection is stored as five tubes, each containing 12 buds (Forsline et al., 1995). Ten buds from one tube are grafted to assess initial bud recovery after 1 month of cryogenic storage. With a recovery level of 40%, the probability that at least one viable bud from each of the other four tubes in storage will be recovered is 99.9%, assuming no deterioration on cryogenic storage. Accessions not meeting the 40% viability level in the initial recovery are reprocessed. Controls such as the use of consistent standards for periodic assessment should alert us to loss of viability over the long term. Through 1997, 1100 accessions have been processed and stored in the base collection, from which 750 accessions have been tested for recovery; over 90% have shown acceptable initial recovery levels (Forsline et al., 1996b).

Effect of scion collection date. Scions from six accessions were collected on three different dates. Bud recovery across storage treatments was significantly lower for the 22 Jan. 1990 vs. the 12 Dec. 1988 collection date for 'McIntosh Summerland Red', 'Cox's Orange Pippin', and 'Jonagold' (Table 2). The former date was preceded by significant deacclimating conditions, with maximum temperatures ≥10 °C 4 d before collection, followed by another 3-d cold period immediately before and during collection. The scions may not have sufficiently reacclimated, since many genotypes do not reacclimate to a cold-hardy state after satisfaction of chilling requirement (Forsline, 1983) and thus may be more difficult to cryopreserve (Stushnoff, 1991). Significant differences in bud recovery for 'McIntosh Summerland Red', 'Cox's Orange Pippin', and 'Jonagold' were observed only between different dormant seasons. No significant differences were observed between two dates in the same dormant season (12 Dec. 1988 and 7 Feb. 1989) (Table 2). Cold tolerance of woody temperate plants varies annually depending on the physiological state of the plant entering dormancy. This state is greatly affected by presence or absence of biotic and abiotic stresses such as crop load, drought, low soil fertility, disease and insect infestation, low light, and other factors in the growing season. Such stresses may reduce accumulation of carbohydrates necessary to attain maximum resistance to subsequent cold (Weiser, 1970). Some stresses may be modified through manipulation of cultural practices which might improve cryopreservability of more cold-tender genotypes.

Stushnoff (1991) suggested that collection of buds early in the dormant season during good acclimation conditions (after a prolonged cold event ≤0 °C) would maximize cryopreservability. Such conditions optimize the level of cold acclimation of temperate woody plant species (Weiser, 1970), and dormant buds in a cold-acclimated state develop increased tolerance to desiccation, a key step for successful cryopreservation (Stushnoff, 1991). However, Forsline et al. (1996a) collected scions multiple times in January and February, even after midwinter thaws, stored the scion wood at –4 °C for 1 to 5 months, and found no effect on cryopreservability of the stored buds. They suggested that buds may sufficiently reacclimate while in –4 °C storage, indicating that collection after and during prolonged cold events may not be as critical for successful cryopreservation as originally hypothesized. This is consistent with results in Table 2 that showed no significant difference for same genotypes collected in the same dormant season.

Effect of bud-graft position. Calculated across all entry and treatment/date combinations including unfrozen controls, bud recovery was 45.7% for bottom-grafted buds and 54.3% for top-grafted buds, significant at $p = 0.0178$, possibly due to apical dominance. Although the results indicate grafting single buds to rootstocks will raise recovery percentage, this incremental increase would require doubling rootstock number and growing space and would not justify additional costs.

Longevity of cryopreserved buds. The longer propagules can be stored until their decreased viability necessitates repropagation, the more cost-effective will be the storage technique. While our initial results for the longevity of cryopreserved apple buds applies to only 4 years of storage, we detected no significant reduction in viability across accessions and LN treatments (Table 3). Similar results have been shown for cryopreservation of dormant mulberry buds for up to 5 years (Niino et al., 1994). When comparing the 4-year recovery test of individual accessions in relation to earlier tests at 1 month, 1, and 2 years, we observed no significant recovery decline in any of the 64 accessions (data not shown).

Implications for collection management. Initial results of this project on *Malus* are extremely encouraging; after 1 month to four years of cryogenic storage, most accessions were successfully recovered and showed high levels of viability. We calculate field maintenance costs to be about $75/accession/year for land, labor, and operating expenses. We estimate a one-time processing cost per accession for cryogenic storage of about $70. Because cryogenic storage tanks are already available at the NSSL, the only additional costs of maintaining an accession in a base collection are LN replacement (about $1 per year per accession), labor, and operating expenses. Thus, cryopreservation of the base collection is much more cost-effective than multiple field backups. Cryopreservation is also a safe, reliable, and convenient procedure.

Cryopreservation has considerable potential for use in an active collection. While germplasm curators are responsible for ensuring that all accessions are readily available, most germplasm collections contain some accessions that are seldom distributed. Since the PGRU *Malus* collection was established in 1986, >1000 accessions (40% of the collection) have never been requested or distributed. These seldom-distributed accessions are candidates for cryopreservation in the active collection so that they can be eliminated from field maintenance, and 350 of them are now in active cryogenic storage at the PGRU (Forsline et al., 1996b). Of 250 accessions tested, over 95% have an initial recovery ≥40%, meeting our viability guideline. Cryopreserving these accessions at Geneva reduces maintenance costs, yet allows easy and reliable access to buds for distribution to the user community. Resources heretofore devoted to field maintenance can be reallocated to improve characterization, evaluation, and documentation, thereby contributing to more effective use.

With every batch of accessions cryopreserved for the base or active collection, we include seven cultivars representing a range of cryopreservability (Table 1): 'Novosibirski Sweet', 'Bisbee', 'Demir', 'Burgundy', 'Empire', 'Ein Shemer', and 'Golden Delicious'. We also include an extremely recalcitrant accession of *M. florentina* (Zuccagni) C. Schneider which has had a recovery rate ≤5% (Forsline et al., 1995, 1996b). These eight standards will be periodically tested for recovery. This approach avoids the expensive step of testing each accession and provides an estimate of the longevity of a given batch. Currently we cryopreserve ≈350 accessions annually in the base collection. Within four years the USDA–ARS *Malus* base collection at Ft. Collins will be complete.

Although we can successfully cryopreserve most *M. ×domestica* accessions, further research is necessary to establish protocols for recalcitrant genotypes. Forsline et al. (1995 and 1996b) demonstrated that many wild *Malus* species of most interest to scion and rootstock breeding programs are of mild temperate or subtropical origin and do not cryopreserve well with the current protocol. We are studying various cultural practices to manipulate the physiological health of the source plants. We are also testing modified bud desiccation procedures as well as alginate coating of buds in combination with sugar imbibition (Seufferheld, 1995).

Finally, although our results show that cryopreservation can expedite conservation of *Malus* germplasm in both active and base collections, it is imperative to monitor the genetic integrity of accessions over time. While our initial observations of a long term experiment with trees of 'Jonagold' and 'Empire' from buds of cryogenic origin indicate no morphological irregularities in 3-year-old trees, this work is ongoing.

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