Cryopreservation of Fruit Crop Genetic Resources— Implications for Maintenance and Diversity during Conservation

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Even though the importation of fruit crop germplasm to North America coincided with and perhaps reached a zenith during the earliest phases of colonization and settlement, a national organized system of conservation did not emerge until the late 1970s. Serious concern that the maintenance and conservation of this important resource required national coordination and commitment led to the establishment of several clonal repositories to accommodate most of the fruit crops with production potential in North America (Brooks and Barton, 1983).

In recent years, it has become apparent that a comprehensive conservation program for fruit crop genetic resources will probably best be accomplished by developing storage systems for organs and tissues under controlled conditions to complement the clonal repositories. Cryopreservation of appropriate tissues and organs is one option. The potential for long-term storage in a stable condition using cryopreservation is appealing for several reasons, including improved efficiency and the potential for long-term storage. However, there is a need for more research to answer questions pertaining to long-term viability, genetic stability with various systems and tissues, improved protocols, and basic cell survival mechanisms at the biochemical and biophysical level. Accordingly, in this report, I will initially attempt to outline some current conservation strategies in light of present programs and conclude with comments on recent research on cryopreservation of fruit crop genetic resources and the potential application to conservation of these resources.

CONSERVATION STRATEGIES

Base collection

Conservation of agronomic, seed-propagated crops generally employs the strategy that long-term conservation goals will be met by establishing a base collection to cover genetic diversity for each crop. Seed stored in a base collection is intended to provide long-term conservation and to serve as a backup to other collections and is not intended for frequent distribution on a regular basis (Towill, 1988).

Active collection

An active collection, in contrast, is intended to serve breeders and others requiring genetic diversity on a regular basis. In these collections, new accessions are obtained regularly, regeneration is more frequent, characterization of genetic diversity is attempted, and enhancement has become a goal in some programs.

In the case of fruit crops, the clonal repositories have served primarily as active collections, but because they represent the major conservation attempt with these crops, they also have been considered to serve a dual role as base collections. Accordingly, there presently is no provision for a backup base collection, in the same sense, as there is for seeds at the National Seed Storage Laboratory at Fort Collins, Col. Recently, however, initiatives have been made to conduct research to support establishment of base collections using low-temperature and cryogenic storage of seed, pollen, and

dormant buds of vegetatively propagated crops.

The core concept

The underlying premise of a core collection is to identify a representative core of germplasm that contains maximum diversity for the target species. During the past two decades, collection and conservation of seed have expanded from service to breeders to the broader concerns of conserving biological diversity (Strauss et al., 1988). They have shown that, in some cases, the result has been to gather many accessions with rare consideration to assess diversity, thus creating large, difficult-to-manage collections that can be redundant and that may not reflect the desired level of genetic diversity. Strauss et al. (1988) contend that implementation of a core concept in managing collections could avoid the need to maintain many accessions for every species, thereby reducing redundancy and the possibility of overloading the system. They cited data showing up to five duplicates for some of the large collections of cereal crops. Although many of the fruit crops appear to be vastly undercollected in North American repositories (C. Sperling, 1988, personal communication), an examination of the *Prunus* data base in Europe by Van Sloten and Holle (1988) revealed up to 16% duplication of some cultivar collections. Genetic diversity of fruit crops must include species and primitive selections, as well as valuable old cultivars; accordingly, fruit and nut crops were included in the 1980 International Board for Plant Genetic Resources (IBPGR) global germplasm program (Van Sloten and Hone, 1988). However, this action does not relieve fruit research scientists of the responsibility to develop methods and data bases to establish the characterization of genetic diversity on a solid basis. In addition to facilitating an efficient germplasm conservation system, characterization of genetic diversity should provide a sharper focus to fruit breeding. Hansche (1983) made an excellent case for emphasizing the development of biological systems to facilitate "genetic engineering" of fruit crops. Characterization of the genetic diversity in fruit crop germplasm would seem to be an essential step toward this objective.

CONSERVATION ALTERNATIVES FOR CLONALLY PROPAGATED CROPS

Ex situ collections

Up to and including recent times, ex situ collections of plants grown in field gene banks (IBPGR term), clonal repositories (USDA term), living collections (redundant term), arboretum specimens, breeder collections, and archival collections by private groups and individuals represent the major intent of horticulturists to conserve genetic resources of fruit crops for future generations. These collections are of obvious immediate benefit to plant breeders in that they provide immediate access to flowering plants for crossing purposes, they maintain genetic integrity (barring chance mutations), and the phenotypic response to environmental fluctuations can be observed and monitored regularly. However, the need for land; constant maintenance; vulnerability to insects, diseases, cold, heat, drought, wind, fire, flood, and pollution; and low visibility in programming priority, creates considerable uncertainty with respect to long-term security of genetic resources.

In situ conservation

In situ conservation, enabling environmental evolutionary forces to continue to generate and direct diversity as they have for cen-

Support for this research, provided by Agricultural Canada, IBPGR, and by USDA, NSSL, is gratefully acknowledged. I thank Nancy Tyler, Carlos Fear, Karen Caswell, Laurie Freisen, Ken Friesen, Rick Sawatzky, M.P. Dwivedi, Gayle Berry, and Tom Ward for their research contributions.

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turies, would appear to be prudent. Any workable attempt in this direction would be welcomed and viewed as good stewardship, as well as scholarly. Unfortunately, if past history is any indicator, mankind is controlled by less reliable forces than stewardship of our natural resources and scholarly interests. Accordingly, long-term dependence on in situ conservation as the sole strategy likely would not produce the desired results. Indeed, there is already plenty of evidence that substantial losses have occurred from natural centers of origin and diversity (Brooks and Barton, 1983; Zagaja, 1983).

Ex situ collections as living plants are important and must be maintained with vigorous support, but there is also a need to consider alternatives that are less vulnerable to environmental, pathological, and financial strain. Several authors have suggested that research is needed to develop techniques so that complimentary and backup systems can be put in place to meet the goals of recognized conservation strategies (Brooks and Barton, 1983; Zagaja, 1983).

There are several potential alternatives using both low-temperature and cryogenic storage for germplasm maintenance. Seeds and pollen can be used to store genes. Various meristematic tissues can be used to provide clonal integrity. The method chosen for a particular crop will depend on the desired conservation goals, reliability, simplicity of the method, and benefits to be attained in view of client needs and long-range plans for germplasm conservation.

Seed

The seed, as a relatively small, embryonic, dormant, and anhydrous organ, is one of the most convenient systems available for prolonged storage of genetic information. Many seeds can be stored in a small space, and genetic diversity is easily captured. Low-temperature (-20°C) and cryogenic systems (Stanwood, 1985) can be used with moisture control for orthodox seeds to prolong storage life, but, because fruit seeds have not been targeted in the past, records on viability and ideal conditions for each species are sparse. Also, seed of many tropical fruit and nut crops is recalcitrant to desiccation and low-temperature storage and cannot be included in these generalizations.

Seed storage should be ideal to conserve a pool of genes in a base collection, especially for wild species in imminent danger of loss and where clonal integrity is less critical or where clonal storage systems are lacking.

Not all fruit crops have orthodox seeds and those recalcitrant to desiccation and low temperature have a very short life span, making them unsuitable for storage using current technology. Seeds from many fruit crops have long periods of dormancy with complicated stratification and germination requirements. Furthermore, most fruit crops have distinct and unpredictably long juvenility periods that would create enormous problems in seed regeneration. Another regeneration problem is the bias that results in genetic diversity of population structure from the necessity to choose a cross-pollenizing parent because of self-incompatibility or dioecy.

Perhaps the most important fundamental difference between vegetatively propagated fruit cultivars and seed-propagated crops is the capability to maintain clonal integrity for the benefit of producers and consumers. Desirable gene combinations, sought by breeders to enhance production and consumer acceptance, are readily identifiable and can be accessed as required. For this very same reason, and because desirable gene combinations are not usually easily recovered from heterozygous parental material, also complicated by long periods of juvenility, breeders wish to have access to germplasm with clonal integrity. This is especially pertinent when the germplasm has been well characterized for desired traits. Seeds are not as suitable for this purpose as are vegetative propagules; thus, their use is probably more appropriate in a base collection to conserve a gene pool than for routine use in working collections.

Pollen

Provision of pollen to breeders from active collections seems to have several potentially positive features. Pollen is produced yearly from plants in clonal repositories and is easily collected, stored, and shipped. Breeders could significantly broaden their access to genetic diversity, especially from species that are difficult to main-

tain in a particular location, for example, cold-tender, high-quality cultivars in northern locations or high chilling germplasm in regions with short, mild winters.

If suitable protocols were developed to maintain longevity, under cryogenic conditions for example, collections should be infrequent and the space requirements for storage minimal. A backup collection also would not be difficult to develop.

Pollen provides only a source of nuclear genes and is subject to some of the same restrictions as seeds in terms of recalcitrant behavior with respect to drying and freezing. There is good evidence for achievable results with some fruit crops (Towill, 1988), and more research will surely broaden the diversity of materials available for successful conservation.

In vitro systems

In recent years, micropropagation of vegetatively propagated crops, based on in vitro tissue culture, has been widely researched and broadly applied in commerce. In addition to propagation, tissue culture has been used to eliminate viruses and maintain virus-free stock, to rescue embryos from abortion in wide crosses, to facilitate physiological studies, and as an integral step in plant biotechnology. Accordingly, these techniques provide many important attributes and also some problems in the conservation of fruit crop genetic resources (Brooks and Barton, 1983; Stushnoff and Fear, 1985).

The primary goal of conserving clonal integrity with capability for rapid increase is generally attainable, but there is evidence that prolonged culture produces variants. This may be tolerable if the frequency is low, but because the techniques are still largely empirical, one cannot yet be entirely comfortable in terms of long-term reliability for conservation of genetic resources. Additional problems are the need for precise accession-specific media and protocol for propagation and storage, the long time required to recover flowering plants, and the potential for loss of an entire collection because of mechanical malfunction.

In addition to rapid clonal multiplication, in vitro techniques possess significant potential advantages, such as small space requirements, attainment of disease-free status, ease of international transportation, and low cost.

There is also significant potential to reduce growth by using low-temperature and cryogenic storage and by applying physiological growth controls (Stushnoff and Fear, 1985; Towill, 1988). Cold acclimation of in vitro-produced meristems has improved survival at cryogenic temperatures (Caswell et al., 1986; Reed, 1988).

However, application to conservation of gene resources on a broad scale still requires development of universal procedures with more predictable responses for most fruit crops. Some of the small-fruit crops, e.g., strawberries, are, perhaps, most amenable to improved management of active collections with present technology.

Cryopreservation of dormant buds

Recently, techniques have been developed that result in 80% to 100% viability and recovery of cryopreserved dormant vegetative buds from *Malus* (Stushnoff, 1987; Tyler and Stushnoff, 1988a, 1988b; Tyler et al., 1988); *Amelanchier* (Friesen, 1987); and *Crataegus*, *Sorbus*, and *Prunus* (C. Stushnoff and L.J. Friesen, unpublished data). This research was based on an initial report by Sakai and Nishiyama (1978), who showed that winter buds of apple could be manipulated to survive immersion in liquid nitrogen by providing an initial exposure to subzero temperatures at slow freezing rates.

Cryopreservation of dormant vegetative buds provides several advantages as a method of conserving fruit crop gene resources: a) clonal integrity is maintained; b) the technique is simple and space-efficient; c) low-maintenance requirements consist of liquid nitrogen as a coolant in storage vessels; d) very long storage is theoretically possible; e) recovery of flowering plants for crossing is possible within ≈ 2 years after grafting to a dwarf rootstock grown in a greenhouse. The system now has been tested with a reasonable number of temperate deciduous tree fruits, including ≈ 30 cultivars of cold-hardy and cold-tender apples; a few cultivars of apricot, sweet and sour cherry, and peach; and 11 other species in the

Rosaceae (C.S., unpublished data).

There are also limitations, as with any system. These include: a) lack of data on long-term viability and genetic stability; b) a requirement for harvesting buds during dormancy and in the acclimated state or the potential to induce controlled cold acclimation; and c) a requirement for facilities to provide controlled desiccation and freezing conditions, including liquid nitrogen storage. Also, compared to in vitro systems, international shipment is not as easily accommodated.

The concept appears to have potential for establishing a base collection and for providing a space- and cost-saving backup to field maintenance of trees. A summary of the protocol and critical steps required to attain 80% to 100% recovery is as follows:

a) Although some success was possible using only a prefreezing treatment before immersion in liquid nitrogen, incorporation of controlled desiccation greatly improved viability and expanded both the sampling interval and the range of cultivars that could be cryopreserved (Tyler and Stushnoff, 1988a, 1988b).

b) Periodic sampling revealed there is a defined time window when buds will tolerate desiccation. Generally, buds taken in August were intolerant of desiccation, but those taken in September and October were attaining tolerance, and by November and December, they were fully tolerant (Fig. 1A). Furthermore, some taxa were more and some less tolerant of desiccation than others (Tyler and Stushnoff, 1988a). The hardest and most northerly distributed species were also most tolerant to desiccation at the onset of resistance in the fall. Field moisture content ranged from 48% to 60%

(fresh-weight basis). Desiccation to 20% to 30% was required; generally, below 20% was damaging even when maximum tolerance had been attained, but with a few very tolerant species, desiccation below 10% water content, on a fresh-weight basis, was possible on some sampling dates (Figs. 1B and 2).

c) An initial slow freezing step (refreezing) before immersion in liquid nitrogen was essential. The optimum rate of prefreezing tolerance was 2C/hr to -16C then 10C/hr to -20, -30, or -40C, after which the samples were held for 5 to 96 hr before being plunged into liquid nitrogen. Pulsed phase nuclear magnetic resonance studies showed that some water remained unfrozen even to -50C in apple (Tyler et al., 1988). The optimum holding temperature was -30C.

d) Recovery from liquid nitrogen involves thawing overnight at 0 to +5C and rehydration at 0 to +5C in moist peatmoss before placing a patch bud or a chip bud (Bienz, 1980) onto al-to 1.5-cm-diameter dwarfing rootstock under greenhouse conditions. These plants have produced flowers $\approx$ 2 years after recovery from liquid nitrogen.

Several criteria are important in attempting to define the limits of successful cryopreservation of dormant buds, including physiological development that imparts seasonal tolerance of desiccation and genetic background that controls the limits of desiccation tolerance and cold hardiness. An attempt has been made to classify the species we have studied in terms of response to desiccation, seasonal adaptation, and cryopreservation viability (Table 1). Undoubtedly, amendments and modifications will be necessary as more research is done, but several common elements are apparent. *Prunus virginiana* Linn. was placed in class I because it tolerated severe desiccation even in September and consequently survived cryopreservation directly from the field and were also tolerant of desiccation stress (Fig. 2).

Species in class II are also very cold-hardy in their native habitat, but are not as tolerant of desiccation stress, eg, *Prunus besseyi* Bailey. Species placed in this class can be cryopreserved without desiccation if harvested in midwinter at maximum cold hardiness following a period of extreme cold exposure outdoors, but otherwise desiccation is beneficial.

Species in class III are less cold-hardy, tolerate desiccation even less, and must be desiccated to attain survival in liquid nitrogen at any time they are harvested. Month-to-month variability, perhaps associated with fluctuation of outdoor temperatures or depth of rest in dormancy, was also encountered as shown for *Prunus nigra* Ait. (Fig. 3).

Those species normally considered as cold-tender were placed in class IV. In this case, cold acclimation under controlled conditions, by freezing at 1C/hr and holding for 10 to 14 days at -20 and -25C, was required. Controlled desiccation, as in class III, was

A. RESPONSE TO DESICCATION

B. RESPONSE TO CRYOPRESERVATION

Fig. 1. Seasonal response of dormant vegetative apple buds to (A) controlled desiccation and (B) cryopreservation in liquid nitrogen. Data are means for two cold-hardy cultivars, Dolgo and Rescue.

Fig. 2. Relationship between moisture content and survival following cryopreservation of dormant vegetative buds from *Prunus virginiana* sampled in September (S), October (O), and November (N) after 0, 6, or 12 days of controlled desiccation at -4C.

Table 1. Classification of cold hardness, dehydration tolerance, and cryopreservability of dormant buds for several fruit crop species.

Class	Characteristics	Species
I	Very cold-hardy (-60°C or colder); acclimate very quickly; resistant to dehydration injury; can cryopreserve without dehydration	<i>Amelanchier alnifolia</i> Nutt., <i>Prunus virginiana</i> L.
II	Very cold-hardy (-60°C or colder); acclimate quickly; susceptible to dehydration injury; can cryopreserve without dehydration	<i>Malus baccata</i> Borkh., <i>P. besseyi</i> Bailey, <i>P. pennsylvanica</i> L.f., <i>P. tenella</i> Batsch.
III	Cold-hardy (-40°C or colder); acclimate quickly; susceptible to dehydration injury; cannot cryopreserve without dehydration	<i>M. pumila</i> Mill., <i>P. nigra</i> Ait., <i>P. fruticosa</i> Pall., <i>P. tomentosa</i> Thunb., <i>P. salicina</i> Lindl. (Pittsin a cold-hardy cultivar)
IV	Cold-tender (-40°C or warmer); acclimate slowly; susceptible to dehydration injury; cannot cryopreserve without controlled acclimation and controlled dehydration	<i>P. armeniaca</i> L., <i>P. avium</i> L., <i>P. cerasus</i> L., <i>P. persica</i> L. Batsch.
V	Very cold-tender (-20°C or warmer); acclimate slowly and very little; dehydration tolerance unknown; no known cryopreservation of in vivo-derived bud meristems	Numerous species of evergreen and some deciduous species

also required to attain survival in liquid nitrogen. Dormant buds of *Prunus avium* Linn., *P. cerasus* Linn., *P. armeniaca* Linn., and *P. persica* Linn. taken in Dec. 1987 survived 80% to 100%, but samples from all other months did not survive, indicating that there is a rather narrow sampling time for species in class IV. No survival was attained unless controlled acclimation was incorporated. Apparently, the need for acclimation is critical, suggesting that best results will be obtained from samples grown in northern locations subject to maximum cold acclimation.

Class V category is reserved for nontemperate and marginally hardy species. Manipulation will be possible for some species and not for others. Little or no research has been done to establish or refute the validity of this class at this time.

Consideration of water status relative to dormancy, cold hardness, and genetic background is useful in developing reliable cryopreservation protocols. Recent studies using differential scanning calorimetry (DSC) (C. Stushnoff and C.W. Vertucci, unpublished data) have provided evidence for different functions of water in dormant vegetative buds. One component of water in dormant buds can be frozen extracellularly and is detectable as typical first-order exotherms that are not related to discernible tissue damage. Another component remains unfrozen. There is evidence to suggest that the genetically hardiest cultivars and cultivars in the acclimated condition retain more unfrozen water than nonacclimated buds (Stushnoff et al., 1988; Vertucci et al., 1988).

There appears to be a relationship between desiccation tolerance, cold hardness, and ease of cryopreservability among dormant buds of species and cultivars of temperate fruit crops. This relationship could lead to inadvertent selection for specific genotypes and shift genetic diversity in collections, especially if the process led to mutations. However, knowledge of this information can be used to avoid this problem. Also, there is no evidence to assure us that any other system that relies on manipulation of any sort is devoid of this potential problem.

Research on low-temperature storage and cryopreservation of fruit crop genetic resources is required to develop more efficient active collections and as a basis for establishing base collections. While there now is evidence that some systems can be used, there is a need for continued research, particularly to establish long-term viability, to assess stability, and to characterize genetic resources.

Fig. 3. The relationship between moisture content and survival following cryopreservation of dormant vegetative *Prunus nigra* buds sampled in September (S), October (O), and November (N) after 0, 6, or 12 days of controlled desiccation at -4°C .

CRYOGENIC STORAGE AS A CONSERVATION STRATEGY

Storage of germplasm with liquid nitrogen as a cryogen in the liquid phase (-196°C) or in the vapor phase (about -150°C) is attractive as a technology for conservation of genetic resources. Details on research for many systems can be found in a recent book edited by Kartha (1985) and a review by Towill (1988).

Although a few species of plants are capable of acclimating to very low temperatures under natural conditions, survival at cryogenic temperatures depends entirely on contrived protocols. Recently, plant scientists have begun to seek evidence from biophysics and biochemistry to support the hypothesis that biological material can be stored much longer, with genetic stability and reduced mortality at cryogenic temperatures (-150 to -196°C) than at ambient or subfreezing temperatures. Low temperatures extend seed storage potential because seed metabolism is slowed. Conventional refrigeration conveniently provides -20°C storage, but there is no literature to suggest that -20°C is ideal. Indeed, for dormant apple twigs, -20°C was shown to be inferior to -3 , -30 , or -70°C (Sakai and Nishiyama, 1978). Evidence that cryogenic seed storage can improve seed conservation has been summarized recently (Stanwood, 1985).

Two general situations can be considered in cryogenic storage: the desiccated state and the hydrated state. Organs that can tolerate desiccation, many seeds for example, can generally be stored at cryogenic temperatures without cryoprotectants or special manipulation. On the contrary, tissues that are hydrated, such as in vitro meristems and shoots, require precise manipulation of protocol and cryoprotectants that are taxon specific. Protocols permitting survival have been derived largely by empirical trials, especially with hydrated samples, and are not generally applicable to diverse taxa and systems.

Theoretically, survival in cryogenic storage depends on: a) the potential of tissues to tolerate removal of freezable water by desiccation or by freeze-induced desiccation, b) the potential for tissues to undergo transitions to the vitreous state, and c) rewarming to return to ambient and hydrated conditions without death.

The vitreous state is characterized as a metastable state with translational and rotational motion that is so slow that molecular reactions dependent on diffusion are stopped, thus imparting extreme stability (Franks, 1982). Another characteristic expected to contribute to the stability of aqueous systems in the vitreous state is their reduced water vapor pressure, compared to crystalline ice, thereby inhibiting desiccation (Burke, 1987).

Transitions from the vitreous or glassy state, produced by slow rewarming, are viscous liquids with some translational and rotational motion; these are detectable by differential scanning calo-

rimetry as endothermic base-line shifts. In hydrated systems, endothermic transitions may be followed by devitrification exotherms (crystallization) and finally by endothermic melting. Transitions to the vitreous state during cooling and desiccation are also detectable as endotherm. Further details on the basis for the formation of glass transitions and the vitreous state can be found in Franks (1982).

Glass transitions have been detected in cold-hardy, dormant vegetative buds of *Malus* and *Amelanchier* (Stushnoff et al., 1988). Although not studied in depth, floral buds that supercool, as with most *Prunus* species, probably do not respond similarly. We believe that the vitreous state detected in dormant vegetative buds enables survival at cryogenic temperatures without damage from freeze-induced desiccation and other events, perhaps associated with freezing of aqueous fractions. If glass transitions to the vitreous state are not the basis for survival, then the tissue must be capable of tolerating removal of most freezable water to survive, which was not the case following desiccation of unfrozen buds (Figs. 1-3).

A thorough understanding of the conditions that promote transitions to and from the vitreous state should be extremely useful as we attempt to develop cryogenic storage systems for fruit crop genetic resources. In all likelihood, this knowledge will also provide important clues to the basis for plant survival at low temperatures, especially to assist plant breeders in the development of cold-hardy cultivars.

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