LONG-TERM PRESERVATION OF CLONAL GERMPLASM: ADVANCES AND CONCERNS

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ABSTRACT

Numerous crops are maintained as clones in field, greenhouse or *in vitro* plant culture. Long-term germplasm conservation for these species is needed and may be accomplished by cryogenic preservation of seeds, pollen, and shoot tips or buds. Seed and pollen can be used to preserve genetic diversity of the crop. These two propagules from many clonal species are desiccation tolerant and survive low temperature exposure. Thus, methods already exist to apply long-term storage to certain crops. Cryopreservation of shoot tips or buds is needed for long-term conservation of the clone. Substantial progress has occurred in defining two-step cooling and vitrification protocols, such that application to certain plant collections is now possible and feasible. For example, long-term storage of apple has been initiated at the National Seed Storage Laboratory using dormant vegetative buds collected in winter from the National Clonal Germplasm Repository at Geneva, NY. Survival after cryogenic exposure was found in all lines tested to date, with cold-hardy lines exhibiting a greater percentage of viability. Cold-tender lines will require a more complex protocol. Vitrification (application of cryoprotectants followed by rapid cooling such that a glass forms within the cells) is an easier method to apply than two-step cooling. Survival was found with apices from many species. A practical project using vitrification to preserve mint is underway. Survival for some species, using either vitrification or two-step cooling methods, still appears genotype-specific. Will a single method be applicable to all genotypes within a collection? A strategy to deal with diversity is needed to minimize the effort required to achieve practical cryopreservation.

***Additional index words:* germplasm preservation, cryopreservation, pollen, shoot tip, vitrification, clones.**

LONG-TERM CONSERVATION

The function of the National Seed Storage Laboratory (NSSL) within the U.S. National Plant Germplasm System (NPGS) is long-term conservation for crop plant genetic resources. Most of these crops are seed-maintained and easily stored; however, many others are maintained as clones in the field, greenhouse or *in vitro*. These require considerable space and labor for maintenance, and are at risk of loss to disease or catastrophe. There are seven National Clonal Germplasm Repositories maintaining a diverse array of species ranging from hardy, temperate plants

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Table 1. Species held as clones within the National Plant Germplasm System.

Location	Crops maintained
National Clonal Germplasm Repositories	
Brownwood, TX	pecan, hickory
Corvallis, OR	bramble fruit, strawberry, filbert, pear, mint, hops
Davis, CA	*Prunus*, grape, kiwifruit, persimmon, walnut, olive, pistachio
Geneva, NY	apple, hardy grape
Hilo, HI	papaya, pineapple, macadamia, passionfruit, guava, rambutan
Mayaguez, PR & Miami, FL	bamboo, cassava, mango, litchi, sugarcane, banana, coffee, cacao, carambola
Riverside, CA	citrus, date palm
Other Facilities	
Sturgeon Bay, WI	potato
Griffin, GA	sweet potato, peanut
Ames, IA	some ornamentals
National Arboretum	ornamentals

to tropical/subtropical plants (Table 1). Clonal plants are also maintained at other locations, for instance potato in Sturgeon Bay, WI, sweet potato in Griffin, GA, and numerous ornamentals at the National Arboretum, Washington, DC. All these collections are active genebanks performing functions of acquisition, propagation, maintenance, distribution, characterization, and information dissemination. Long-term conservation is needed and best accomplished using cryogenic conditions, preferably with storage at a different site other than that of the active collection. The NSSL can fulfill this latter function by the preservation of seed, pollen and/or shoot apices.

I have been examining long-term preservation for clonal crops, concentrating on cryopreservation of the clone and on studies which may facilitate implementation of pollen cryopreservation. Here I discuss new procedures that might be useful to achieve the goal of long-term conservation, particularly for vegetative propagules, and give my assessment of the current status of cryopreservation for clonal crops.

POLLEN PRESERVATION

Most clones in the NPGS are heterozygous. Seed and pollen preservation would not preserve the genotype but would preserve the genes in altered combinations. Thus, genetic diversity for the species could be preserved. The main advantages for pollen and seed preservation are ease of handling, processing and storage since most pollen and seed are desiccation-tolerant and have been shown to survive cryogenic exposure and storage. But if grow-out is eventually needed, maintaining gene frequen-

Table 2. Examples of pollen survival after exposure to liquid nitrogen (LN).

Species	Average percent germination	
	Control	LN (storage time)
pine	78	83 (6 mo)
spruce	83	78 (6 mo)
black walnut	54	41 (4 da)
pecan	51	46 (6 mo)
apple	47	28 (4 da)
pear	57	59 (7 da)

cies would require extreme care. Problems for maintenance over generations increase as the gene diversity from the original clone is handled as a seed (or pollen) population. These problems may be minimal for some clonal crops but would be difficult for woody plants, some of which require several years to flower. Intercrossing to maintain gene frequencies would be extremely difficult.

Cryopreservation of clonal propagules is difficult and it will take several years before practical methods are developed. Seed and pollen preservation, thus, has the benefit that it can be done now and can provide security against loss until clonal cryopreservation is routine.

Pollen from different species can be classified as desiccation-tolerant or desiccation-sensitive (Towill, 1985; Hoekstra, 1986), similar to that for seeds. Desiccation-tolerant pollen can be dried to low moisture content and retain viability if properly handled; whereas, desiccation-sensitive pollen loses viability when dried below some value unique to the species. Fortunately, many temperate zone fruits and nuts and other clonal crops possess desiccation-tolerant pollen. These pollens have been shown by many researchers to survive cryogenic exposure (Towill, 1985; Akihama and Omura, 1986). Some examples are given in Table 2.

Longevity at temperatures of about –160°C to –196°C has not been studied for clonal materials but should be substantial. Cryogenic storage of pollen is not yet utilized routinely for long-term conservation mainly because of the additional labor needed to implement the system. The steps in a practical preservation system have been identified to coordinate the process between active collections and the NSSL (Table 3). Critical

Table 3. Processes identified as important for the development of long-term pollen preservation.

Pollen preservation:	collection
	shipping
	handling
	viability testing
	packaging
	storage
	periodic viability tests
	distribution
	information

issues are how and when pollen is collected from the flower, how and what moisture level it is dried prior to storage, and how viability is tested. *In vitro* germination or fluorescein diacetate staining usually provide an accurate assessment of viability. Seed set using treated pollen is the definitive test for fertility but is difficult to perform routinely throughout the year and does not give an estimate of viability per se.

Pollen desiccation tolerance and longevity characteristics at different storage temperatures have not been assessed for most clonal crops. Whereas temperate zone species have desiccation-tolerant pollens and have been better studied, characteristics of many tropical and subtropical crop species are not known. Some tropical and subtropical species are thought to have desiccation-sensitive pollen. Pollen from the Gramineae, tricellular and often desiccation-sensitive, may serve as a model for these species, but, as with seed, the reasons for desiccation sensitivity appear quite complex such that artificial groupings may not be helpful. The evaluation of desiccation sensitivity is an important research area.

CLONAL CRYOPRESERVATION

The diversity of species held as clones within the NPGS and the range of physiological characteristics that they possess preclude use of a single method for cryopreservation. A generalized protocol for processing a vegetative propagule is shown in Figure 1. Details for each step are usually empirically determined for the species of interest. Optimization for each step is often difficult to achieve because of variability in survival among experiments. Two variations of this scheme are proving valuable and are described below.

Dormant vegetative buds

Some species cold acclimate to a considerable extent, although within a clonal repository there may be considerable variation among accessions in that ability. Several reports showed that buds and bark from hardy twigs can survive cryogenic exposure if slowly cooled (Sakai and Nishiyama, 1978; Katano et al., 1983; Moriguchi et al., 1985; Tyler and Stushnoff, 1988). The NSSL has examined use of dormant vegetative buds for preservation of *Malus* species and has held twigs in storage for about 5 years. Twigs from field-acclimated apple are collected after a suitable cold period (December to January) and shipped from the Geneva, NY, clonal repository to NSSL where they are cut into 2 bud sections and partially desiccated. Desiccation has been reported to increase cold acclimation in dogwood (Chen et al., 1975). Sections are then slowly cooled to –30°C (1–2°C hr^{-1}) and transferred to the vapor phase above liquid nitrogen. After different storage periods, sections are thawed at 2°C overnight and then shipped to Geneva where they are rehydrated and grafted to determine viability. Sixty-four accessions have been examined and survival has been found in each. Buds from cold-hardier accessions survived in greater percentages than those from cold-tender ones. In examining 99 fire-blight sensitive accessions this past year, similar results were obtained. Thus, in light of the low percentages obtained with some clones, alternative (or supplemental) methods still must be developed to deal with diversity.

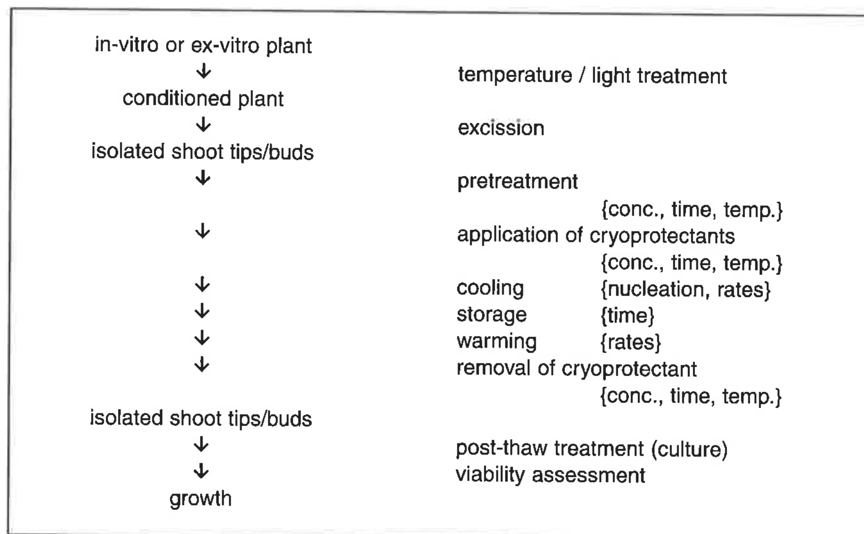

Figure 1. Generalized flow chart for cryopreservation of shoot tips.

Nevertheless, the dormant vegetative bud process is now being used to preserve apple lines held in the Geneva, NY, repository, and the project is the first for cryopreservation of clonal germplasm in the National Plant Germplasm System (Forsline et al.,1993).

Less-hardy and non-cold acclimatable species

Several methods have been examined to cryopreserve shoot tips and buds from these species. Two-step cooling and vitrification methods are commonly cited (Sakai, 1986; Chen and Kartha, 1987; Withers, 1987; Towill, 1991) and have been analyzed in greater detail. The former method is more studied, and a brief synopsis suggests that this method is useful for some materials but not others, for reasons not readily apparent. The latter method, not nearly as well studied, utilizes concentrated cryoprotectant(s) which form a glass upon being rapidly cooled; that is, the system vitrifies (Fahy et al., 1987; Fahy, 1988; Macfarlane and Forsyth, 1990; Macfarlane et al., 1992; Steponkus et al., 1992). Vitrification may avoid some damaging events which occur during ice formation in the two-step cooling method. The advantage to the user of vitrification is simplicity of the technique (no cooling rate apparatus required) and potential to use larger pieces of tissue. Tests have shown that survival can be obtained after vitrification in protoplasts (Langis and Steponkus, 1990; Langis and Steponkus, 1991; Steponkus et al., 1992), cells (Langis et al., 1989; Sakai et al., 1990; Sakai et al., 1991), somatic embryos (Uragami et al., 1989) and shoot tips (Towill, 1990; Kohmura et al., 1992; Niino et al., 1992a; Niino et al., 1992b; Towill, 1990; Towill and Jarret, 1992) when these materials are treated appropriately.

Table 4. Survival of excised shoot tips after exposure to vitrification solution and then liquid nitrogen (LN) immersion.

Species/line	Method[b]	Survival[a] + vitrification solution	
		not cooled	LN-exposed
Mentha sp.			
M. aquatica × *M. spicata*	A	97	80
	B	92	83
M. aquatica	B	58	33
M. arvensis	B	50	39
M. canadensis	A	75	19
	B	83	62
M. × *dumetorum*	B	73	54
M. × *gracilis*	B	69	45
M. maximiliana	A	90	96
	B	92	4
M. niliaca	A	100	83
	B	90	100
M. × *piperata*	B	100	81
M. spicata	A	100	96
	B	100	76
M. × *verticillata*	B	92	83

[a] Values presented are the percentage of shoot tips that survived as assessed by regrowth.
[b] Method A is according to Towill, 1990; Method B is slightly modified from that of Sakai et al., 1990.

Other methods have been reported to give fairly high levels of survival after low temperature treatment. Some of these involve encapsulation of shoot tips in alginate beads with exposure to dehydrating conditions prior to using rapid cooling (Tannoury et al., 1991; Paulet et al., 1993). In these studies pretreatment of the shoot tip with elevated levels of sucrose is essential to maintaining viability. The combination of sugar, desiccation, and rapid cooling probably leads to vitrification in the shoot tips.

Two-step cooling also have been successfully applied to many species and still is a feasible method for cryopreservation. Indeed, survival after two-step cooling is probably due to the vitrification of the cellular contents during the immersion step from about −35°C to liquid nitrogen (LN).

Work at NSSL has examined vitrification. Stock plants for species used are maintained *in vitro* and were either initiated using standard techniques or obtained from sources within the NPGS. Exposure of the plants to acclimation conditions *in vitro* for 1–2 weeks is beneficial for those species that cold acclimate (Reed, 1988). Shoot tips (ca. 0.5–1 mm in length, containing 2–4 leaf primordia) are excised from either apical or axillary buds and subjected to the vitrification procedure. Viability of the treated shoot tip is assessed by examining the retention of green color and growth on an agar-solidified growth medium over a 1 to 3 month period.

The standard vitrification procedure consists of applying the cryoprotectant solution to the shoot tips, exposing the samples to liquid nitrogen

Table 5. Survival of excised shoot tips after exposure to vitrification solution and liquid nitrogen (LN) immersion.

			Survival[a] + vitrification solution	
Species/accession	Method[b]	Control	Not cooled	LN-exposed
Herbaceous species				
Arachis glabrata	A	—	54	62
	B	—	75	56
Carica papaya	A	—	96	40
	B	—	98	39
Ipomoea batatas	B	100	100	75
Phytolacca dodecandra	A	—	88	56
	B	—	75	17
Woody species				
Malus domestica				
"Golden Delicious"				
not acclimated	A	100	92	25
cold acclimated	A	100	75	20
not acclimated	B	100	50	49
cold acclimated	B	100	92	87
Prunus fruiticosa				
not acclimated	B	—	100	55
Prunus pensylvanica				
not acclimated	B	—	75	72
Vitis sp.				
"Valiant"	B	100	100	38
"Thompson Seedless"	B	100	8	0
"French Colombard"	B	100	28	0

[a] Values presented are the percentage of shoot tips that survived as assessed by regrowth in culture.

[b] Method A is according to Towill, 1990; Method B is slightly modified from that of Sakai et al., 1990.

(LN), warming, and removing the vitrification solution. The details of vitrification often vary with species and will not be extensively discussed. A key issue is the application of the cryoprotectant solution. This is usually done in two steps. Shoot tips are first exposed to a dilute solution of a permeating cryoprotectant, such as dimethylsulfoxide or ethylene glycol. They are then exposed to a concentrated solution which serves mainly to dehydrate the shoot tip and is of a composition that vitrifies during cooling. Steponkus et al. (1992) give an excellent discussion of what strategy might be used to arrive at useful solution(s) which involves an analysis of permeation and dehydration characteristics. For many materials permeation is not needed and, indeed, may be detrimental. In these cases survival with vitrification is obtained using the desiccation phase alone, although pretreatments with sugars still are required to obtain survival.

Table 6. Storage of *Mentha* sp. shoot tips in the vapor phase over liquid nitrogen (LN) after vitrification.

Storage time	% Survival of accessions (% forming shoots)[a]					
	312		573		130	
Unfrozen	100	(95)	83	(89)	100	(100)
LN, 1 hr	70	(94)	42	(70)	56	(78)
1 wk	81	(85)	13	(67)	38	(100)
1–2 mo	91	(90)	28	(83)	52	(83)
3–4 mo	68	(93)			43	(80)
7–9 mo	65	(87)	37	(67)	48	(90)
12–13 mo	80	(100)	54	(100)		

[a] Values are the percentage of shoot tips that exhibit growth; the number in parentheses is the percentage of those growing shoot tips that develop into a normal shoot.

Other issues are important in developing a practical method of cryopreservation. One is whether a given method can be applied to diversity within a germplasm collection. Some vitrification methods can be applied to a wide array of species. For example, two methods, one a method devised in my laboratory for mint and the other a slight modification of the method of Sakai et al. (1991), have given useful levels of survival in several species of mint (Table 4), in hardy woody plants, such as apple and *Prunus*, and in nonhardy plants such as papaya, potato and sweet potato (Table 5). Other modifications, mainly of the time of exposure to either the permeation phase or the dehydration phase, have increased survival percentages. Obviously, one sequence of application times does not work for all materials. *Vitis vinifera* has proven difficult to vitrify even though many variations of the procedure have been tried. A hardy grape hybrid, however, has shown survival after vitrification. The encapsulation method has been reported to give survival for some *V. vinifera* cultivars (Plessis et al., 1991). The reduced viability of shoot tips exposed to the vitrification solution (but not cooled) illustrates that injury can occur in the exposure phase.

Another issue is longevity during cryogenic storage. Cryogenic storage should allow materials to retain viability for extremely long periods of time. There is no comprehensive information about storage of shoot tips at low temperatures in plant systems. At the NSSL, samples of three accessions of mint preserved by vitrification have been held for about 1 year with no loss of viability (Table 6). These data and those in Table 7, however, illustrate a problem observed in many samples and that is the variation in survival found from time to time and from experiment to experiment. Reasons for this variation are unknown. Physiological or biochemical status of the shoot tip (or stock plant) may vary from experiment to experiment and be partially a factor. Cryoprotectant exposure and cooling and warming rates undoubtedly are injurious to some degree and, thus, the degree to which a tissue or cell recovers from these stresses during subsequent culture would introduce a source of variation, especially if the culture conditions themselves are not optimal. In addition, cellular concentrations of sugars are not defined in shoot tips and their contributions to glass formation

Table 7. Example of variation in survival among experiments using vitrification of *Mentha* sp.

Species	Control[a]		LN[a]	
M. spicata	100	(100)	91	(100)
	100	(92)	62	(87)
	100	(100)	42	(70)
M. × smithiana	100	(73)	74	(67)
	100	(91)	50	(100)
	87	(100)	23	(100)
M. dahurica	100	(83)	82	(100)
	100	(100)	72	(92)
	100	(92)	61	(100)
	83	(84)	42	(70)

[a] % survival (% shoots)

within the cell are unknown. Differences in concentrations, whether endogenous or resulting from the permeation or dehydration phases, may make some shoot tips vulnerable to minor variations in cooling or warming rates.

STATUS OF CRYOPRESERVATION

Long-term storage is an important facet of germplasm preservation. As far as I am aware, cryopreservation for clonal collections is not routinely performed. Desiccation-tolerant seed and pollen usually survive low-temperatures and probably little research is needed to apply the information to diversity in many species, particularly temperate zone fruit and nuts. Pollen would be the preferred propagule for storage of some species, for example in walnut where seed are large and have poor storage characteristics but where pollen is desiccation-tolerant. Seed storage is an advantage for other species, for example in citrus where many lines have low pollen fertility, but where seed preservation is feasible—even though many citrus species have a difficult-to-store seed, or in some grasses where seeds store well but pollen shows a desiccation sensitivity. In either case, although the genotype would not be preserved, the genes would be. Seed and pollen storage could be established and provide safe, long-term conservation until clonal cryopreservation is more reliable and applicable to diversity, both within and among collections.

The dormant vegetative bud method now has been applied to apple and should be feasible with several other temperate zone species, including the large *Pyrus* and *Prunus* collections. The process with apple is implemented at NSSL for routine preservation, but handling diversity still requires use of more than one technique. One that may prove advantageous is vitrification. Higher levels of survival were obtained in buds from tender apple lines (from *in vitro* culture) by vitrification than by the dormant vegetative bud method.

Results with vitrification obtained in this laboratory as well as those reported by others are encouraging and show the potential of the method

for long-term clonal preservation. The main advantage of vitrification is simplicity in handling and exposing samples to low temperatures, such that it can be easily done wherever a suitable cryogen is available. The major impediment to application of cryopreservation is the variable results obtained with diversity. The selection of a given method for long term conservation relates to the somewhat arbitrary decision of what is a useful survival percentage. I usually consider greater than 50% survival to be a criterion. A strategy that might be employed is to utilize a method with diverse lines and to accept the method if the survival percentage after liquid nitrogen exposure is greater than 50%. Alternative methods will be then examined for those lines with less than 50% survival. This strategy could be wasteful if a large percentage of lines fall below 50%. There is not enough experience, however, to state whether any one solution (or slight modifications of it) may have general applicability across either species or lines within a species. Obviously, lack of knowledge about permeation, cellular contents and concentrations, and dehydration tolerance and kinetics, and about differences in these traits with genotype hinders application. Cryopreservation of clonal propagules still requires considerable research and development before practical use is feasible for many species.

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