

I.2 Implementing Cryopreservation for Long-Term Germplasm Preservation in Vegetatively Propagated Species

BARBARA M. REED

1 Introduction

Vegetatively propagated fruit, vegetables, or forestry related crops are important in the agriculture of every country. Maintaining the genetic diversity of these crops is more demanding than for most seed-producing plants, because the specific genotype must be maintained. Some plants are maintained as clones because: they do not form viable seeds; seeds are short lived or do not tolerate drying; seeds are large and require too much space to store; long juvenile periods limit usefulness of seedlings; clones are heterozygous and do not produce true to type seed; select clones are more productive than seed-derived lines (Towill 1988). Vegetatively propagated crops are normally stored as clonal material; sometimes, however, seed-derived materials may be used as well. Primary collections of clonal crops are in a field or screened enclosure; however, backups for these materials are needed to provide security in case of a disease or environmental disaster. In vitro gene banks provide alternative storage for a number of crops. Active in vitro gene banks exist for temperate and tropical crops in several countries (IPGRI/CIAT 1994; Ashmore 1997; Engelmann 1999). These in vitro gene banks, however, are not ideal for a base collection, as the plantlets require repropagation at 6-month to 4-year intervals and may be lost due to contamination or technical difficulties. Cryopreservation is the preferred option for the long-term storage of clonal germplasm (Engelmann 2000). Cryopreservation may also be the best option for long-term germplasm storage of some seed-propagated species (e.g., coconut, coffee, papaya). Cryopreservation is an excellent storage method for genetic variants with special medicinal or industrial value, recalcitrant seed, rare germplasm, disease-free plants, pollen, and embryogenic cultures (Bajaj 1995; Razdan and Cocking 1997).

Cryopreservation techniques have been developed over the last 25 years and can be implemented for routine storage of germplasm (Reed et al. 2000a,b). Techniques of controlled freezing, vitrification, encapsulation-dehydration, dormant bud preservation, and combinations of these are now directly applicable with plant genotypes representing hundreds of species.

USDA-ARS National Clonal Germplasm Repository, 33447 Peoria Rd., Corvallis, Oregon 97333-2521, USA

Biotechnology in Agriculture and Forestry, Vol. 50
L.E. Towill and Y.P.S. Bajaj (Eds.) Cryopreservation of Plant Germplasm II
© Springer-Verlag Berlin Heidelberg 2002

Unfortunately, few laboratories are actually instituting the techniques. Initial implementation of cryopreservation procedures can be daunting where financial and human resources are lacking. The experimental protocols tested on one or a few genotypes in a genus will provide a starting place for storage, but may require modification before application to a wide range of germplasm such as that available in national or breeder collections. Initial steps must be taken to adapt these protocols and set up procedures for testing, screening, and ultimately storing the range of genotypes in each collection (Reed et al. 1998a).

The cooperation of government and foundation administrators is important in providing the necessary infrastructure, labor, and international collaboration necessary for the security of a base clonal collection. Interest in germplasm security at the highest levels of government is often needed to secure stable funding and provide adequate resources to realize secure long-term germplasm storage (Benson 1999b).

2 Initial Planning

Early decisions in planning for plant germplasm cryopreservation include the choice of accessions to be stored, number of each accession per storage unit, number of replicates, protocol development, location of storage, viability testing, records, and proper control groups (Fig. 1). Emphasis should be placed on selecting a secure storage site and compiling complete records needed for the recovery of plant material. Secure remote storage, duplicate locations, and secure, accurate records are all important in insuring the safety and usefulness of base collections. Evaluation of cryostored collections should be initiated to determine the longevity of plants and stability of storage conditions.

2.1 Cryopreservation and Storage Records

Storage records must be designed to link cryopreserved propagules to the original plant accession and to all information related to that original plant (passport information). Important cryogenic information must also be linked because propagules are to be retrieved 50, 100, or 500 years in the future. Each cryopreserved accession must have information on preparation, pretreatment, cryopreservation method, thawing method, and the recovery medium. Thawing methods and recovery media are especially critical to recovering the germplasm. These two items should be readily accessible in the accession database records for easy access by future scientists wishing to recover plants. Complete protocol information that is not critical to recovering plants but may be of scientific interest could be stored in a secondary database.

**DECISION FLOW CHART FOR CRYOPRESERVED
STORAGE OF CLONAL GERmplasm**

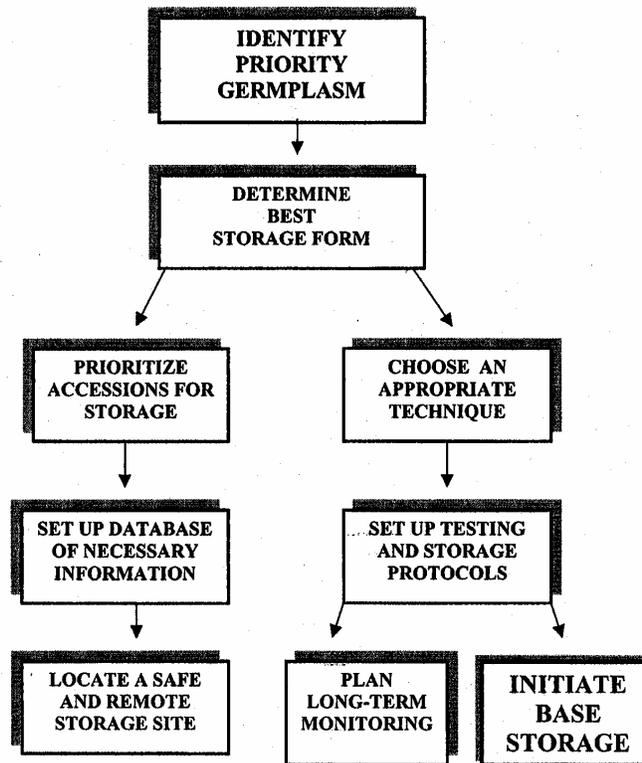


Fig. 1. Flow chart for cryopreserving clonal germplasm

2.2 Choice of Accessions

The choice of material to store will vary with the genus involved. Material presently stored in cryopreserved collections varies from randomly chosen selections to carefully chosen clones that represent morphological and genetic variation as well as specific geographic criteria, sometimes referred to as "core" collections (IPGRI/CIAT 1994; Reed et al. 1998a,b; Golmirzaie and Panta 2000). Priority accessions will vary with the crop, country, and facilities available. For any collection early attention should be paid to "at risk" accessions. These "at risk" accessions may be those susceptible to diseases, climatic conditions, insect damage, or other environmental conditions that increase the

chance of loss from a collection. Plant material from areas where changes in farming practices or clearing of wild land may cause loss of local cultivars might also be placed in this category. Plants listed as “threatened” or “endangered” in their wild or native range should be conserved.

2.3 Storage Form

Seed, pollen, shoot apices, dormant buds, excised embryonic axes, zygotic or somatic embryos may be the form of choice depending on the species involved (Table 1). Pollen provides a way to store part of the genetic diversity in a form

Table 1. Storage forms for cryopreserved germplasm

Best Storage Form	Plant groups	Advantages	Disadvantages
Seed	Small seeded, desiccation tolerant, cold tolerant, not clonally propagated	Easy to use for appropriate seed types (orthodox)	Not useful for large, cold sensitive or desiccation intolerant seeds (recalcitrant) or clonally propagated plants
Pollen	Many	Easy for many plant genera, useful for plant breeding	Preserves only half of the genome
Dormant buds	Temperate woody plants	Dormant buds are readily available from field genebanks	Degree of cold hardiness varies with season and genotype, requires more storage space than other techniques, requires grafting and budding expertise for recovery
<i>In vitro</i> shoot tips	Many	Available at any time of year, easy to manipulate physically and physiologically	Techniques are not developed for all plants, requires a laboratory and skilled workers
Embryogenic cultures	Various plant groups	Callus is generally easy to cryopreserve	Not all plants produce somatic embryos, techniques are not broadly applicable across species/accessions
Embryonic axes	Some marginally recalcitrant seed types	Easy to remove and process	<i>In vitro</i> systems are needed to recover whole plants
Zygotic embryos	Some marginally recalcitrant seed types	Removal of the seed coat may improve recovery	Time consuming and often technically difficult

easily accessible and useable by plant breeders and may be a quick first step for starting a cryopreserved gene bank (Towill and Walters 2000). Dormant buds of cold hardy plants that can be grafted or budded may be a good option for temperate trees and shrubs (Forsline et al. 1998; Niino et al. 2000). Shoot apices are successfully recovered in vitro for many plant types, require little room for storage, and preserve the exact genotype of both tropical and temperate plants (Reed and Chang 1997; Benson 1999a). For some species the storage of excised embryonic axes provides the only long-term storage option, while for others the production of somatic embryos provides large amounts of material for preservation (Engelmann 2000).

2.4 Numbers, Numbers, Numbers

The number of propagules needed for storage will vary with the plant type and the recovery potential of the accession. Ideally, enough propagules should be stored in each container to produce several living plants and enough containers to allow for several recoveries over time. If periodic testing is anticipated, then additional vials should be included for that purpose so that the vials in the base collection remain untouched. Storage of somatic embryos could include large numbers of vials with large numbers of embryos due to their normally prolific production. Dormant bud wood is easy to collect; however, it may require more storage space so the number of replicates may be limited. In vitro shoot tips are relatively easy to produce but require excision before freezing, thus limiting the number available for storage at one time. At the extreme, zygotic embryonic axes require removal from the seeds, which may be difficult, and the seed may also be limited in availability, so few may be available for storage at one time.

2.5 Protocol Testing

The amount of initial protocol testing varies greatly from facility to facility. If methods development involved several species and cultivars, then less testing may be needed at the storage phase. There are two schools of thought on the amount of testing needed for each accession prior to cryopreserved storage. The first uses several storage trials and their controls as the testing phase. In this scenario a laboratory might store five vials of 10 propagules and use one as the control. If the control recovers with a high percentage, then four vials (40 propagules) remain in storage. If the control shows low recovery, then another group of vials should be stored to increase the number of viable propagules in storage (Schafer-Menuhr 1996). The second method stores 20–50 propagules in vials and thaws them after short storage. If the regrowth percentage is greater than a set percentage (i.e., 40%–50%), then base-storage shoot tips are processed and stored. If the percentage is lower, improvements are made to the culture or cryopreservation protocols to improve performance before storage (Reed et al. 1998a; Golmirzaie and Panta 2000).

2.6 Viability Testing and Controls

Both internal and external controls are needed for any storage format. Internal controls include regrowth of plants at critical stages of the protocol. For shoot tip cryopreservation this might occur after dissection from the mother plant, after cryoprotection, and after liquid nitrogen (LN) exposure. If cryopreserved vials are transferred from one storage container to another or from one location to another, a control vial should be regrown to monitor the effect of those transfers. No studies are available on the effect of insertion and removal of samples from the dewar on viability of stored propagules; however, frequent removal and replacement of storage racks may impact the storage life of cryopreserved plants. Proper techniques for inserting and removing vials from storage containers should be developed before storage begins. Improper handling at the storage or removal point can easily kill propagules.

2.7 Storage Location

The location of the storage dewar is very important for long-term storage. As a container for a base collection of important germplasm, the dewar should be located at a site remote from the field gene bank. The storage site needs to be secure and under the control of dependable management to insure that LN is added to the dewar as needed. Alarms should be installed to monitor the LN level in the dewars to insure constant temperature in the storage container. A remote storage location is especially important when field collections are at risk due to environmental or political problems (natural disasters, severe weather, civil unrest).

2.8 Genetic Stability

Limited data is available on the long-term storage of clonally propagated plants. Plant tissues were first stored in the 1970s and initial tests with short-term storage of pea (2 years), strawberry (10 years) and potato (1 year) shoot tips indicated no loss of viability (Haskens and Kartha 1980; Kartha et al. 1980; Towill 1981). With strawberry, fluctuations in viability were noted after various LN storage periods, but the changes were attributed to differences in the physiological status of the shoot tips used in different experiments, not to LN storage. Three generations of field-grown strawberries produced runners, exhibited vigorous growth, and produced normal fruit (Kartha 1985). Normal potato plants were recovered from cryopreserved shoot tips (Towill 1981) and strawberry plants of 10 cultivars evaluated in the field following LN storage all produced normal leaves, flowers and fruit (Reed and Hummer 1995; Reed and Chang 1997). An 8-year study of dormant buds from mulberry cv. Kenmochi found no change in shoot formation with increased length of -135°C storage (Niino et al. 2000). Nearly 74% of 376 mulberry cultivars preserved for 5 years had 50% or more bud regrowth when grafted onto 1-year-old

seedling rootstocks. Only 6% had regrowth of less than 30% of buds (Niino et al. 2000).

Genetic change of plants grown in tissue culture is termed somaclonal variation (Larkin and Scowcroft 1981). These variations are most common in plants regenerated from single cells or callus. Variation occurs, but is not common, in micropropagated or shoot tip propagated plants derived from existing shoot tips. If care is taken in the clonal multiplication of plant materials, the frequency of variation in propagules should be similar to that of field-propagated plants. Genotypes that often produce mutations/sports will also produce them *in vitro*. Familiarity of germplasm scientists with the plants in question can assist in identifying variable genotypes so that they can be monitored carefully prior to the cryopreservation process. The establishment of descriptor characteristics for each genotype allows for roguing of off types upon regrowth. Any genotype with known variability should be flagged in the database and propagules carefully selected for storage. Field and genetic analyses are needed to determine whether instability is a problem; however, several studies have shown little need for concern (Towill 1990; Harding 1991, 1994; Harding and Benson 1993, 1994; Kumar et al. 1999).

2.9 Physical and Biochemical Stability

Another factor relating to viability of propagules stored for long periods involves the physical and biochemical stability of the system. Vitrified solutions are known to crack from physical shocks or in response to certain warming procedures. Thermal stress-induced fractures of biological materials may cause serious damage to stored samples. Slow cooling rates minimize thermal stress from non-uniform temperature distribution, and cryoprotectants contribute to reduce stress by changing the microstructure of the ice formed (Gao et al. 1995). Fractures can also occur as random events during cryopreservation (Najimi and Rubinsky 1997). Fractures typically occur in large organs such as whole seeds and are less common in cell suspensions and shoot tips. Most reports of physical cracking are with animal organs (liver slices, veins, and arteries) rather than plant specimens.

The stability of vitrified biological materials (liposomes and red blood cells), held at temperatures above the glass transition, was predicted to decrease by a factor of 10 for every 15°C rise in temperature (Sun 1998). As a glassy (vitrified) system cools toward its glass transition temperature, processes become increasingly slow or nearly arrested because of high viscosity, which also slows molecular movement. Additional studies of the relationship between the glass transition temperature and the stability of cryopreserved organisms are needed to adequately predict storage life of biological collections (Sun 1998).

Cryopreserved samples held in dewars are exposed to warming and cooling cycles as other samples are added or removed. There are no studies that quantify the effects of these temperature variations on storage life. The stability principles discussed above could be applied to this phenomenon

and with the resulting hypothesis that these fluctuations in temperature would have an impact on the storage life of a cryopreserved sample. It is not known if stability varies among the various procedures used to cryopreserve a propagule.

Biochemical stability of cryopreserved plant cells is well documented through the analysis of cell cultures that produce secondary products (Chen et al. 1984a,b; Yoshimatsu et al. 2000). Studies of cell and shoot tip cultures also suggest that the stability of these systems is maintained by cryopreservation (Harding 1991, 1994; Harding and Benson 1993, 1994).

3 Implementation

Curators, with teams of expert advisors such as crop germplasm committees, will need to determine the amounts and types of germplasm to be stored based on plant characteristics. A rough measure of the number of propagules needed should be based on the expected percent recovery following cryopreservation, the ease of regrowth of the plant, and the number of times samples will be removed from storage. For most clonally propagated crops recovery of five or more plants from a vial would provide adequate material for micropropagation. If the control recovery were 50% or more, then 10 shoot tips per vial would be adequate for long-term storage. If more individuals are desired or the recovery percentage is lower, 25 shoot tips per vial might be warranted. Most accessions could be stored as four or five vials, thus allowing four or five uses over 100 years. Any accessions used for viability testing over time should include more vials. One test vial for each 5–10 years of proposed storage might be a good guideline (i.e., 10–20 vials for testing a clone over 100 years). Designated accessions should be used for viability tests, so only a few genotypes out of hundreds would be stored in larger quantities. These test accessions should have high recovery percentages so any losses of viability over time would be readily apparent.

3.1 Existing Cryopreserved Storage

Cryopreserved storage of important crop plants has begun in many countries throughout the world. The storage form varies with the crop, facilities, and expertise available (Table 2). Additional genera (mint, strawberry, sweet potato, and taro) are currently being tested prior to LN storage, and while not yet officially stored, they will be in the near future. Cryopreserved storage comes in many forms and involves many techniques with varied advantages (Table 1).

Cryopreservation of *in vitro* shoot tips is frequently recommended because they are easy to multiply, available any time of year, easy to manipulate physically and physiologically, and can be recovered in culture. While in

Table 2. Cryopreserved collections of clonally propagated plant germplasm stored as dormant buds, *in vitro* shoot tips, or excised embryonic axes

Taxon	Country/Institute	Technique	Number Accessions/Replicates
<i>Dormant Buds</i>			
Apple	USA (NSSL)	E-D + CF	2100 accessions
Elm	France (AFOCEL)		101 accessions
Mulberry	Japan (NIAR)	CF	45 accessions
<i>In Vitro Shoot tips</i>			
Apple	China (CI)	CF/E-D	20 accessions/50 shoot tips each
Blackberry (species and cultivars)	USA (NCGR)	CF	17 accessions/100 shoot tips
Cassava	Columbia (CIAT)	E-D	95 accessions/30 shoot tips
Grass	USA (NCGR)	CF/E-D	10 selections/100 shoot tips
Hops	USA (NCGR)	CF	2 accessions/100 shoot tips
Pear	USA (NCGR)	CF	106 accessions/100 shoot tips
Potato	Germany (DSM/FAL)	Droplet	219 accessions/40-350 shoot tips
	Peru (CIP)	Vit	197 accessions/250 shoot tips
Currant/ Gooseberry	USA (NCGR)	Vit	5 accessions/100 shoot tips each
	Scotland (UAD)	E-D	31 accessions/25-30 shoot tips
<i>Embryonic Axes</i>			
Almond	India (NBPGR)	D-FF	29 accessions/20 axes each
Citrus	India (NBPGR)	D-FF	12 accessions of 6 species/50-100
Hazelnut	USA (NCGR)	D-FF	5 species/100-300 axes each
Jackfruit	India (NBPGR)	D-FF/Vit	3 accessions/25 axes each
Litchi	India (NBPGR)	D-FF	2 accessions/30 axes each
Tea	India (NBPGR)	D-FF	85 accessions/25 axes each
Trifoliolate Orange	India (NBPGR)	D-FF	1 accession/30 axes each

Facilities: AFOCEL-Association Forêt-Cellulose; CI - Changli Institute of Pomology; CIAT - International Center for Tropical Agriculture; CIP - International Center for the Potato; DSM/FAL - Deutsche Sammlung von Mikroorganismen und Zellkulturen/Institute für Pflanzenbau, Bundesforschungsanstalt für Landwirtschaft; NBPGR - National Bureau of Plant Genetic Resources. NCGR - National Clonal Germplasm Repository-Corvallis; NIAR - National Institute of Agrobiological Resources; NSSL - National Seed Storage Laboratory; UAD - University of Abertay-Dundee.

Techniques: CF - Controlled Freezing; D-FF - Dehydration-Fast Freezing; Droplet - Droplet Freezing; E-D - Encapsulation-Dehydration; Vit - Vitrification.

in vitro systems require some additional input before storage, the ease of recovering and propagating the shoot tips has many advantages. Storage space required is very small so many accessions can be stored in a small dewar. Shoot tips taken directly from source plants are less desirable as they may carry contaminants, are more difficult to handle, and are less uniform in their recovery from storage.

Embryonic axes are stored for some vegetatively propagated species and for some recalcitrant seeds. Removal and drying of the axes is time consuming but not usually difficult, and the resulting propagules can be stored in a small space and recovered *in vitro*. Embryonic axes are best used for preserving the genetic diversity of a species when seeds cannot be stored.

Somatic embryos are a good form for cryopreserved storage of selected genetic lines. Many forest-tree production systems depend on embryogenic

callus cultures to produce plants from somatic embryos for field-testing. Over 5000 genotypes of 14 conifer species are cryostored in one facility alone. Storage of these cultures allows for use of an embryogenic culture line after an extended period of testing in the field (Cyr 2000). Continued subculturing of embryogenic cultures can lead to somaclonal variation or loss of embryogenic potential, so cryopreservation of important lines from freshly initiated callus is a high priority.

Pollen storage in liquid nitrogen is an important tool for plant breeders. Pollen storage requires drying pollen to a low moisture content. Many plant pollens are easily stored and can be available at any time of year for crosses that are not possible with fresh pollen due to timing constraints (Inagaki 2000; Towill and Walters 2000).

4 Conclusions

The first cryopreserved collections of clonally propagated germplasm are now established for at least 20 economically important crop genera. Cryopreservation is an ideal backup for collections in field gene banks, but is not intended to be the only form of a clonal plant accession. Bull semen was first cryopreserved in the 1940s and the production of healthy calves from semen stored for 60 years shows the reliability of this storage method. Long-term cryostored-plant viability studies monitoring the recovery of designated plant accessions will provide additional information on the stability and viability of this storage form.

Curators planning cryostorage for their crops should first determine the most practical technique for their facility and the crops involved. Off-site storage should be arranged well in advance with a trusted facility. Information management should be an important consideration because the recovery of plants from cryostorage will depend on knowing the proper techniques for thawing and regrowth of each accession.

When considering cryopreservation for long-term storage of germplasm collections, curators should determine the best storage form for the crop in question, prioritize accessions to be stored, determine the best technique to apply to these accessions, set up a database for needed information, make arrangements for offsite storage, plan long-term monitoring, and finally initiate storage.

References

- Ashmore SE (1997) Status report on the development and application of in vitro techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome, Italy

- Bajaj YPS (1995) Cryopreservation of plant cell, tissue and organ culture for the conservation of germplasm and biodiversity. In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry. Cryopreservation of plant germplasm I*, vol 32. Springer, Berlin Heidelberg New York
- Benson EE (1999a) Cryopreservation. In: Benson EE (ed) *Plant conservation biotechnology*. Taylor and Francis, London
- Benson EE (ed) (1999b) *Plant conservation biotechnology*. Taylor and Francis, London
- Chen THH, Kartha KK, Constabel F, Gusta LV (1984a) Freezing characteristics of cultured *Catharanthus roseus* (L). G. Don cells treated with dimethylsulfoxide and sorbitol in relation to cryopreservation. *Plant Physiol* 75:720-725
- Chen THH, Kartha KK, Leung NL, Kurz WGW, Chatson KB, Constabel F (1984b) Cryopreservation of alkaloid-producing cell cultures of periwinkle (*Catharanthus roseus*). *Plant Physiol* 75:726-731
- Cyr DR (2000) Cryopreservation: roles in clonal propagation and germplasm conservation of conifers. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome
- Engelmann F (ed) (1999) Management of field and in vitro germplasm collections. Proceedings of a consultation meeting, 15-20 Jan 1996, CIAT, Cali, Columbia. International Plant Genetic Resources Institute, Rome
- Engelmann F (2000) Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome, Italy
- Forsline PL, Towill LE, Waddell J, Stushnoff C, Lamboy W, McFerson JR (1998) Recovery and longevity of cryopreserved dormant apple buds. *J Am Soc Hortic Sci* 123:365-370
- Gao DY, Lin S, Watson PF, Critser JK (1995) Fracture phenomena in an isotonic salt solution during freezing and their elimination using glycerol. *Cryo Lett* 32:270-284
- Golmirzaie AM, Panta A (2000) Advances in potato cryopreservation at CIP. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome
- Harding K (1991) Molecular stability of the ribosomal RNA genes in *Solanum tuberosum* plants recovered from slow growth and cryopreservation. *Euphytica* 55:141-146
- Harding K (1994) The methylation status of DNA derived from potato plants recovered from slow growth. *Plant Cell Tissue Organ Cult* 37:31-38
- Harding K, Benson E (1993) Biochemical and molecular methods for assessing damage, recovery and stability in cryopreserved plant germplasm. In: Grout BWW (ed) *Genetic preservation in vitro*. Springer, Berlin Heidelberg New York
- Harding K, Benson EE (1994) A study of growth, flowering, and tuberisation in plants derived from cryopreserved potato shoot-tips: implications for in vitro germplasm collections. *Cryo Lett* 15:59-66
- Haskens RH, Kartha KK (1980) Freeze preservation of pea meristems: cell survival. *Can J Bot* 58:833-840
- Inagaki M (2000) Use of stored pollen for wide crosses in wheat haploid production. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome
- IPGRI/CIAT (1994) Establishment and operation of a pilot in vitro active genebank. Report of a CIAT-IBPGR Collaborative Project using cassava (*Manihot esculenta* Crantz) as a model. International Plant Genetic Resources Institute and International Center for Tropical Agriculture, Rome
- Kartha KK (1985) Meristem culture and germplasm preservation. In: Kartha KK (ed) *Cryopreservation of plant cells and organs*. CRC Press, Boca Raton
- Kartha KK, Leung NL, Pahl K (1980) Cryopreservation of strawberry meristems and mass propagation of plantlets. *J Am Soc Hortic Sci* 105:481-484

- Kumar MB, Barker RE, Reed BM (1999) Morphological and molecular analysis of genetic stability in micropropagated *Fragaria* \times *ananassa* cv. Pocahontas. *In Vitro Cell Dev Biol Plant* 35:254-258
- Larkin PJ, Scowcroft WR (1981) Somaclonal variation - a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197-214
- Najimi S, Rubinsky B (1997) Non-invasive detection of thermal stress fractures in frozen biological materials. *Cryo Lett* 18:209-216
- Niino T, Seguel I, Murayama T (2000) Cryopreservation of vegetatively propagated species (mainly mulberry). In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome
- Razdan MK, Cocking EC (1997) Biotechnology in conservation of genetic resources. In: Razdan MK, Cocking EC (eds) *Conservation of plant genetic resources in vitro*. Science Publishers, Enfield
- Reed BM, Chang Y (1997) Medium- and long-term storage of in vitro cultures of temperate fruit and nut crops. In: Razdan MK, Cocking EC (eds) *Conservation of plant genetic resources in vitro*, vol 1. Science Publishers, Enfield
- Reed BM, Hummer K (1995) Conservation of germplasm of strawberry (*Fragaria* species). In: Bajaj YPS (ed) *Biotechnology in agriculture and forestry, cryopreservation of plant germplasm I*, vol 32. Springer, Berlin Heidelberg New York
- Reed BM, DeNoma J, Luo J, Chang Y, Towill L (1998a) Cryopreservation and long-term storage of pear germplasm. *In Vitro Cell Dev Biol Plant* 34:256-260
- Reed BM, Paynter CL, DeNoma J, Chang Y (1998b) Techniques for medium-and long-term storage of (*Pyrus* L.) genetic resources. *Plant Gen Res Newslett* 115:1-4
- Reed BM, Brennan RM, Benson EE (2000a) Cryopreservation: an in vitro method for conserving *Ribes* germplasm in international gene banks. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome, Italy
- Reed BM, DeNoma J, Chang Y (2000b) Application of cryopreservation protocols at a clonal genebank. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome, Italy
- Schafer-Menuhr A (1996) Refinement of cryopreservation techniques for potato. Final report for the period Sept 1991-1993 Aug 1996. International Plant Genetic Resources Institute, Rome
- Sun WQ (1998) Stability of frozen and dehydrated cells and membranes in the amorphous carbohydrate matrices: the Williams-Landel-Ferry kinetics. *Cryo Lett* 19:105-114
- Towill LE (1981) *Solanum etuberosum*: a model for studying the cryobiology of shoot-tips in the tuber-bearing *Solanum* species. *Plant Sci Lett* 20:315-324
- Towill LE (1988) Genetic considerations for germplasm preservation of clonal materials. *HortScience* 23:91-95
- Towill LE (1990) Cryopreservation of isolated mint shoot tips by vitrification. *Plant Cell Rep* 9:178-180 (Vitrification of shoot tips)
- Towill LE, Walters C (2000) Cryopreservation of pollen. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome
- Yoshimatsu K, Touno K, Shimomura K (2000) Cryopreservation of medicinal plant resources: retention of biosynthetic capabilities in transformed cultures. In: Engelmann F, Takagi H (eds) *Cryopreservation of tropical plant germplasm. Current research progress and application*. Japan International Research Center for Agricultural Sciences/International Plant Genetic Resources Institute, Rome