Chapter 1
Cryopreservation—Practical Considerations

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1.1 Introduction

Over the past 40 years individual scientists developed and tested a range of cryopreservation techniques for preserving plant cells and tissues, but routine storage of plant germplasm other than seeds in liquid nitrogen (LN) is a relatively new practice (Engelmann 2004). Controlled rate cooling, vitrification, encapsulation dehydration, dormant bud preservation, and combinations of these techniques are now directly applicable for plant genotypes representing hundreds of species. An increasing number of laboratories are instituting these techniques for secondary genetic resource preservation. Cryopreservation is commonly used for storage of suspension and callus cultures, and is now becoming more useful for organized tissues. Many protocols are available in the literature; however they are often difficult to interpret for everyday use (Towill 2002). The procedures themselves are not difficult, but initial implementation of cryopreservation procedures can be daunting where financial and human resources are limited. Often the initial steps to define protocols and set up procedures for testing, screening, and ultimately storing the range of genotypes in each collection are

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*

beyond the scope of many plant laboratories (Reed et al. 1998b). Cryopreserved storage is now in place for many important collections of algae, cell cultures, and organized plant tissues, and techniques are now available for many more. The aim of this book is to provide starting points for a wide range of plant types and to make cryopreservation an easier technique to use for long-term storage by providing step-by-step instructions. The experimental protocols presented here were developed for, and in some cases already used with a range of genotypes. They will provide a starting point for application to additional germplasm such as that available in national or breeder collections. The protocols were provided by the scientists who developed them, and the underlying literature was reviewed by expert scientists working with each particular plant system.

1.2 Planning for a Liquid Nitrogen Stored Collection

There are many practical considerations that need to be resolved before storage is initiated. The actual storage process in the laboratory is the culmination of a planning process that is as important as the technique used. Storing a collection involves not only the proper cryopreservation technique, but also a number of practical issues. Because cryopreservation is labor intensive, the order of plants must be prioritized for storage. Preplanning for storage vessels, storage location, number of replicates, viability testing and types of records to be kept are all vital to successful storage. A secure storage site and complete records are vital for the successful recovery of stored plants when needed, perhaps far into the future. Long-term evaluation of the stored plants is also a necessary part of the planning process. Cryopreservation is valuable as a secondary backup for primary collections of clonally-propagated plants or as a secure system for maintaining the embryogenic potential or metabolic potential of important cultures. In either case, cryopreservation as a secure backup for living plant material is invaluable.

1.2.1 Plant Materials

Decisions concerning which plants to store will vary with the plant type and the reason for storage. Curators need to make decisions on which plants to store based on their knowledge of plant vulnerability. Priority plants may be based on plant availability or on specific criteria. Criteria used for choosing plants may be to represent important morphological and genetic variation, geographic criteria, secondary metabolite production, or

risk factors. These plant materials may make up a core collection that represents a larger assemblage of plants (Golmirzaie and Panta 2000; IPGRI/CIAT 1994; Reed et al. 2003). At-risk plant materials include endangered species or those susceptible to diseases, climatic conditions, insects or other environmental conditions that might lead to death of the plant. Clearing of wild areas, changes in farming practices, loss of crop diversity, heirloom varieties, and wild ancestors might put other plant types at risk and these may also be on the priority list.

Individual curators or scientists need to make these decisions based on their particular plant collection. In the case of a collection where all accessions in the facility will be conserved, storage can progress in whatever order the plants are available, but prioritization can still take place. In this type of situation, a triage system can be implemented. The plants can be tested with the chosen technique as they are available, and stored if the initial test meets a standard criterion. Those that are not up to standards can be put aside for further research while those that cryopreserve well are stored. In the storage of any collection curators should not allow the poor response of one genotype to stop or delay the testing and storage of the rest of the collection.

Cryopreservation can be thought of as either a primary or a secondary storage form. For embryogenic cultures that may lose their capacity for embryo formation with time, cryopreservation provides primary storage, with cultures revived and used to produce more embryos at a later date. Cryopreservation used in the conservation of plant genetic resources is normally seen as a secondary storage form designed as a secure backup for living collections. In general, cryopreservation is not considered a sole source of clonal plant preservation because evaluation and identity verification require a growing plant.

Often the decision of which plant to store depends on the growth condition of the plants in question. General experience shows that plants that are diseased or not thriving for any reason are generally poor candidates for cryopreservation. Plants or cultures should be in an optimal growth phase, and dormant materials should be fully dormant, and where appropriate, fully cold acclimated.

1.2.2 Storage Records

Accurate records are vital to a cryogenic storage plan. Storage records must link cryopreserved propagules to all information related to the original plant (passport information). Important cryogenic information must be linked to standard plant records because propagules may not be retrieved

until far into the future. Information on pregrowth of the plant material, pretreatments, cryopreservation protocol, thawing method, and the recovery medium are vital to recovering living plants. Future scientists wishing to recover plants will require complete rewarming methods and recovery media, so these records must be readily accessible. Complete protocol information that is not critical to recovering plants, but may be of interest, could be stored as well. A logbook kept with the Dewars should be a first record of all storage with a backup in a computerized database.

Some items needed for the database

1. Accession identifying number or code
2. Accession name
3. Dewar number
4. Cane or rack location
5. Number of vials stored
6. Storage date
7. Standard culture medium (reference)
8. Preconditioning conditions
9. Preculture conditions
10. Osmoprotection
11. Cryopreservation technique (reference to the technique)
12. Cryoprotectant
13. Rewarming protocol
14. Regrowth medium
15. Data from control regrowth

1.2.2.1 Sample Labeling

Labeling and numbering of stored accessions is a critical issue especially for large collections. Mistakes in numbering and naming occur despite the best of intentions. Double checking of numbering and labeling are needed to offset the expected amount of human error possible in these types of situations. A protocol should be developed to insure accuracy in labeling and computer entry before any accessions are stored. Labels should include accession number, name, and date as a minimum. A reference to the storage or rewarming technique would be useful if there is room on the label. Bar-coded labels are now available for cryovials.

1.2.2.2 Dewar Organization

Organization of the storage Dewar is an important but little thought of detail in cryopreserved storage. Grouping of related samples may make recovery

of items easier. Clear labeling and numbering of canes or boxes is important for easy retrieval of specimens. Long-stored accessions should not be combined with those that will be frequently sampled, to avoid any warming of the vials. If storage is to be at two locations, samples should be divided when initially placed in the Dewar, as moving samples from one Dewar to the next can potentially decrease viability.

There are many factors to consider when choosing a storage Dewar. Seed samples that are rewarmed at room temperature can be safely stored in boxes or trays in Dewars. Biological samples that require fast rewarming (i.e. in 40°C water) require fast transfer from LN into the warming solution. Finding a sample in a box or tray held under LN may be difficult, and storage on canes may be the best solution.

1.2.3 Storage Form

Cryopreservation techniques are well developed for most types of plant propagules. The chosen storage form may be seed, pollen, shoot apices, dormant buds, excised embryonic axes, zygotic or somatic embryos, callus or cell cultures depending on the species involved. The form is of course dependent on the reason for preserving a plant collection. Plant breeders store pollen as a way to keep part of the genetic diversity of a crop in a form easily accessible and directly useable. Dormant bud cryopreservation is best used with cold-hardy woody plants that can be grafted or budded (Forsline et al. 1998; Niino et al. 2000). *In vitro*-grown shoot apices can be stored in a small space, and can preserve the exact genotype of both tropical and temperate plants (Benson 1999; Reed and Chang 1997). Orthodox seeds (seeds that survive at low moisture contents) in general respond well to LN storage, but for species with recalcitrant seeds the storage of excised embryonic axes often provides the only long-term storage option. Callus tissues and somatic embryos provide large amounts of material for preservation and are often easy to store (Engelmann 2000). Cryopreservation of algae and lower plants is useful for both environmental and experimental purposes (Day et al. 1999).

1.2.4 How Many to Store

Determining how much plant material to store depends on the storage purpose and plant availability. The recovery potential of the plants may also be an important factor in making this determination. With the exception of some pollen and orthodox seeds, vials of cryopreserved samples can not be rewarmed and then returned to LN. Generally each container or vial should

hold enough propagules to produce several living plants or cultures and enough vials should be stored to recover the accession several times. Additional vials of some accessions should be included for periodic viability testing. The plant form may also influence the amount of material stored. Dormant buds require more space than small vials of somatic embryos or cell cultures. Easily regenerated cultures may require fewer vials than difficult-to-grow accessions. Zygotic embryos require removal from seeds, limiting the number that can be stored at one time. The recovery potential of a stored accession is an important factor, but one that is often difficult to address. A formula for determining the number of propagules needed to obtain long-term recovery of plant materials in culture was proposed by Dussert et al. (2003) based on binomial distribution using the number of controls tested, the recovery of those control plants, the number stored, and the probability of recovering one growing sample. Calculations of this kind can be used to determine how many propagules to store, how many rewarmed vials are required to recover growing plants, and how many times a sample can be retrieved. The number of propagules stored may vary from fewer than 100 for dormant buds to many thousands for pollen grains. The final number stored will be based on the planned future use of the material, the ease of storage, the expected success of the technique, the availability of propagules and the characteristics of the plant.

1.2.5 Protocol Testing

During the development of cryopreservation protocols extensive testing was needed to develop workable techniques for new types of plants. With the developments of the last 15 years it is now possible to take a previously developed protocol and apply it to new plant materials with little or no modification (Reed 2005; Reed et al. 2006). A standard method can often be applied to a new plant group with only minor modifications and limited testing. It is possible to do preliminary tests with 3-5 genotypes and, if successful, apply the technique to the remainder of the collection. This can be approached in several ways. Curators can begin storage of the collection with small amounts of each accession and repeat the process several times to reach the desired amount of material in storage (Schafer-Menuhr 1996). Another approach is to test the material in question and judge its suitability for storage (Reed et al. 1998a). For successful accessions all the plant material can be prepared for storage. Accessions with low recovery will require improvement in the plant materials or modifications of the technique to improve recovery following LN exposure. Protocols for most plant forms are found in the second section of this book.

1.2.6 Storage Controls

Each protocol has critical stages that can be used to indicate the viability of the propagule as it moves toward storage in LN. Testing at these points in the protocols will provide important information for future storage. For example with shoot tip cryopreservation it is important to monitor viability after dissection from the mother plant, preconditioning or preculture, cryoprotectant exposure, and LN exposure, by growing explants directly following each step. With shoot tips, ten would be regrown after each step. Significant decreases in recovery following a step in the protocol would indicate where changes need to be made for successful storage. Transfer of storage vials from LN in one container to another or from one location to another should be carefully monitored, as improper handling can result in loss of viability. Removal of samples from a cane, tray or drawer in a Dewar must be managed to keep the vials under LN until properly rewarmed. Even short exposure to warm conditions is detrimental to most cryostored plants.

1.2.7 Recovery

Recovery of viable propagules (growing shoots, germinating pollen, multiplying cells) is the ultimate goal of all cryopreserved storage. Development of the best rewarming techniques and growth media are crucial to the success of any protocol. For protocols that include an *in vitro* culture phase, it is vitally important to optimize the recovery medium for the plant in question. Recovery of true-to-type plants and actively growing cultures requires the best regrowth conditions. Suboptimal environmental conditions or growth media may limit recovery even though the cryopreservation procedure was done correctly. Recovery of clonally propagated shoots should be without a callus phase, through the optimization of both the cryopreservation protocol and the recovery stages. Studies have found that the removal of auxins from the recovery medium is sometimes required to eliminate or reduce callus formation during the regrowth phase (Chang and Reed 1999). Testing of genetic stability can be instituted at any step; however, studies to date have shown no instability in cryopreserved samples. A large number of studies using morphological, cytological, biochemical and molecular techniques indicate that stability is the norm (Harding 2004; Harding and Benson 2000; Harding et al. 1997). In all cryopreservation protocols the regrowth of propagules is an important factor. Regrowth of organized shoots without a callus component provides safe recovery of stored materials. Storage in LN should result in fewer mutations than materials held either as plants in field collections, or as long-term *in vitro* cultures.

1.3 Standard Protocols

Three standard protocols are used as the basis for most plant cryopreservation. Each has some basic steps that can help make them effective for all users. The choice of technique is related to the needs of the facility involved. Personnel, equipment, expertise, plant type, and available facilities may influence which technique is most appropriate (Table 1.1).

Table 1.1 Advantages and disadvantages of some commonly used cryopreservation techniques. (From Reed 2001)

Technique	Advantages	Disadvantages
Controlled rate cooling	Stability from cracking, relatively nontoxic cryoprotectants, takes little technician time	Requires equipment, low applicability to tropical species
Vitrification	No special equipment needed, fast procedure, fast recovery	Vitrification solutions are toxic to many plants, cracking is possible, requires careful timing of solution changes
Encapsulation dehydration	No special equipment needed, nontoxic cryoprotectants, simple thawing procedures, fast recovery	Requires handling each bead several times, some plants do not tolerate the high sucrose concentrations
Dormant bud preservation	Easy, useful for many temperate tree species	Requires freezing equipment, larger storage space, recovery requires grafting or budding, works best in cold temperate regions

Controlled rate cooling (discussed in Chap. 5) was the first technique developed. In most cases it is dependent on cold acclimation of the source plants (Chang and Reed 2000), either *in vitro* or *in vivo*. Many types of temperate plants can be cryopreserved with this method using a relatively standard technique (Chaps. 6, 8, 9, 11, 15). It is an excellent technique to use for storing large plant collections because large numbers of samples can be processed at one time and it takes little technician time. Dormant bud techniques normally involve a controlled rate cooling step (Chap. 15).

PVS2 vitrification (Sakai et al. 1990) is commonly used and is effective for both temperate and tropical plants (discussed in Chap. 3). It requires a preconditioning stage for the source plants, preculture, and osmotic conditioning before PVS2 treatment (Chaps. 11–15). Timing of the PVS2 treatment is critical to the success of the procedure.

Encapsulation dehydration (Dereuddre et al. 1990) is suitable for most plant types (discussed in Chap. 4). It requires preconditioning or preculture, osmotic conditioning and desiccation to ~20% moisture content (Chaps. 7, 11–15).

Transferring protocols from one laboratory to another is often problematic. However, most protocols can be successfully completed if care is taken to follow protocols to the letter (Reed et al. 2004). Careful attention to the details of the protocols provided in this book should provide good starting points for most plant materials. Simplified slide presentations of the three most used techniques are demonstrated on the Web site: http://www.ars-grin.gov/cor/cryo.html.

1.4 Conclusions

Cryopreservation is now a viable option for storage of plant cells, tissues, seeds and embryos. Consideration of the details of storage will make the cryopreserved collections both useful and more valuable. Although the particulars of each stage of planning will vary with the institution, the general principles apply to all types of storage. By carefully considering the materials to use, storage records, storage form, amounts needed, and protocols available, each facility can develop a useful storage system for important plant materials. The protocols in this volume, and the general planning considerations outlined in this chapter, should provide starting points for most plant scientists who wish to cryopreserve a cell or shoot culture, dormant bud, pollen, or seed.

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