Barbara M. Reed
Editor

Plant Cryopreservation

A Practical Guide

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ISBN 978-0-387-72275-7 e-ISBN 978-0-387-72276-4

Library of Congress Control Number: 2007935104

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Printed on acid-free paper.

9 8 7 6 5 4 3 2 1

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This book is dedicated to my late parents, Mary and Howard Baltensperger. Their longstanding belief that goals should be viewed as “when” rather than “if” made many of my dreams possible. It is also dedicated to my husband for his constant support and encouragement.

Preface

The alarming loss of plant biodiversity both in nature and within agricultural systems has led the plant biology community to look for alternatives to *in situ* conservation. Although cryopreservation by itself is not a panacea for the global loss of biodiversity, it is a useful tool for long-term maintenance of select plant germplasm. The development of plant cryopreservation techniques for cell cultures in 1968 has led us now, 40 years later, to the stage where cryopreservation of organized tissues is a reality.

I came to the field of cryobiology through the need to conserve crop germplasm for future generations. At the time I began the field was still young and the techniques were being applied mostly to unorganized tissues and cells. The first applications of cryopreservation to organized tissues by Dr. Kutty Kartha and Dr. Akira Sakai showed the promise of the technique for the storage of plant diversity. With this encouragement I started toward my goal of storing the unique and invaluable plant germplasm at the USDA Agricultural Research Service's National Clonal Germplasm Repository, Corvallis, Oregon. I started my studies with the initial guidance of Dr. Bernard Finkle and picked up tips along the way from Dr. Lyndsey Withers, Dr. Akira Sakai and Dr. Jean Dereuddre.

While long-term storage of clonally propagated plants or those with recalcitrant seeds was once a dream, that dream became a reality by the mid 1990s. Cryopreserved collections, now located in several countries around the world, are a testament to the utility of cryopreservation. Now the challenge is to expand the utility of these techniques by making them available to laboratories that do not specialize in cryopreservation, but rather wish to use it as a safe backup for valuable plant materials.

The availability of well-tested and widely-used protocols makes the development of a book of this type possible. The first cryogenic technique, "controlled rate cooling" (also called slow cooling and two-step cooling), was the only available protocol for many years. This technique is very successful for a wide range of plant materials and is widely used for callus and suspension cell cultures. It is also easily applicable to the shoot tips of temperate plants. With the aid of a programmable freezer, relatively large amounts of plant material can be stored at one time with little technical input. At the end of the 1980s the

development of vitrification techniques provided a second approach that is applicable even to tropical plants. Several techniques were developed, but the development of Plant Vitrification Solution number 2 (PVS2) by Dr. Akira Sakai, led to the wide use of vitrification for plant tissues. Soon thereafter encapsulation dehydration was developed in the laboratory of Dr. Jean Dereuddre. This technique is also widely used and highly successful for a wide range of plants. Modifications of all these techniques are available as well. With the wide choice of techniques available it should be possible to store most types of plants.

The choice of a technique for storing a particular plant should be based on several factors. Laboratories that wish to store an occasional plant or a tropical plant will choose vitrification or encapsulation-dehydration techniques. These protocols require little more than a standard tissue culture laboratory. The techniques can be adapted to the plant material with a few simple experiments. Facilities with large amounts of temperate plant materials may wish to use controlled rate cooling to more efficiently store larger quantities of plants at one time.

This book was developed to aid in the use of cryopreservation techniques throughout the world, for the conservation of all forms of plant biodiversity. It is hoped that this volume will provide the step-by-step instructions needed to transfer cryopreservation technology to general plant biology laboratories that might make use of these protocols to store important plant materials. Often, published techniques are difficult to interpret and apply in a laboratory that is not familiar with cryopreservation. The protocols presented in this volume were tested on a range of genotypes and should be suitable for storing additional materials. By using the complete and tested protocols presented here, laboratories will have a starting point and may only need to make slight modifications before storing their valuable plant materials.

This volume was written for those active in cryobiology, and also for those who are not cryobiologists, but in need of a long-term storage method. The volume is divided into two parts. The first section introduces the reader to cryopreservation and the main techniques used. The second combines literature reviews of plant groups with defined step-by-step protocols. It is hoped that these techniques will be directly useable by the scientific community.

Corvallis, Oregon
April 2007

Barbara M. Reed

Acknowledgements

The Editor gratefully acknowledges the support and cooperation of the chapter authors and the many protocol contributors. I am also appreciative of the staff of the National Clonal Germplasm Repository for their assistance in keeping the laboratory running while I was occupied with this project. I especially acknowledge the invaluable assistance of my husband Ralph, for his final reading of the chapters and protocols. Without his eagle eye and scientific insight, many errors and inconsistencies might have slipped by unnoticed.

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Section I: Introduction to Cryopreservation

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.*
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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 cryo-stored 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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Chapter 2
Cryopreservation Theory

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2.1 Introduction

Cryopreservation protocols contain components which are usually developed empirically using plant-specific strategies that enhance survival. The theory of cryopreservation encompasses several interconnected disciplines ranging from the physiological to cryophysical. Water status and cryoprotection are the most influential determinants of survival in combination with physiological factors. This chapter introduces the basic principles of cryopreservation theory and aspects of biological chemistry pertinent to protocol development. This review is targeted at researchers new to the field and emphasis is placed on understanding cryoprotection, the physiological role of water and the manipulation of its different states. A theoretical understanding of cryobiology will help cryoconservationists to optimize their storage methods and enhance the long-term preservation of plant and algal collections in a stable cryogenic state.

2.2 The Biological Chemistry of Water

Water, nature's biological solvent, is highly influential in determining survival after exposure to liquid nitrogen (LN). For its small size, H_2O is a complex and behaviourally anomalous molecule, possessing special properties without which the existence of life would not be possible. These

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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pertain to its polar, electrostatic, cohesive, solvent, buffering and thermal stabilizing characteristics.

The behaviour of water in liquid and frozen states is largely dictated by its *bipolar chemistry*. Its hydrogen atoms carry a slight net positive charge and act with unpaired "negatively charged" electrons of oxygen to make water dipolar. The different parts of its *tetrahedron geometry* carry both positively and negatively charged regions. The lone pairs of electrons on water's oxygen atom and its two polarized hydrogens enable the creation of *hydrogen bonds.* How water molecules associate with one another is influenced by H-bonding. Neighbouring molecules orient so that partially positively charged H atoms align with the partially negatively charged oxygen. As liquid water participates in electrostatic interactions with other neighbouring water molecules it is less disordered than most liquids.

Molecules drawn to water are hydrophilic; whilst those that are repelled are *hydrophobic*. The organization of hydrophobic lipids in membranes dictates the compartmentalized structure of cells. Water bodies are highly cohesive, having a robust capacity to resist rupturing if placed under tension, due to H-bonds. In plants these *cohesive forces* support the integrity and movement of water in vascular tissues; these are biological pipelines which, via transpiration and surface evaporation, deliver nutrients from the soil to the plant.

Because of its electrical nature, water clusters in "hydration shells" around individual ions or molecules; this specifies the *hydration state,* the number of H_2O molecules associated with solutes. Water thus maintains dissolved substances in a fluid state. The relationship between water and solutes defines the *colligative properties* of a system and specifically the *concentration of dissolved solutes* in the cell.

In contrast, the *osmotic properties* of a system are defined by flow of a solvent across a *semipermeable membrane,* which permits the passage of the solvent, but not the solutes. Osmotic pressure is the pressure that must be applied to a solution to prevent the flow of a solvent across the semipermeable membrane. Solutions separated in this way must equilibrate with respect to their molecular concentration. Water flows from a weaker to a stronger solution: when equilibrated, solutions are termed *isotonic*. Tonicity measures the extent to which a concentrated solution causes water to move in or out of cells. Solutions that cause cells to swell are *hypotonic*, as the bathing solution has a lower solute content than the cell. In plants, cellular swelling caused by hypotonic solutions creates *turgor pressure*. This is observed as an expansion of the cytoplasm and the pushing of the plasma membrane against the rigid cell wall. This process is affected by the swelling of the central vacuole which gains water. Unlike animal cells, hypotonic plant cells do not usually burst because the cell wall holds

them in place. Turgor pressure is structurally important in plants because it maintains them in an erect condition. Solutions that cause cells to shrink are *hypertonic*; they have a higher solute level than the cell. When a plant cell is placed in a hypertonic solution the plasma membrane recedes from the wall as the vacuole loses water; this process is plasmolysis.

To summarize, H_2O, water has unique physio-chemical properties essential for cell function. Cryobiological manipulations influence the different states of water placing it in an important and central role in cryopreservation and storage stability (as summarised in Fig. 2.1).

2.3 Thermal Properties of Water and Ice

The temperature of a given substance may be considered a measure of its molecular motion or "energy". High numbers of H-bonds in liquid water "buffer" large intracellular thermal fluctuations and stabilize the impacts of temperature on organisms living in aquatic environments. Liquid water is dynamic and, at its interface with other molecules or at exposed surfaces, H-bonds constantly break and re-form. If a large amount of energy impacts the system or it is exposed to differential humidities (e.g. powerful desiccants and air-drying), H-bonds remain broken and individual molecules are expelled by evaporation. The energy applied converts liquid water to vapour and when molecules are discharged they take energy with them and this lowers surface temperature.

In contrast, at temperatures at or below 0°C, H_2O resists the breaking of H-bonds and the molecules lock together in a lattice-like symmetry. This creates pockets of "open space" between more tightly associated parts of the structure. Ice is formed, which is less dense than liquid water. The anomalous density of solid, compared to liquid water is the reason why ice sheets occur near the surface of aquatic bodies. Ice acts as a "frozen duvet" insulating the liquid phase of water beneath and protecting aquatic organisms from freezing.

2.3.1 Ice Nucleation

How liquid water associates to form ice is a point of debate (Benson et al. 2005; Mazur 2004; Franks 1972). Critical to the development of cryopreservation protocols is the control and/or avoidance of intracellular *ice nucleation.* This is also termed "*seeding*", the point at which ice crystals are initiated. It occurs when local thermal properties of a system make it energetically possible for H_2O molecules to come together to form sufficient H-bonds

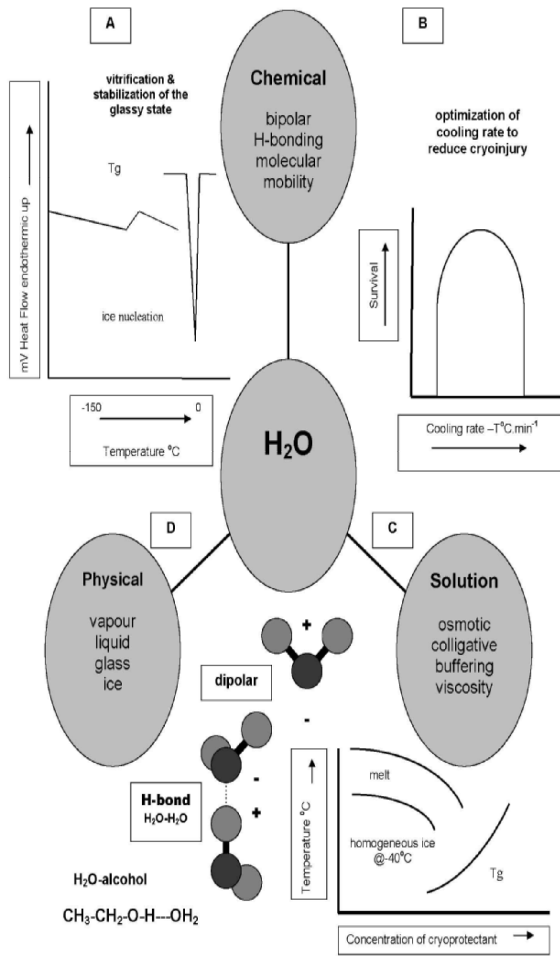

Fig. 2.1 Properties of water and their relevance to cryoprotection: **(A)** Thermal analysis used to confirm stabilities of vitrified states, ice nucleation on cooling and rewarming, and glass transition temperatures (Tgs); **(B)** Optimization of cooling rate to reduce cellular damage and enhance survival as depicted by the inverted "U" and two-factor theory of cryoinjury proposed by Mazur (2004); **(C)** Water as a biological solvent exemplified by a phase diagram demonstrating the effects of cryoprotectant concentration on ice nucleation, melting and *T*g; **(D)** Chemical properties, of water, bipolar characteristics, and hydrogen bonding (H-bonds) with self and other molecules (e.g. cryoprotective additives such as alcohols, polyols and sugars)

to enable initiation of an embryonic ice crystal. It is popularly believed that water freezes at 0°C, but this is rarely the case. In the absence of templates that allow the coming together of H_2O molecules, water supercools to freezing points below zero. The lowest possible supercooling temperature in most biological systems is the point of homogeneous ice nucleation, around or at –40°C. At this temperature, water molecules form an "ice embryo" of a critical size that is thermodynamically capable of growing a crystal. This creates an ordered matrix and energy is released as the latent heat of fusion and as ice is formed heat is produced.

Ice nucleation has important consequences. When liquid water is removed from the system to form ice, solutes become more concentrated. This lowers the temperature at which further ice is formed (Fig. 2.1a, c). One way of considering this is that the solute molecules become increasingly concentrated and impede the ability of the remaining water molecules to interact to form crystals. Above the temperature of homogeneous ice formation the freezing temperature of water becomes increasingly depressed to a point known as the eutectic. At this stage, the whole system solidifies and there is no further change as all available water has "frozen out". The eutectic has coordinates for temperature and concentration and it applies to two or more substances that are able to form solid solutions with one another and lower each other's freezing point to a minimum temperature.

After the initial ice nucleation event, ice crystals have the capacity to grow into complex clusters forming networks that can grow exponentially as more and more water molecules participate in complex alignments with each other. The implications of both extra-and intracellular ice nucleation were discussed by Muldrew et al. (2004) and Mazur (2004). Ice nucleation affects the structural, osmotic and colligative integrity of cells causing physical ruptures and mechanical injury. Colligative damage results from excessive solute concentrations that jeopardize cellular function.

In cryopreserved plants, the dynamics of the two factors involved in cryoinjury are moderated by differentials in the ability of the intracellular and extracellular components to initiate ice crystals. Freezing usually occurs when it is energetically and physically (in terms of the availability of ice templates) more favourable. With the exception of very rapid freezing temperatures, ice preferentially forms *extracellularly*. In this scenario, a deficit is created between the partially frozen outside of the cell and the unfrozen inside and water molecules migrate to the outside to establish osmotic equilibrium. Cells continually exposed to this process shrink and the rate of water loss can be expressed as a function of cell size. This is more applicable to animal as compared to plant cells which normally have a rigid cell wall that defines size and shape. The osmotic gradient and

permeability of the plasma membrane also affect water movement, through diffusion and the passage of water molecules across aquaporin pores (Tyerman et al. 2002).

2.3.2 Controlled Rate Cooling

Controlled cooling aims to optimize two injurious components: damaging colligative solution effects and ice formation (Fig. 2.1b). These are moderated by the precise control of cooling rate, terminal freezing parameters (transfer and holds at intermediate subzero temperatures), ice nucleation and the application of colligative protectants (Chap. 5). The underlying principle that determines cell survival in this type of protocol is the rate of cooling (Fig. 2.1b), figuratively described as the inverted "U" of cryoinjury (Mazur 2004). If the rate is too fast or too slow, the cells do not survive and balancing these two cryoinjuries is the key to success. When a controlled, gradual rate of cooling is applied to cells, extracellular ice first forms, a differential water gradient is created across the cell membrane and intracellular water moves to the outside. This has an important cryoprotective effect as it reduces the amount of water available to form ice. Although it is a counterintuitive concept, extracellular ice has a vital cryoprotective role as its formation indirectly reduces the potential for ice nucleating inside the cell. Cells can survive extracellular ice formation, although it can be harmful, particularly for complex multicellular tissues. Intercellular ice is almost always lethal. This accounts for one arm of the "U" (Fig. 2.1b), but if cooling is too slow, the cell's solutes become excessively concentrated causing colligative damage and this accounts for the other side. The key is to balance the two injuries by optimizing cooling rates so that just the right amount of water is removed to prevent colligative injury. Any water that does remain is so minimal that ice formation is inhibited and the cytoplasm most likely vitrifies.

Generally, methods for controlled rate cooling include a sequence of manipulations by which the sample is precultured or acclimated and then cryoprotected. Usually, DMSO is the main penetrating colligative cryoprotectant and it may be combined with non-penetrating osmotically active additives such as sugars or polyethylene glycol. Samples are exposed to a specific cooling rate (range –0.1 to 5°C·min^{-1}) usually called a "ramp" during which a step may be incorporated for the operator or the machine to "seed" extracellular ice, and initiate an exothermic reaction. The ramp then continues to an intermediate or *terminal transfer temperature* (usually at or above the temperature of homogeneous ice nucleation, –35 to –40°C). One or more ramps with different rates of cooling may be incorporated into a

protocol. Samples may be plunged at this point, or a *hold* (of 30–45 min) at the terminal transfer temperature may be instituted, followed by a direct plunge into LN. Hence, this type of protocol is also called *two-step cooling* or freezing. The slow rates of cooling may also be achieved using alcohol baths, or self-contained units (containing methanol or isopropanol) placed in a –20 or –80°C freezer. For example, isopropanol contained in a Mr Frosty unit provides a 1°C·min^{-1}cooling rate. These methods are sometimes termed traditional because they were some of the first techniques developed (Benson 2004; Day and McClellan 1995; Kartha 1985; Sakai 1966; Uemura and Sakai 1980; Withers 1975; Withers and King 1980). However, this is a general term that could also apply to early vitrification techniques, so it should be avoided.

2.4 Vitrification: The Glassy State

So far cryopreservation has been considered in terms of the liquid and solid (ice) phases of water. It is also possible to cryopreserve plants by the process of "*vitrification*", the solidification of liquids without crystallization. This comprises a "glassy state" as the system is *amorphous,* lacks organized structure but possesses the mechanical and physical properties of a solid (Taylor et al. 2004). Vitrification of water in biological systems is dependent on increased cell *viscosity,* occurring as cell solutes become concentrated. Increased viscosity inhibits the coming together of water molecules to form ice. The vitrified state is *metastable*, meaning that it can relatively easily revert back to a liquid and/or devitrify to form ice (Fig. 2.1a, c). Vitrified systems are particularly unstable during rewarming as changes in molecular mobility and energy may be sufficient to allow water molecules to rearrange (relax) and form ice. Vitrification can be achieved in a number of ways but all usually have the end result of increasing solute concentration to a critical viscosity. For temperature-induced glasses, the point at which this occurs is called the *glass transition temperature (Tg);* molecular motion nearly ceases and the liquid becomes a glassy solid (Fig. 2.1a).

Ice-free cryopreservation was pioneered for animal cells (Fahy et al. 1986) and extensively applied to plants by Sakai (2004). This cryoprotective regime completely eliminates ice formation inside and outside the cell. Vitrification is used to advantage in cryopreserving complex, heterogeneous tissues for which it is difficult to achieve optimum cooling rates. Plant materials may be preconditioned on special media or with specific growth conditions, precultured with cryoprotective chemicals just prior to cryopreservation, and protected with vitrification solutions prior to plunging in

LN (Chap. 3). Alternatively, plant samples are encased in an alginate solution, osmoprotected with sugar solutions, and dried to low moisture contents before plunging in LN (Chap. 4).

2.5 Water State Transitions and Instabilities

Thermal analysis using *differential scanning calorimetry* (DSC) is used in fundamental cryobiology to assist cryopreservation protocol development (Fig. 2.1a). Heat flow and water state transitions are monitored in DSC samples as a function of time and temperature, and changes can be detected during the cooling and heating of samples. This approach is used to evaluate the efficacy of cryoprotectants, particularly in stabilizing the glassy state (Benson et al. 2005) and the development of protocols for highly recalcitrant plants. *Cryomicroscopy* is also a useful tool which explores real-time transitions between water states in the presence of cryoprotectants (Fleck et al. 2006).

The behaviour of water in different state transitions and its influence on survival was evaluated by Taylor et al. (2004) with respect to the relative stabilities of amorphous, liquid and solid phases and their potential for causing thermo-mechanical stresses in biological tissues. Glasses are usually considered to be more unstable and their labile properties are often observed as cracking, or fractures which are particularly damaging to larger organs and tissues such as seeds. Glasses can also undergo relaxation during rewarming, which can be innocuous, but may lead to devitrification and ice crystal formation. This is observed in cryovials as the transparent glasses become opaque when ice is formed. For many vitrification systems, rapid rates of rewarming are prescribed to avoid the possibility of ice nucleation when the samples pass through their Tg. This practice should be undertaken with caution as stress cracks and fractures can occur if rewarming is too rapid. Two phases may be required, first a short (e.g. 1–2 s) slow phase (e.g. at ambient temperatures) to allow for glass relaxation without stress fracturing, followed by a rapid warming (in a water bath at 45°C) to ensure the speedy transition from glass to liquid without passage through an ice phase. Empirical optimization of these phases may be required for each biological system and its cryoprotective regime.

Liquid-ice-liquid transitions common to controlled rate cooling protocols have different biological consequences compared to those of totally vitrified systems. It is important to insure the initial transition from liquid to frozen state by manually or automatically "seeding" ice nucleation. This nucleation prevents supercooling of the sample with resulting spontaneous nucleation at –40°C and death of the sample. Control of the point at which

extracellular ice is first formed consequentially controls the sequence of events key to colligative cryoprotection and freeze dehydration.

Rewarming can also affect ice stability and is dependent upon the type of tissue, rates of cooling and the cryoprotective additives used. In general, higher survival is achieved when slow cooling is followed by rapid rewarming (e.g. in a water bath at 40–45°C). Very small and innocuous ice crystals may grow into larger, more damaging crystals if a frozen system is rewarmed too slowly. These ice-liquid transitions can be potentially damaging as expansions and contractions may occur non-uniformly across cells, tissues and organs; these can be induced by a combination of thermal, colligative and osmotic stresses (Taylor et al. 2004).

2.6 Cryoprotection

Many temperate and cold-adapted plants are naturally equipped to withstand freezing in their natural environments (Hirsh 1987; Meryman and Williams 1985; Pearce 2004; Sakai 1956, 1960) and an appraisal of their behaviours provides a logical starting point.

2.6.1 Biophysical Aspects of Natural Freezing Tolerance

In the absence of ice nucleators, it is potentially possible for water in biological systems to supercool to around –40°C, the point of homogeneous ice formation. This is an avoidance strategy used by plants to resist freezing at natural subzero temperatures. Thus supercooling is an important adaptation in cold-tolerant tree species and it accounts for the location of tree line on mountains, the point above which vegetation cannot survive (–40°C). This adaptation allows survival of *dormant* tissues. It is termed *cold hardening* or *acclimation* and generally involves the synthesis of intracellular solutes which increase cell osmolality and reduce plasmolysis injury at freezing temperatures.

Meryman and Williams (1985) cautioned that freeze avoidance by these mechanisms would only permit tolerance to temperatures around –15°C. It was therefore necessary to explore how, and why, cold acclimation (CA) adaptations might be applied to recover plants from much lower cryogenic temperatures (as demonstrated by Sakai 1956, 1960). Hirsh et al. (1985) pioneered fundamental DSC and freeze-etch/fracture electron microscopy studies on a cultivar of *Populus balsamifera.* They formulated the first definitive explanation as to why resistance to LN was possible in naturally cold-acclimated woody plants. Their findings support their hypothesis that

aqueous glasses form intracellularly. Three distinctive Tgs (glass transitions) were detected using thermal analysis, these remained stable at relatively high, subzero temperatures and vitrification was concomitant, with hardened tissue having high levels of free sugars (Hirsh 1987). Hirsh went on to postulate that proteins in conjunction with sugars caused the solutions to have Tgs at temperatures lower than those of water and sugars alone. This pointed the way towards a bio-molecular understanding of natural cold tolerance in plants.

2.6.2 Aspects of Natural Freeze Tolerance

It is outside the scope of this chapter to provide a comprehensive review of the molecular biology of natural freezing and CA responses. The reader is directed to Thomashow (1999), Xin and Browse (2000), Pearce (2004), Thorlby et al. (2004) and Chen et al. (2006) for further insights. Xin and Browse (2000) report the involvement of reduced hydration status; growth; increased antioxidants, osmotic regulation; the accumulation of polyols, sugars, proline, betaine, increased transcription, stability and down regulation, changes in hormone status, and lipid composition of membranes; fatty acid unsaturation and cell wall modification from CA. Pearce (2004) proposed that an array of molecular mechanisms is involved in CA and that these are likely to be related to dehydration tolerance. This is not a surprising assumption, considering the links between freezing injury, colligative and osmotic damage. Gaining an understanding of natural cold-acclimation mechanisms is highly pertinent to cryopreservation protocol development.

In temperate species and naturally cold-hardy plants, acclimation can enhance the ability to withstand LN and is usually achieved by exposing donor plants to low, near zero temperatures. Standard CA is often at 4°C in the dark, but CA is more effectively applied in a diurnal temperature cycle (Chang and Reed 2000; Reed 1988). CA is also affected by environmental parameters other than cold *per se,* as demonstrated by Bourion et al. (2003), who demonstrated that CA in *Pisum sativum* L. plants did not occur at low temperatures if light intensity was low. However, light was not a significant factor for the CA of pear or blackberry shoots. Shoots developed significantly better cold tolerance when grown under a 12–16 h cold period and a short warm period than with a constant temperature, either with a light cycle or in the dark (Chang and Reed 1999, 2000). Future explorations of CA will undoubtedly be dependent upon molecular and transgenic technologies (see Chen et al. 2006).

2.6.3 Artificial Cryoprotection

The cryobanking era commenced with the serendipitous and milestone discovery of Polge et al. (1949) that glycerol protects avian sperm cells against freezing. After this breakthrough, mammalian and health care cryobiological research explored the mode of action of cryoprotectants in terms of colligative theory (Lovelock 1953; Meryman and Williams 1980, 1985). In Lovelock's key study, red blood cell injury occurred when a critical concentration of applied extracellular salt was exceeded, independent of glycerol being applied or the freezing temperature required for achieving the critical salt concentration. This was interpreted that glycerol penetrates the cell, and on a colligative basis reduces the amount of ice formed at any temperature. That is, it acts as antifreeze by reducing the concentration of extracellular salt and water lost due to osmosis. The cell is protected against reaching a *minimum lethal cell volume* with the additional benefit of lowering the temperature at which freezing actually occurs. A *colligative property* of a solution depends only on the ratio of the number of particles of solute and solvent in the solution, not the identity of the solute. The *theory of colligative cryoprotection* has two essential attributes:

1. Cryoprotectants must be able to penetrate the cell, or else they will cause osmotic dehydration and cause the very injury they are used to protect against.
2. Cryoprotectants must be non-toxic to the cell at the concentrations required for their efficacy.

Penetrating cryoprotectants make a contribution to the overall osmolality of the cell. A colligative additive such as glycerol increases the initial osmolality of the cell before the freezing process is initiated. Hence, the level of water that needs to be frozen out to achieve osmotic equilibrium is far less, and the extent of the dehydration that occurs in the cell is better tolerated (Meryman and Williams 1985). There is an additional protective advantage in that this occurs at a lower temperature as the additive depresses the freezing point. The most common colligative (penetrating) cryoprotectants used in plant cryopreservation are glycerol, dimethyl sulphoxide (DMSO), methanol (and sometimes ethanol) and the smaller molecular weight glycols.

It is important to consider colligative cryoprotection in relation to the dynamics of the freezing process and revisit (Fig. 2.1b) Mazur's "inverted U theory" of cryoinjury (Mazur 2004). The aim of controlling cooling rate is to optimize cell dehydration between too little (resulting in ice damage) and too much (resulting in colligative damage). Colligative agents moderate

the extent to which excessive and insufficient dehydration occurs. For example, if glycerol is present, damage to the cell will only occur at a higher osmolality. It is necessary to apply a sufficiently slow cooling rate to ensure water has enough time to exit the cell. Operator-induced extracellular seeding of ice is important and creates a gradient. A "hold" in the programme at a terminal transfer temperature (the end of the slow cooling phase) allows for the completion of water efflux before exposure to LN. Colligative additives also enhance viscosity of the cell and reduce the efflux of water from the cytoplasm. Cell viscosity may be elevated to such a level that ice nucleation is inhibited (Meryman and Williams 1980, 1985). Thus, although extracellular freezing occurs, cells do not freeze intracellularly and they are preserved in a "glassy state".

Completely ice-free cryogenic storage is the newest approach to plant cryoconservation (Benson 2004; Sakai 2004). The key to developing cryoprotective vitrification strategies is to increase cell viscosity to the point at which ice formation is inhibited and water becomes vitrified on exposure to cryogenic temperatures. Cell viscosity enhancement is achieved using two main approaches:

1. Addition of cryoprotective additives at very high concentrations.
2. Water removal by evaporative desiccation and osmotic dehydration.

In practice, many plant vitrification protocols integrate both. Vitrification achieved through the use of additives can result in cryoprotectant toxicity, osmotic injury (for non-penetrating components) and devitrification. In the case of dehydrating processes where water is removed through osmotic dehydration and/or by evaporative means (air drying or drying agents such as silica gel), there is the additional problem of desiccation sensitivity. It is advantageous to use a mixture of different additives (Fahy et al. 1986). This reduces the toxicity of any single additive, limits the impacts of extreme evaporative drying and helps to stabilise the glasses formed. It is recommended that mixtures of penetrating and non-penetrating cryoprotectants are used (Fahy et al. 1984). Most vitrification solutions applied to plants include a mixture of penetrating and non-penetrating cryoprotectants.

Whereas cooling rate is critical to the success of colligative cryoprotection, it is rewarming and glass stabilisation that is key to successful vitrification (Fahy 1987). Glass stability may be dependent upon how the glass was initially formed, particularly in relation to the water potential within the whole system. In the case of plants it may be expected that cells that have been vitrified at very low water contents through evaporative desiccation will have fewer water molecules available to participate in nucleation,

devitrification and ice growth on rewarming. In contrast, those vitrified using a combination of penetrating and non-penetrating cryoprotectants may form less stable glasses on rewarming. Comparative thermal analysis studies of the stabilities of glasses in dehydrated, desiccated alginate beads and Plant Vitrification Solution 2 (PVS2) provides some evidence for this (Benson et al. 1996). In vitrification by alginate encapsulation dehydration, glasses remained stable on passive rewarming at ambient temperatures. For vitrification solutions, the stringently controlled addition and removal of cryoprotectants and rapid rewarming is required to achieve thermal and osmotic stability.

2.7 New and Retrospective Insights in Cryoprotection

Research on both plants and animals has resulted in a number of fundamental investigations, which question and expand upon existing cryoprotection theory (Fuller 2004; Benson 2004). It is important to integrate physical and biological knowledge of cryoconservation particularly for difficult-to-preserve plants. As reviewed by Fuller (2004), polyols and sugars (particularly sucrose and trehalose) increase the glass-forming tendency of aqueous solutions which, when applied in combination with other cryoprotectants, reduce the cryoprotectant concentrations required to achieve a stable glass. Sugars offer several advantages in that they form H-bonds with water and have high molecular weights, contributing substantially to the elevation of cell viscosity. For example, desiccation tolerance in seeds has been associated with glass-formation tendencies of sugars (Koster 1991) even at ambient temperatures.

Turner et al. (2001) explored an alternative theory as to the basis of cryoprotectant efficacy by comparing different polyols and sugars with regard to the stereochemical arrangement of their hydroxyl groups. They postulated that the mode of action is based not on molarity but on the total number of OH groups present in the medium. Not too surprisingly, glycerol was the most effective. Similarly, Fahy et al. (2004) present a new theory pertaining to the basis of non-specific cryoprotectant toxicity. Their studies on mammalian cells showed that cryoprotectant solutions designed to be at the minimum concentrations needed for vitrification at moderate cooling rates were toxic in proportion to the average strength of water H-bonding by the polar groups on the permeating cryoprotectants in the system. This finding is highly relevant to plant cryobiologists, particularly for those working on desiccation-sensitive plants. The theory proposes that vitrification solutions based on achieving a minimal disturbance of intracellular

water may be superior and Fahy et al. (2004) provide an elegant explanation for this. This starts with the premise that for a solution to vitrify at attainable cooling rates, water–water interactions must be inhibited. It also considers the observations that despite the equivalence of the physical tendencies of different types of vitrification solutions to inhibit ice nucleation, they do have very different levels of toxicity. Fahy et al. (2004) propose that the toxicity of vitrification solutions increases as the strength of the water-cryoprotectant H-bonding increases. The non-specific toxicity of vitrification solutions is dictated by the availability (using a statistical prediction model) of water molecules for hydrating bio-molecules. Competition between cellular components and cryoprotectants for hydration will affect water availability for cellular constituents and will damage the cell; although how this occurs is yet to be elucidated. The future development of controlled rate cooling and vitrification protocols will benefit from this type of fundamental research.

In plants, progress in cryoprotection must also take into account their special properties. Tao and Li (1986) present the distinctive attributes of plant, compared to animal cells, classifying a range of frequently used plant cryoprotectants on their ability to penetrate cells:

1. *Unable to penetrate the cell wall* (high molecular weight polymers (PEG_{6000}, PVP), polysaccharides and proteins)
2. *Penetrable through the cell wall only* (oligosaccharides, mannitol amino acids (proline) and low molecular weight polymers PEG_{1000})
3. *Penetrable through the cell wall and membrane* (DMSO and glycerol)

Importantly, the plant cell wall is often overlooked, particularly as animal models of freezing and cryoprotective theory are presented as standard. Tao and Li (1986) propose that each classification of cryoprotectant has a different function and to provide optimum protection, cryoprotectants should be used in combination in plants. Penetrating cryoprotectants act colligatively, but they may also produce temporary plasmolysis as they penetrate the wall and loosen adhesion between the wall and cell membrane. Those that penetrate the wall, but not the plasmalemma prevent ice crystals from damaging the cell membrane and also buffer the cytoplasm from excessive dehydration. Cryoprotectants that remain in the intercellular spaces and do not penetrate either structure become concentrated at the ice crystal front, inhibit the rate and extent of ice growth and protect against mechanical deformation.

A similar approach is now required to critically evaluate the *modus operandi* of vitrification in plants (Sakai 2004). There are now a number of permutations of the two main approaches to creating a glassy state in plants. Usually, these are alginate encapsulation dehydration (using osmotic dehydration with sugars and evaporative desiccation) and the application of additives such as PVS2. Volk and Walters (2006) recently applied DSC to help elucidate the mode of action of the PVS2 cryoprotectant mixture developed by Sakai and colleagues (Sakai 2004). They propose that PVS2 operates through two cryoprotective mechanisms: (1) it replaces cellular water, and (2) it changes the freezing behaviour of water remaining in the cells. Volk and Walters (2006) also put forward the theory that the penetration of some of the components (e.g. DMSO) of PVS2 into the cell is central to its cryoprotective efficacy. Significantly, the assumption that the mode of action of PVS2 is primarily due to osmotic dehydration cannot therefore constitute a full explanation for its high efficacy. Rather, cell-penetrating constituents of PVS2 replace water as the cells become dehydrated and prevent injurious cell shrinkage caused by dehydration (Volk and Walters 2006). Penetrating PVS2 cryoprotectants therefore have a colligative role, moderating the effects of osmotically active, non-penetrating components of the mixture, which includes high concentrations of sucrose and glycerol. The solution is effective because protection is imparted before exposure to cryogenic treatments as some components of PVS2 are sufficiently mobile to permeate cells at 0°C and displace water. When the temperature is subsequently reduced, the penetrating components of PVS2 cryoprotect the cells by restricting the molecular mobility of water molecules and prevent them from nucleating ice crystals. Buitink and Leprince (2004) present a similar view, such that to fully understand the glassy state it is important not only to measure thermal behaviour (e.g. as Tgs) but also to elucidate the molecular mobility and H-bonding properties of the systems.

Cryoconservationists are increasingly exploiting and amalgamating cryoprotectant modalities which combine various vitrification, colligative and biophysical/molecular attributes. These resulted in the cryopreservation of a diverse range of species. Recent studies of plant cryoprotectant behaviour suggest that a greater theoretical understanding of their mode of action is required. This is particularly important for affirming the long-term stability of stored plants using different cryoprotective strategies, which may potentially and differentially affect their stability.

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Chapter 3

Development of PVS-Based Vitrification and Encapsulation–Vitrification Protocols

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3.1 Introduction

Cryopreservation is a very important tool for the long-term storage of plant genetic resources for future generations, requiring only a minimum of space and maintenance. With increasing interest in the genetic engineering of plants, the preservation of cultured cells and somatic embryos with unique attributes is assuming greater importance. Recently, cryopreservation was reported to offer real hope for enhancing the preservation of endangered and rare plants (Touchell 1995; Touchell and Dixon 1996). This chapter describes protocols for successful vitrification using plant vitrification solution 2 (PVS2), and highlights some of the factors contributing to high levels of post-LN recovery. The development of a simple and reliable method for cryopreservation would allow the widespread storage of cultured cells, meristems, and somatic embryos. Vitrification involving vitrification solutions (Langis et al. 1990; Sakai et al. 1990; Yamada et al. 1991) and encapsulation–dehydration techniques (Fabre and Dereuddre 1990) were developed in the 1990s, and the number of cryopreserved species has increased markedly since then (Sakai 1995, 1997; Engelmann and

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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Takagi 2000). A vitrification procedure using an ethylene glycol-based vitrification solution and French straws was presented by Steponkus and colleagues. They reported successful cryopreservation of *Dianthus* and *Chrysanthemum* (Langis et al. 1990; Schnabel-Preikstas et al. 1992), and potato (Lu and Steponkus 1994). This chapter will outline the development and uses of PVS2 developed by Sakai and associates.

3.2 The Concept of Vitrification

To maintain the viability of hydrated cells and tissues it is essential to avoid the lethal intracellular freezing that occurs during rapid cooling in LN (Sakai and Yoshida 1967). Cells and tissues that are to be cryopreserved in LN need to be sufficiently dehydrated before being immersed in LN.

There are two types of liquid–solid phase transitions in aqueous solutions. Ice formation is the phase transition from liquid to ice crystals, and vitrification is a phase transition from a liquid to amorphous glass that avoids crystallization. Water is very difficult to vitrify because the growth rate of crystals is very high, even just below the freezing point. However, highly concentrated cryoprotective solutions such as glycerol are very viscous and are easily supercooled below –70°C. This allows them to be vitrified on rapid cooling. Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass without undergoing crystallization at a practical cooling rate (Fahy et al. 1984). Vitrification was proposed as a method for the cryopreservation of biological materials because it would avoid the potentially detrimental effects of extracellular and intracellular freezing (Luyet 1937). Thus, vitrification is an effective freeze-avoidance mechanism. As glass fills space in a tissue it may prevent additional tissue collapse, solute concentration, and pH alteration during dehydration. Operationally, glass is expected to exhibit a lower water vapor pressure than the corresponding crystalline solid, thereby preventing further dehydration. Because glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to dormancy and stability over time (Burke 1986).

In controlled rate cooling methods, slow freezing to about –40°C results in sufficient concentration of the unfrozen fraction of the suspending solution and cytosol to enable vitrification upon rapid cooling in LN (partial vitrification). Vitrification can also be achieved by direct immersion in LN without the freeze-concentration step by exposing cells and tissues to extremely concentrated solutions (7–8 M) of cryoprotectants. This technique is referred to as complete vitrification, and is distinct from controlled rate cooling methods.

3.3 Vitrification Procedure

In vitrification procedures, cells and meristems must be sufficiently dehydrated with a vitrification solution to avoid lethal injury from immersion in LN. We used glycerol-based vitrification solutions designated PVS2 (Sakai et al. 1990, 1991b) and PVS3 (Nishizawa et al. 1993). Plant vitrification solution 2 contains 30% glycerol (w/v), 15% ethylene glycol (w/v), and 15% dimethylsulfoxide (DMSO; w/v) in basal culture medium (without growth regulators) containing 0.4 M sucrose (pH 5.8), and PVS3 consists of 40% glycerol (w/v) and 40% sucrose (w/v) in basal medium. PVS2 easily supercools below –100°C upon rapid cooling and solidifies into a metastable glass at about –115°C. Upon subsequent slow warming, differential scanning calorimetry records the vitrified PVS2 as displaying a glass transition (Tg) at about –115°C, with an exothermic devitrification (crystallization) (Td) at about –75°C and an endothermic melting (Tm) at about –36°C (Sakai et al. 1990). In mulberry apical meristems treated with PVS2, no freezing exotherm occurred during cooling. During the subsequent warming (10°C min^{-1}) a series of changes in the thermal behavior of the vitreous solid were observed. Such changes included glass transition, exothermic devitrification, and endothermic melting (Niino et al. 1992b). These results indicate that meristems sufficiently dehydrated with PVS2 became vitrified during rapid cooling. Crystallization during the warming process can be prevented if warming occurs rapidly. The complete vitrification method for unencapsulated tissues involves the following steps:

A. Preculture of excised meristems on solidified medium with 0.3 M sucrose for a specified duration at 25°C or 0°C (Fig. 3.1A).

B. Osmoprotection (loading treatment) (Fig. 3.1B). Precultured meristems are placed in a 2 ml cryotube and osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20–30 min at 25°C. An osmotic loading treatment increases the osmolarity of the cell and minimizes osmotic damage caused by the vitrification solution.

C. Dehydration with a vitrification solution (PVS2 or PVS3; Fig. 3.1C). After removing the osmoprotection solution using a Pasteur pipette, 1–2 ml of PVS2 is added and gently mixed. Five minutes later, PVS2 is replaced with 1–2 ml of fresh PVS2 and held at 25°C or 0°C for different periods of time. Wrapping several dissected meristems together in a small piece of tissue paper (1.5 cm × 1.5 cm) may facilitate the implementation of the osmoprotection, dehydration, cooling, warming, and dilution steps (Thinh 1997).

D. Rapid cooling (Fig. 3.1D). The meristems are suspended in 0.5 ml PVS2 in cryotubes, and the cryotubes are then directly plunged into LN (cooling rate: about 300°C min^{-1}) and held at –196°C for a minimum of 1 h.

E. Rapid warming (Fig. 3.1E). Cryotubes are rapidly transferred to sterile distilled water in a water bath at 40°C (warming rate: 250°C min^{-1}). The cryotubes are vigorously shaken during rewarming for 1.5 min.

F. Dilution (unloading, dilution of the vitrification solution) (Fig. 3.1F). Immediately after warming, the PVS2 is drained from the cryotubes, replaced with 2 ml of basal culture medium with 1.2 M sucrose (Sakai et al. 1991b), and held for 20 min.

G. Plating (Fig. 3.1G). Meristems are then transferred onto a double sterile filter paper disc over a Petri dish filled with culture medium. After 1 day, the meristems are transferred to fresh filter paper in a Petri dish containing the same medium. Shoot formation is recorded as the percentage of the total number of meristems forming normal shoots 3–4 weeks after plating.

Table 3.1 presents (nonexhaustive) lists of plant species to which PVS2 or PVS3 vitrification was successfully applied. Table 3.2 lists encapsulation–vitrification successes.

Table 3.1 Examples of plant species cryopreserved using vitrification techniques. Unless specified otherwise the developed protocols are for shoot tips

Plant species	Treatments	Recovery	Reference
Allium porrum	CA, PC, LD, PVS2, RF	80%	Niino et al. 2003
Allium sativum	PC, PVS2, RF	100%	Niwata 1995
Allium sativum	PC, LD, PVS3, RF	0–100%	Makowska et al. 1999
Allium sativum	PC, PVS3, RF	72–95%	Kim et al. 2004
Allium sativum	PC, LD, PVS2, RF	0–75%	Volk et al. 2004
Allium wakegi	CA, PC, PVS2, RF	80%	Kohmura et al. 1994
Ananas comosus	PC, LD, PVS2, RF	25–65%	Gonzalez-Arnao et al. 1998
Ananas comosus	PC, LD, PVS2, RF	35.7%	Thinh et al. 2000
Arachis spp.	PC, LD, PVS2, RF	40–75%	Gagliardi et al. 2003
Armoracia rusticana	PC, PVS2, RF	69%	Phunchindawan et al. 1997
Artocarpus heterophyllus (EA)	PC, LD, PVS2, RF	50%	Thammasiri 1999
Asparagus	PVS2, RF	90%	Kohmura et al. 1992
Asparagus officinalis (EC)	LD, PVS3, RF	80–90%	Nishizawa et al. 1993
Atropa belladonna(R)	PC, PVS2, RF	50%	Kamiya et al. 1995
Beta vulgaris	PC, LD, PVS2, RF	60–100%	Vandenbussche et al. 2000.
Bletilla striata (ZE)	PC, LD, PVS2, RF	60%	Ishikawa et al. 1997
Brassica campestris (CS)	LD, EG+sor+BSA, RF	40%	Langis et al. 1989

Camellia sinensis	CA, PC, PVS2, RF	60%	Kuranuki and Sakai 1995
Castanea sativa (SE)	PC, PVS2, RF	68%	Corredoira et al. 2004
Citrus madurensis (EA)	PC, LD, PVS2, RF	85%	Cho et al. 2002
Citrus sinensis (NC)	PVS2, RF	70–75%	Sakai et al. 1990
Citrus 3 species (NC)	PVS2, RF	90–97%	Sakai et al. 1991b
Citrus spp.	PC, LD, PVS2, RF	70.5–95.6%	Wang and Deng 2004
Chrysanthemum	CA, EG+sor+BSA, RF	84%	Schnabel-Preikstas et al. 1992
Colocasia esculenta	PC, LD, PVS2, RF	80%	Takagi et al. 1997
Cymbidium spp.	PC, LD, PVS2, RF	93.3%	Thinh and Takagi 2000
Cymbopogon	PC, LD, PVS2, RF	56.6%	Thinh et al. 2000
Daucus carota (CS)	PC, LD, PVS2, RF	83.3%	Chen and Wang 2003
Daucus carota (PP)	PC, LD, PVS2, RF	47%	Chen and Wang 2003
Dendranthema	PC, PVS2, RF	31.8%	Fukai 1992
Dianthus caryophyllus	CA, LD, EG+sor+BSA, RF	100%	Langis et al. 1990
Dianthus caryophyllus	PC, PVS2, RF	50.9%	Fukai 1992
Dioscorea spp.	CA, PC, LD, PVS2, RF	0–50%	Leunufna and Keller 2005
Dioscorea rotundata	PC, LD, PVS2, RF	63%	Takagi et al. 1998
Diospyros kaki	PC, LD, PVS2, RF	89%	Matsumoto et al. 2001
Doriteanopsis (CS)	PC, LD, PVS2, RF	64%	Tsukazaki et al. 2000
Doritis pulcherrima (S)	PVS2, RF	62%	Thammasiri 2000
Fragaria x ananassa	CA, PC, LD, PVS2, RF	93%	Niino et al. 2003
Gentiana spp.	CA, PC, LD, PVS2, RF	16.7–76.7%	Tanaka et al. 2004
Grevillaria scapigera	PC, PVS2, RF	65%	Touchell and Dixon 1996
Hyosciamus niger (R)	PC, LD, PVS2, RF	93.3%	Jung et al. 2001
Ipomoea batatas	LD, PVS2, RF	0–64%	Towill and Jarret 1992
Ipomoea batatas	PC, LD, PVS2, RF	93%	Pennycooke and Towill 2001
Lilium japonicum	PC, LD, PVS2, RF	80%	Matsumoto et al. 1995b
Limonium	PC, PVS2, RF	75%	Matsumoto et al. 1998
Macropidia fuliginosa (SE)	PC, PVS2, RF	90.6%	Turner et al. 2000
Malus spp.	CA, PC, PVS3, RF	27–94%	Wu et al. 1999
Malus spp.	PVS3, RF	60%	Wu et al. 2001
Manihot esculenta	PC, LD, PVS2, RF	75%	Charoensub et al. 1999
Mentha aquatica x M. spicata	PC, LD, EG+DMSO+PEG, RF	31–75%	Towill 1990
Mentha spp.	PC, LD, PVS2, RF	8–89%	Towill and Bonnart 2003
Morus bombycis	CA, PC, PVS2, RF	65%	Niino et al. 1992b
Musa spp.	PC, LD, PVS2, RF	70%	Takagi et al. 1998
Musa spp.	PC, LD, PVS2, RF	69%	Thinh et al. 1999
Musa spp.	PC, LD, PVS2, RF	0–85%	Agrawal et al. 2004
Nicotiana tabaccum (CS)	PC, PVS2, RF	55%	Reinhoud 1996
Oryza sativa (EC)	PC, LD, PVS2, RF	45%	Huang et al. 1995
Oryza sativa (CS)	LD, DMSO+sorbitol, RF	1%	Watanabe and Steponkus 1995
Panax ginseng (R)	PC, PVS2, RF	69%	Yoshimatsu et al. 1996
Picrorhiza kurroa	CA, PC, PVS2, RF	35%	Sharma and Sharma 2003
Populus alba	CA, PC, LD, PVS2, RF	60%	Lambardi et al. 2000
Prunus domestica	PC, LD, PVS2, RF	57%	De Carlo et al. 2000
Prunus dulcis	CA, PC, LD, PVS2, RF	10%	Shatnawi et al. 1999
Prunus spp.	CA, PC, PVS2, RF	80%	Niino et al. 1997
Pyrus spp.	CA, PC, PVS2, RF	80%	Niino et al. 1992a
Quercus robur (EC)	PC, PVS2, RF	70%	Martinez et al. 2003
Quercus suber (SE)	PC, PVS2, RF	88–93%	Valladares et al. 2004
Ribes	CA, DMSO, PVS2	24–57%	Reed 1992; Luo and Reed 1997

Secale cereale (PP)	CA, LD, EG+sorb+BSA, RF	38%	Langis and Steponkus 1991
Solanum spp.	PC, EG+sor+BSA, RF	30–60%	Golmirzaie and Panta 2000
Solemostemon rotundifolius	PC, LD, PVS2, RF	85%	Niino et al. 2000a
Trifolium repens	PC, PVS2, RF	80%	Yamada et al. 1991
Vitis vinifera	PC, LD, 50/100% PVS2, RF	80%	Matsumoto and Sakai 2003
Wasabia japonica	PC, LD, PVS2, RF	85%	Matsumoto et al. 1998
Xanthosoma spp.	PC, LD, PVS2, RF	62–67%	Thinh 1997

CA: cold acclimation; *PC*: preculture; *LD*: osmoprotection (loading); *PVS2*: treatment with PVS2; *PVS3*: treatment with PVS3; *RF*: rapid freezing; *CS*: cell suspension; *PP*: protoplasts; *EA*: embryonic axes; *SE*: somatic embryos; *ZE*: zygotic embryos; *S*: seeds; *NC*: nucellar cells; *R*: roots; *EC*: embryogenic cultures; *EG*: ethylene glycol; *sor*: sorbitol; *BSA*: bovine serum albumin; *DMSO*: dimethylsulfoxide; *suc*: sucrose; *PEG*: polyethylene glycol.

Table 3.2 Examples of plant species cryopreserved using encapsulation–vitrification techniques. Unless specified otherwise, the developed protocols are for shoot tips

Plant species	Treatments	Recovery	Reference
Ananas comosus	PC, LD, PVS3, RF	54–83%	Gamez-Pastrana et al. 2004
Fragaria x ananassa Duch	CA, LS, PVS2, RF	70.3–90.0%	Hirai et al. 1998
Gentiana spp.	CA, PC, LS, PVS2, RF	43.3–93.3%	Tanaka et al. 2004
Ipomoea batatas	PC, LD, PVS2, RF	82–95%	Hirai and Sakai 2003
Malus domestica	sucr+EG, RF	64–77%	Paul et al. 2000
Manihot esculenta	PC, PVS2, RF	57–86%	Charoensub et al. 2004
Mentha spicata	LD, PVS2, RF	73–97%	Hirai and Sakai 1999
Olea europaea (SE)	LD, PVS2, RF	64%	Shibli and Al-Juboory 2000
Poncirus trifoliata x Citrus sinensis	LD, PVS2, RF	100%	Wang et al. 2002
Prunus domestica	PC, PVS2, RF	47.5%	De Carlo et al. 2000
Solanum tuberosum	PC, LD, PVS2, RF	41–71%	Hirai and Sakai 1999
Wasabia japonica	LD, PVS2, RF	95%	Matsumoto et al. 1995a

CA: cold acclimation; *PC*: preculture; *LD*: osmoprotection (loading); *PVS2*: treatment with PVS2; *PVS3*: treatment with PVS3; *RF*: rapid freezing; *CS*: cell suspension; *PP*: protoplasts; *EA*: embryonic axes; *SE*: somatic embryos; *ZE*: zygotic embryos; *S*: seeds; *NC*: nucellar cells; *R*: roots; *EC*: embryogenic cultures; *EG*: ethylene glycol; *sor*: sorbitol; *BSA*: bovine serum albumin; *DMSO*: dimethylsulfoxide; *suc*: sucrose; *PEG*: polyethylene glycol.

3.4 Preconditioning

In vitrification methods cells and meristems must be sufficiently dehydrated with PVS2 at 25°C or 0°C without causing injury to enable vitrification upon rapid cooling in LN. Many papers demonstrated that cells and meristems conditioned to withstand the dehydration of PVS2 (the treated

control without cooling to –196°C) survived subsequent rapid cooling and rewarming during the vitrification procedure with little or no additional loss (Yamada et al. 1991; Niino et al. 1992a, b; Matsumoto et al. 1994; Reinhoud 1996). It is still unclear if the constituents of PVS2 penetrate cells during the dehydration process. Steponkus et al. (1992) stated that neither sucrose nor glycerol could penetrate the cytoplasmic domain with such a short duration of loading treatment. Kim (2004) indicated that DMSO might penetrate the cell wall and plasmalemma: it was unclear if DMSO penetrated the cytoplasm during treatment with PVS2.

In any vitrification method, procedures such as preconditioning, preculture, osmoprotection (loading treatment), PVS2 exposure time, and post-LN handling are vital. The cells and tissues to be cryopreserved must be in a physiologically optimal status for the acquisition of dehydration tolerance and to produce vigorous recovery of growth (Withers 1979; Dereuddre et al. 1988). Thinh (1997) demonstrated the importance of the structure of shoot tips used as explants for the cryopreservation of tropical monocot species. Shoot tips with the apical dome partially covered (PC type) are a key for attaining high post-LN regrowth (Fig. 3.2). In addition, the growth stage of shoot tips grown *in vitro* was a key factor in obtaining more regrowth after cryopreservation.

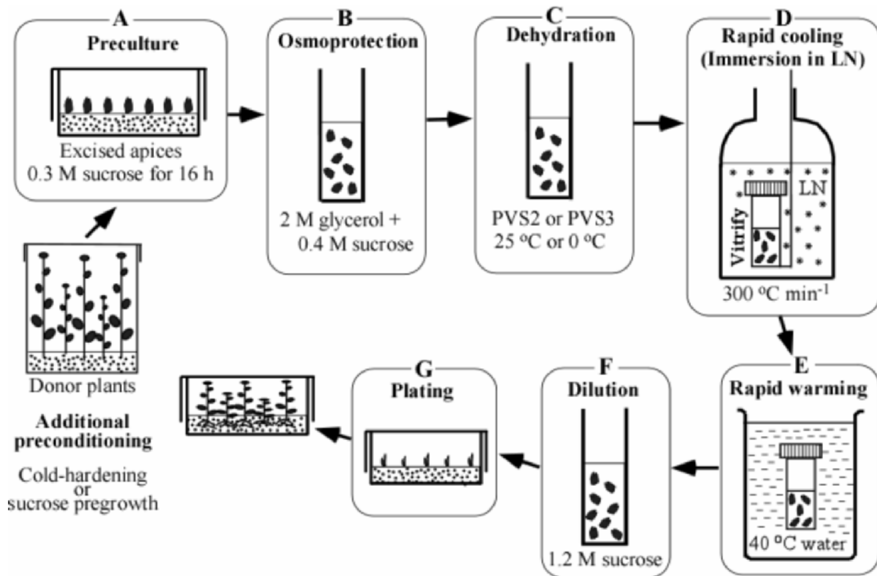

Fig. 3.1 Vitrification procedure for *in-vitro* grown meristems

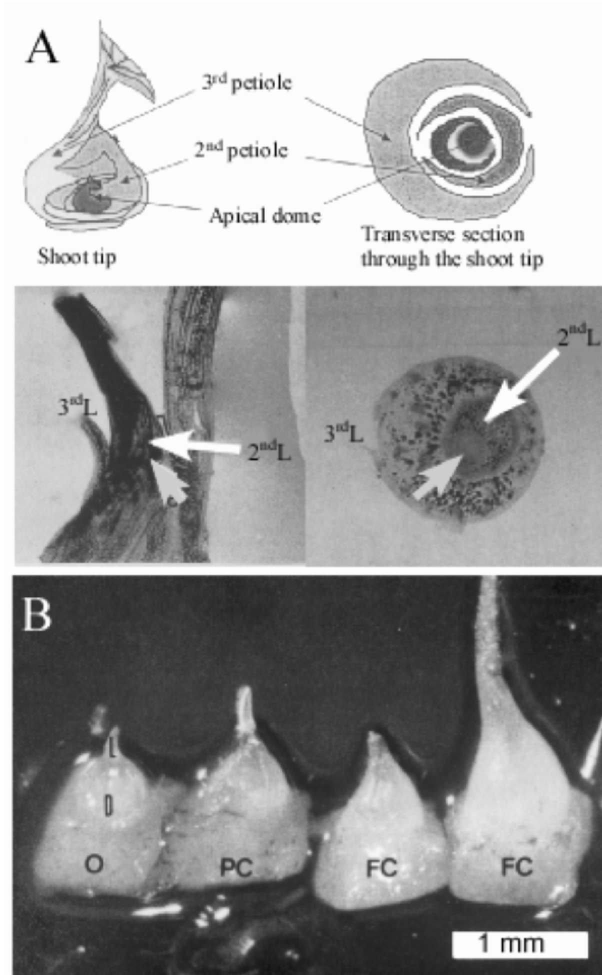

Fig. 3.2 A: Longitudinal (left) and transverse (right) hand-cut sections (lower) and diagrams (upper) across the apical dome of taro. (Thinn 1997)
B: Banana shoot tips. O: Meristems with apical domes uncovered; PC: partly covered; and FC: fully covered. PC-type shoot tips showed the highest rate of dehydration tolerance to PVS2 and recovery of growth after rapid cooling in LN. (Thinh 1997)

3.5 Preculture

3.5.1 Herbaceous Plants

Preculturing meristems excised from plantlets grown *in vitro* on solid medium with 0.3–0.7 M sucrose for 1–2 days was very effective in improving the regrowth of cryopreserved meristems (Dereuddre et al. 1988; Niino et al. 1992a). During preculture the sucrose concentrations in the plants increase significantly (Uragami et al. 1990; Dereuddre et al. 1991). The accumulation of endogenous cryoprotectants such as sugar and sugar alcohol may increase the stability of membranes under conditions of severe dehydration (Crowe et al. 1984a, b). Reinhoud (1996) cryopreserved cultured tobacco cells by vitrification, and clearly demonstrated that the development of tolerance to PVS2 in tobacco cells during preculture with 0.3 M mannitol solution for 1 day was because of the response of cells to mild osmotic stress caused by the preculture. During preculture, there is production of abscisic acid, proline, and certain proteins (late embryogenesis abundant proteins), and the uptake of mannitol. Preculture of innala buds in 0.3 M sucrose for 2 days at 25°C produced the greatest regrowth of shoots (Table 3.3) (Niino et al. 2000a).

Table 3.3 Effect of the duration of preculturing on the survival of lateral buds of innala nodal segments following PVS2 vitrification (Niino et al. 2000a, revised). Nodal segments were precultured with 0.3 M sucrose for various periods of time at 25°C. These precultured segments were osmoprotected with a mixture of 2 M glycerol and 0.4 M sucrose for 20 min at 25°C, and then dehydrated with PVS2 for 18 min at 25°C before being plunged into LN. S.E.: standard error

Preculture duration	Survival (% ± S.E.)
No preculture	0.0 ± 0.0
0.3 M sucrose 1 day	33.3 ± 5.4
0.3 M sucrose 2 days	78.3 ± 2.0
0.3 M sucrose 3 days	40.0 ± 3.5
0.3 M sucrose 4 days	8.3 ± 5.4

3.5.2 Woody Plants

Vitrification was successfully applied to cold-acclimated and precultured apical meristems of woody plants grown *in vitro* (Tables 3.1; 3.2). Cold acclimating at 5°C for 3 weeks under an 8 h photoperiod significantly

improved the recovery of shoot tips from apple, pear, and mulberry cooled to –196°C by vitrification (Niino et al. 1992a, b). Preculture of apple shoot tips on 0.7 M sucrose at 5°C for 1 day after an 8 days cold acclimation was effective at producing high levels of shoot formation (Table 3.4). Five apple species or cultivars, eight pear cultivars (Niino et al. 1992a), *Ribes* (1 week cold acclimation and subsequent 2 days preculture with 5% DMSO; Reed 1992; Luo and Reed 1997), tea plants (Kuranuki and Sakai 1995), five cultivars of cherry (Niino et al. 1997), and 11 species or cultivars of mulberry including southeast Asian cultivars were cryopreserved with PVS2 (Niino et al. 1992b). Thus, in these temperate woody plants, preculturing following cold acclimation appears to be a necessary step in producing a high level of shoot formation.

Table 3.4 Effect of cold acclimation and preculturing on the shoot formation of vitrified apple shoot tips (Niino et al. 1992a, revised). Cold acclimation: 3 weeks at 5°C (8 h day photoperiod). Preculture: on MS medium with 0.7 M sucrose at 5°C. Shoot tips (2 mm in length) were treated with PVS2 for 80 min at 25°C. Shoot formation: percentage of shoot tips producing shoots 40 d after plating. Material: *Malus domestica* cv. Fuji. S.E.: standard error

Preculture (days)	Shoot formation (% ± S.E.)	
	Non hardened	Hardened
0	10 ± 5	62.5 ± 7.5
1	25 ± 5	80.0 ± 0.0
2	10 ± 5	57.5 ± 2.5

Cryogenic protocols for shoot tips were investigated and optimized by Touchell and Dixon (1996) using rare and endangered Australian woody plants such as *Grevillea scapigera*. Meristems were precultured with 0.6 M sorbitol for 2 days at 23°C, dehydrated with PVS2 for 30 min at 0°C, and then plunged into LN. This successful vitrification protocol developed for *G. scapigera* was tested on another 18 rare and endangered Australian species. The shoot tips of 12 species were successfully cryopreserved, with 6 species showing a high capacity to regenerate shoots.

3.5.3 Tropical Plants

In some tropical plants, the preconditioning of meristem-donor shoots on solidified medium enriched with sucrose was very important in producing a high regrowth after cryopreservation (Engelmann 1991; Dumet et al. 1993a, b; Panis 1995). Thinh (1997) demonstrated that taro meristems cryopreserved by vitrification using PVS2 produced nearly 100% recovery of shoot growth when the meristem donor shoots were cultured on MS medium with 60–120 g l^{-1} sucrose for one month, and osmoprotected before dehydration with PVS2.

The same preconditioning was successfully applied to the meristems of banana (two genotypes: Cavendish AAA and Vietnamese Chuoi Huong AAB). The regrowth of vitrified banana meristems cooled to –196°C was 60–80%. Thus pregrowth is an additional promising step to be included in the vitrification procedure of some tropical plants. This vitrification procedure, with slight modification, was successfully applied by Thinh (1997) to about 20 tropical monocotyledonous plants including taro (8 cv.), banana (10 cv.), pineapple (2 cv.), and orchid (2 cv.). It is very interesting to note that the same vitrification procedure, with slight modification, is applicable to a wide range of tropical plants from several genera and families.

3.6 Osmoprotection (Loading Treatment)

For many herbaceous species, preculture with only 0.3 M sucrose for 1–5 days does not produce high recovery following vitrification. A solution of 2 M glycerol and 0.4 M sucrose in growth medium is very effective at inducing tolerance to freeze dehydration or cryoprotectant induced dehydration (Sakai et al. 1991a; Nishizawa et al. 1992). This osmoprotective solution and 50% PVS2 produced the greatest shoot formation in vitrified meristems of wasabi (Table 3.5) (Matsumoto et al. 1994). Similar results were seen with mint axillary shoot-tip meristems cooled to –196°C by encapsulation vitrification. This treatment was successfully applied to other meristems such as lily (Matsumoto et al. 1995b), statice (Matsumoto et al. 1998), hairy roots (Yoshimatsu et al. 1996), taro (Takagi et al. 1997), and innala (Niino et al. 2000a). Pretreatment was very effective in tropical monocotyledonous species (meristems) such as taro, tannia, banana, pineapple, and orchid (Thinh 1997). Several osmoprotectants were effective for *Ribes* shoot tips including sucrose and bovine serum albumen (Luo and Reed 1997).

Table 3.5 Effect of the type of osmoprotective solution used on the shoot formation of PVS2 vitrified wasabi meristems. (Matsumoto et al. 1994, revised). Meristems were precultured with 0.3 M sucrose for 16 h and treated with different solutions for 20 min at 25°C before dehydration with PVS2 for 10 min at 25°C. They were then plunged into LN. S.E.: standard error

Osmoprotection solution	Shoot formation (% ± S.E.)		
2 M glycerol	46.7	±	8.8
0.4 M sucrose	20.0	±	0.0
2 M glycerol + 0.4 M sucrose	86.3	±	3.2
40% PVS2	50.0	±	11.5
50% PVS2	80.0	±	5.8
60% PVS2	33.3	±	14.5

3.7 Exposure Time to PVS2

The one of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity or excess osmotic stresses during treatment with PVS2. Optimizing the time of exposure, or the temperature during exposure to PVS2, is important for producing a high level of shoot formation after vitrification. To determine the optimal time of exposure to PVS2, precultured wasabi meristems (1 mm) were treated with PVS2 at 25°C or 0°C for different periods of time before being plunged into LN. Exposure to PVS2 produced a time-dependent shoot formation rate (Fig. 3.3). With exposure at 25°C, shoot formation increased greatly with increasing dehydration and reached the highest (about 95%) at 10 min. There was a slight decrease at 20 min, followed by a rapid decrease due to toxic effects. In meristems treated at 0°C, the treated controls (without cooling in LN) retained very high shoot formation up to 60 min. In vitrified meristems shoot formation was best during 30–50 min. These results demonstrate that vitrification does not cause additional loss of shoot formation beyond that produced during dehydration by PVS2 (Fig. 3.3). Thus tolerance to PVS2 is sufficient for meristems to survive vitrification under optimized conditions. In the meristems of taro and banana the injurious effects caused by PVS2 were eliminated or reduced by treatment with PVS2 at 0°C (Thinh 1997). In many vitrified cells and meristems, PVS2 treatment at 0°C was required to reduce injurious effects. Such treatment produced high recovery, even in tropical materials (Nishizawa et al. 1993; Kuranuki and Sakai 1995; Yoshimatsu et al. 1996; Thinh 1997). In many herbaceous plants the optimum exposure time to PVS2 is 10–25 min at 25°C (Matsumoto et al. 1994; Takagi et al. 1997). In excised wasabi meristems,

the optimal exposure time to PVS2 was 10 min at 25°C or 30–60 min at 0°C (Fig. 3.3). In apple shoot tips (2 mm long, 1.5 mm base diameter) consisting of the apical meristem and four or five leaf primordia, the optimum exposure time to PVS2 was 80–90 min at 25°C (Niino et al. 1992a) (Fig. 3.4). In tea (*Camellia sinensis*) shoot tips (2 mm long) excised from cold-acclimated plantlets, similar results were obtained (Kuranuki and Sakai 1995). Exposure time to PVS2 may be associated with the size of excised meristems (Niwata 1995; Niino et al. 2000a, 2003a). It also appears to be considerably species specific at the same temperature (Niino et al. 1992a, 1997; Matsumoto et al. 1994). PVS2 treatment to meristems smaller than 0.5 mm often results in toxic effects and meristems larger than 3 mm require a much longer time for dehydration.

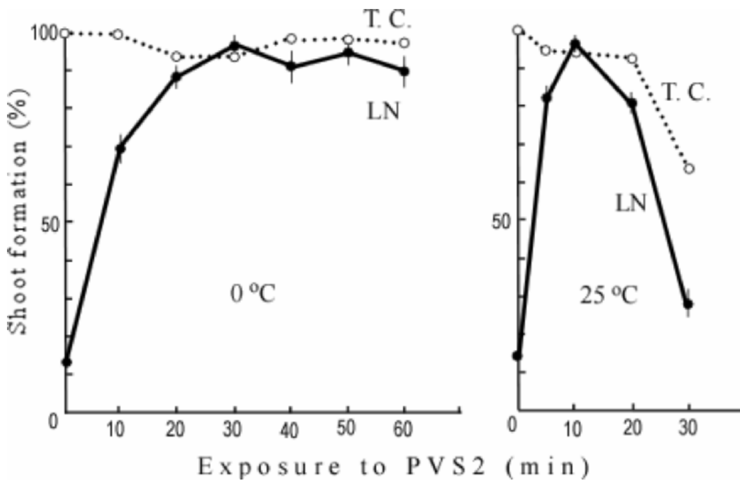

Fig. 3.3 Effect of exposure time to PVS2 at 0°C or 25°C on the shoot formation of wasabi ‘Shimane No.3’ meristems cryopreserved by vitrification. Meristems (1 mm size) were precultured with 0.3 M sucrose and then osmoprotected with a mixture of 2 M glycerol plus 0.4 M sucrose for 25 min at 20°C. These meristems were treated with PVS2 at 25°C or 0°C for different lengths of time before being plunged into LN. TC: treated control, same as treated with PVS2 without cooling to –196°C (Matsumoto et al. 1994, revised). Bars represent standard error

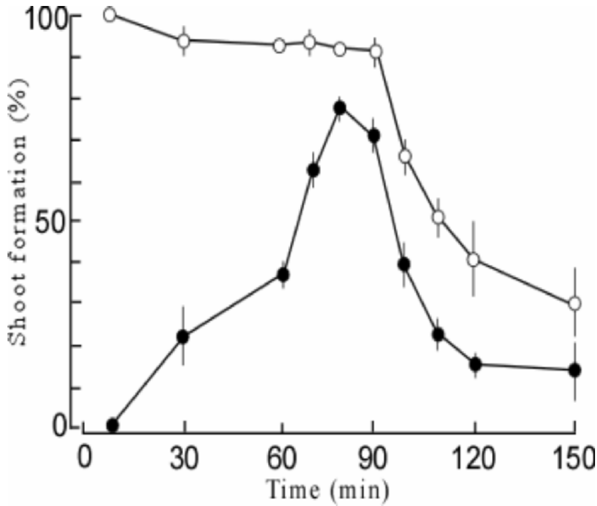

Fig. 3.4 Effect of PVS2 exposure at 25°C on the shoot formation of apple (*Malus domestica* cv. Fuji) shoot tips cryopreserved by vitrification. Cold acclimated (for 3 weeks at 5°C) shoot tips precultured with 0.7 M sucrose at 5°C for 1 day were treated with PVS2 for different lengths of time at 25°C before being plunged into LN (Niino et al. 1992a, revised). o control, • vitrified (LN). Bars represent standard error

3.8 Regrowth

It is particularly important that cryopreserved meristems produce plants identical to their untreated phenotypes. A callus phase before shoot formation is undesirable because callusing potentially increases the frequency of genetic variants. Successfully vitrified and warmed meristems of many plants tested with the PVS-based vitrification protocol remained green after plating, resumed growth within a few days, and developed shoots directly without intermediary callus formation (Yamada et al. 1991; Niino et al. 1992a, b; Matsumoto et al. 1994, 1995b). Fluorescence microscopic examinations of longitudinal sections through the meristematic dome of vitrified meristems after 3 d of reculture revealed that the domes in most meristems appeared to be viable. This was based on fluorescein diacetate staining (Yamada et al. 1991; Matsumoto et al. 1994). In addition, little or no morphological abnormalities were observed in plants that developed from vitrified apical meristems (Yamada et al. 1991; Niino et al. 1992a, b; Matsumoto et al. 1994, 1995a, b). Touchell and Dixon (1996) reported that vitrification of the shoot tips of *G. scapigera* produced high-quality regrowth (70–80%). The same results were obtained with *Dianthus* and *Chrysanthemum* (Fukai 1992).

3.9 Encapsulation Vitrification

It order to carefully treat a large number of meristems at the same time with vitrification protocols, we developed an encapsulation-vitrification method (Matsumoto et al. 1995a). In this procedure, meristems precultured on 0.3 M sucrose were trapped within alginate beads (diameter: 3–4 mm) containing 2–3% (w/v) sodium alginate, 2 M glycerol, and 0.4 M sucrose in culture medium without calcium (Fabre and Dereuddre 1990). Encapsulated meristems were placed in a solution of 2 M glycerol and 0.4 M sucrose in liquid medium for 30 min. They were dehydrated with PVS2 in a 100 ml glass beaker on a rotary shaker at 100 rpm for 70–100 min at 0°C. About 10 encapsulated-dehydrated meristems were suspended in 0.7 ml PVS2 in 2 ml cryotubes and the cryotubes were then plunged into LN. For warming, the cryotubes were transferred to a 40°C water bath for 1 min. PVS2 was drained from the tubes and 2 ml of 1.2 M sucrose solution was added and replaced once with fresh solution. Washed encapsulated–vitrified meristems were transferred onto solidified 1/2 MS medium (Murashige and Skoog 1962) for regrowth (Table 3.6). The encapsulation–vitrification method is easy to manage and eliminates the time required for air desiccation. This method was successfully applied to the meristems of statice (Matsumoto et al. 1998), lily (Matsumoto et al. 1995b), strawberry (Hirai et al. 1998), mint (Hirai and Sakai 1999a), potato (Hirai and Sakai 1999b), gentian (Tanaka et al. 2004), and horseradish hairy roots (Phunchindawan et al. 1997). Thus, the encapsulation–vitrification procedure shows promise as a practical cryopreservation method.

Table 3.6 Shoot formation of wasabi meristems cooled to –196°C by three cryogenic protocols. (Matsumoto et al. 1995a, revised)

Cryogenic protocol	**Shoot formation** (% ± S.E.)	**Shoot length** (mm size ± S.E.)
Vitrification[1]	97.5 ± 1.0	10.6 ± 4.0
Encapsulation–vitrification[2]	96.7 ± 2.9	12.2 ± 3.6
Encapsulation–dehydration[3]	67.1 ± 8.9	6.3 ± 3.6

Preculture: 0.3 M sucrose for 1 d; [1, 2]: Osmoprotected with 2 M glycerol plus 0.4 M sucrose for 20 min at 25°C; PVS2 treatment for 50 and 100 min at 0°C; [3]: treated with 0.8 M sucrose for 16 h before air-drying for about 7 h. Shoot length: 30 d after reculture. S.E.: standard error

3.10 A Personal View of the Development of PVS2 by Akira Sakai

Until about the middle of 1980, the controlled rate cooling method (Sakai 1960; Sakai and Nishiyama 1978) was used for storing cultured cells and apical meristems in liquid nitrogen. The materials were first cooled to –30 to –40°C for sufficient freeze-dehydration before cooling in liquid nitrogen. A programmable freezer is needed in this method because the material needs to be cooled at a constant rate for freeze dehydration. The method could not be used for materials that were not frost hardy. There was thus an urgent need to develop a relatively simple and efficient method that could replace the conventional method, especially for storing the genetic resources of tropical plants.

3.10.1 The Ultimate Goal—Storing Tropical Plants

The practical problem that I set myself to solve was the development of a technique in which 1 mm long apical meristems removed from a cultured plant were dehydrated osmotically for several minutes at room temperature in a concentrated vitrifying solution containing glycerol and sugar, cooled rapidly from room temperature by plunging into liquid nitrogen for vitrification, while they were still alive, and regrown into plants when required. In 1988, as a retired scientist I had no possibility of obtaining government aid for scientific research so I decided to work on this project using my own financial resources. It was the spring of my 68th year (5 years after retirement from Hokkaido Univ.). I had neither a laboratory nor co-workers. The first thing I did after returning was to purchase a PC and software so that I could prepare documents in Japanese and English. I was handling a PC for the first time. At home I read and understood the unfamiliar manual little-by-little and worked on the keyboard with two fingers. I did not want to become a "*Technobsolete*" a name given in Japan to a technologically obsolete person. This effort enabled me to prepare papers in English at home and exchange information with scientists within and outside the country through e-mail.

3.10.2 The Core of the Technology Development

To keep the about 1 mm long apical meristems excised from cultured plants alive at –196°C, they must be vitrified by cooling in liquid nitrogen after dehydration in a concentrated vitrification solution. Two problems

needed to be solved before this could be accomplished. The first was to develop an osmotic dehydrating solution (vitrification solution) that caused minimum chemical damage to the tissue. The other challenge was to provide dehydration resistance to plants that were cultured under optimum growth conditions at 25°C and therefore did not have such resistance.

Thus, firstly I had to find an effective vitrification solution. I sought the assistance of Dr. S. Kobayashi, an old acquaintance of mine working at in Akitsu a substation of the National Fruit Tree Research Station. I went to this substation and made combinations of several solutes in different proportions to prepare several vitrification solutions. I immersed nucellar embryo cells of orange in these solutions for several minutes for dehydration and plunged them into liquid nitrogen for rapid cooling. The suitability of these solutions was assessed from the survival of the cells. (Dr. Kobayashi is a specialist of nucellar cells, of *Citrus*). After cryopreservation of nucellar cells, Dr. Kobayashi determined the viability under the fluorescence microscope. I traveled from Sapporo where I lived, to Akitsu (by air and train) five times in a year, staying in a hotel each time for about 5 days and conducted these experiments. Initially, the experiments were one failure after another, but on the last day of my 5th visit to Akitsu at the end of October, 1988, I could finally get 90% survival. I named the vitrification solution that gave this result Plant Vitrification Solution 2 (PVS2). This was a glycerol-based concentrated (approximately 8 molar) solution.

Luckily the nucellar embryo cells of orange had a high level of dehydration resistance. When the cells were immersed in PVS2 for 2 min for dehydration and then cooled rapidly in liquid nitrogen, both the cells and the PVS2 vitrified. When this was rapidly thawed in warm water, about 90% of the cells were viable and regenerated by Kobayashi. However, many other cultured cells and apical meristems which did not have dehydration resistance could not survive this procedure. The next challenge was to impart dehydration resistance to excised apical meristems. First I tried culturing such meristems for 16 h in an agar medium containing 0.3M sucrose so that a large amount of sugar was taken up by the cells of the meristem. But adding sugar to the culture medium alone was not sufficient to make these cultured meristematic cells withstand the strong osmotic dehydration in the highly concentrated PVS2. I then treated the meristems cultured in the sugar-containing medium, in an osmoprotective solution (LS) with 2 M glycerol and 0.4 M sucrose per liter for 20 min. The dehydration resistance of the apical meristems could be sufficiently increased by this treatment in LS and they could be kept viable at −196°C for the first time. I first tried cryopreservation of apical meristem cultures of white clover by PVS2 protocol. Firstly, meristems (about 1 mm long) were cryopreserved by

PVS2 protocol. About 90% of the meristems were viable and grew normally (90% mean recovery).

3.10.3 Success with Vitrification of Tropical Plants

Unlike those of temperate plants, the cultured apical meristems of tropical plants were considered difficult to store at –196°C. There were only a few cases of success with tropical plants. At the end of March 1995, at the request of Dr. Takagi of the Ishigakijima branch of the Japan International Research Center for Agricultural Sciences (JIRCAS), I delivered a lecture and gave a practical demonstration on vitrification. At that time, one Mr. N.T. Thinh from Vietnam was on a research assignment at the Center as was Mr. Hirai from Hokkaido. About 1 month after that I received a communication from Mr. Thinh that meristems of *Colocasia esculenta* L. taro (an aroid) cooled to –196°C by vitrification could be regrown after warming with good success. Thereafter Mr. Thinh succeeded in liquid nitrogen storage of the meristems of about 30 tropical monocotyledonous crops one after the other, including banana (eight varieties), pineapple, and orchids, using the vitrification method developed by us. With his success, liquid nitrogen storage became possible with tropical plants using the vitrification process. Thus the apical meristems of cultured plants, whether temperate or tropical, could be vitrified by more or less the same method and stored for a long time in liquid nitrogen. In short, it became clear that the dividing tissue in the apical meristem of both tropical and temperate plants could exhibit a high level of dehydration resistance if treated suitably.

3.10.4 Fifteen years of Research

These studies that I started in 1988 without a laboratory or research grant luckily interested about 15 bright young researchers working in agricultural research stations in different regions in Japan and continued for about 15 long years. As a result of these studies more than 200 species and varieties could be successfully stored in liquid nitrogen by vitrification. I believe that these positive results could be achieved because I took the plunge into this line of research and persisted in my efforts. I had the good fortune of having many brilliant research collaborators both in Japan and abroad. I could exchange information with my collaborators in different parts of the world easily through fax and e-mail while sitting at home. These advances in information technology acted like a wind on our back and I felt the force of the changing times. Forty-six years have passed after

I first succeeded, in 1956, in cryopreserving willow shoots in liquid nitrogen, which had opened the possibility of long-term storage of plants.

My wish, as I approach the twilight of my life, is to have a few more years of active life and to contribute to the training of researchers from the tropical regions, including Southeast Asia, and also to engage in international collaborative work in order to contribute to putting international collaborative projects on the storage and development of tropical plant germplasm on proper tracks.

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Chapter 4
The Development of Encapsulation Dehydration

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4.1 Introduction

The application of cryopreservation to plants is relatively recent as the first report of successful cryopreservation was published by Sakai in 1960 with silver birch twigs, and *in-vitro* cultured flax cells were frozen by Quatrano in 1968. The first protocols developed in the 1980s included pre-treatment with cryoprotectants followed by controlled rate cooling. These protocols were based on freeze-induced dehydration (Sakai 1985; Kartha and Engelmann 1994; Engelmann 1997). Such protocols were applied to numerous species, especially from temperate origin; however, there were cases, particularly for plants of tropical origin, where such controlled cooling protocols did not produce good results (Bagniol et al. 1992; Haskins and Kartha 1980). Further research was thus carried out and at the beginning of the 1990s a set of new, vitrification-based protocols became available (Engelmann 2000, 2003). Vitrification can be defined as the transition

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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of water directly from the liquid phase into an amorphous phase or glass, while avoiding the formation of crystalline ice (Fahy et al. 1984). Among these vitrification techniques a new technique termed encapsulation dehydration was developed for cryopreservation of pear and potato shoot-tips (Dereuddre et al. 1990; Fabre and Dereuddre 1990). This method is based on the technology developed for producing synthetic seeds, i.e. the encapsulation of explants in calcium alginate beads (Redenbaugh et al. 1986). Encapsulated explants are then precultured in liquid medium with a high sucrose concentration and partially desiccated before exposure to liquid nitrogen (LN). Encapsulating the explants allows exposure to extreme treatments including preculture with high sucrose concentrations and desiccation to low moisture contents (MCs) that would be highly damaging or lethal to non-encapsulated samples. Due to the extreme desiccation of explants, most or all freezable water is removed from cells, and vitrification of internal solutes takes place during rapid exposure to LN, thus avoiding lethal intracellular ice crystallization (Engelmann 1997). As a consequence, the whole or a large part of the frozen explant is kept intact after rewarming, which results in high survival, rapid and direct regrowth and reproducible results after cryopreservation (Engelmann 2000).

The encapsulation–dehydration technique was applied to other species from temperate climates including shoot tips of eucalyptus (Poissonnier et al. 1991) and grape (Plessis et al. 1991) and carrot somatic embryos (Dereuddre et al. 1991). Later, it was experimented with plants from tropical origin, notably apices of cassava (Benson et al. 1992), sugarcane (Gonzalez-Arnao et al. 1993a, b; Paulet et al. 1993) and coffee (Mari et al. 1995). The technique was successfully extended to over 70 different plant species (Table 4.1). In this chapter we present and discuss the optimal conditions of the successive steps of an encapsulation–dehydration protocol and make a brief overview of the current application of the technique.

Table 4.1 Plant species whose shoot tips (or other explants – specified in the column Plant species) were cryopreserved using the encapsulation–dehydration technique

Plant species	Reference
Acacia mangium	Sudarmonowati and Rosmithayani 1997
Actinidia chinensis	Wu et al. 2001
Actinidia chinensis	Suzuki et al. 1996
Actinidia spp.	Bachiri et al. 2001
Amygdalus communis L.	Al-Ababneh et al. 2003
Anacamptis morio seeds + fungal symbiont	Wood et al. 2000
Anthirrinium microphyllum	Gonzalez-Benito et al. 1998
Armoracia rusticana hairy root cultures	Hirata et al. 1995

Armoracia rusticana hairy root cultures	Phunchindawan et al. 1997
Auricularia	Hirata et al. 1996
Beta vulgaris	Vandenbussche and De Proft 1996
Brassica napus microspore embryos	Uragami 1993
Camellia sinensis L.O. Kuntze	Aoshima 1997
Catharantus roseus cell suspension	Bachiri et al. 1995
Centaurium rigualii Esteve	Gonzales-Benito and Perez 1997
Ceratopetalum gummiferum	Shatnawi and Johnson 2004
Chichorium intybus	Vandenbussche et al. 1993
Chrysanthemum morifolium	Sakai et al. 2000
Citrus aurentium	Al-Ababneh et al. 2002
Citrus madurensis	Cho et al. 2002
Citrus spp.	Gonzalez-Arnao et al. 1998
Citrus spp. Embryonic axes	Santos and Stushnoff 2002
Citrus spp. Ovules and somatic embryos	Gonzalez-Arnao et al. 2003
Cocos nucifera	Hornung et al. 2001a
Coffea racemosa	Mari et al. 1995
Coffea sessiliflora	Mari et al. 1995
Cosmos atrosanguineus	Wilkinson et al. 1998
Cynodon sp	Reed et al. 2006
Dactylorhiza fuchsii seeds + fungal symbiont	Wood et al. 2000
Daucus carota somatic embryos	Dereuddre et al. 1991
Dianthus caryophyllus L.	Tannoury et al. 1995
Dianthus hybridus cv Sakuranadesiko	Fukai et al. 1994
Digitalis obscura	Sales et al. 2001
Dioscorea alata	Malaurie et al. 1998
D. bulbifera	Malaurie et al. 1998
Dioscorea spp.	Mandal et al. 1996
Ekebergia capensis	Perán et al. 2006
Eucalyptus gunnii	Poissonnier et al. 1991
Eucalyptus sp.	Pâques et al. 1997
Eucalyptus grandis x E. camaldulensis	Blakesley and Kiernan 2001
Fragaria x ananassa	Clavero-Ramirez et al. 2005
Holostemma annulare	Decruse et al. 1999
Iopomea batatas	Pennycooke and Towill 2001
Iris nigricans	Shibli 2000
Laminaria digitata L. gametophytes	Vigneron et al. 1997
Lilium	Matsumoto and Sakai 1995
Lolium sp.	Chang et al. 2000
Malus spp.	Zhao et al. 1999
Malus x domestica Borkh.	Paul et al. 2000; Wu 1999
Manihot esculenta Crantz.	Manrique 2000; Escobar-Pérez 2005
Medicago sativa L. cell suspensions	Shibli et al. 2001
Mentha spicata	Sakai et al. 2000
Microalgae	Hirata et al. 1996
Morus bombysis	Niino et al. 1992
Olea europaea somatic embryos	Shibli and Al-Juboory 2000
Oncidium bifolium seeds and protocorms	Flachsland et al. 2006
Paeonia lactiflora Pall. zygotic embryos	Kim et al. 2004
Pelargonium spp.	Grapin et al. 2003
Polygonium aviculare cell suspension	Swann et al. 1998

Poncirus trifoliata (L.) Raf. *x Citrus sinensis* (L.)	Wang et al. 2002a
Populus tremula x alba	Accart et al. 1993
Primula pubescens	Hornung et al. 2001b
Prunus dulcis	Shatnawi et al. 1999
Pyrus communis	Dereuddre et al. 1990
Pyrus communis cv Beurré Hardy	Scottez et al. 1992
Pyrus syriaca	Tahtamouni and Shibli 1999
Quercus ilex embryonic axes	Gonzales-Benito et al. 1999
Quercus suber embryonic axes	Gonzales-Benito et al. 1999
Ribes spp.	Reed and Yu 1995; Reed et al. 2005
Rhododendron simmsii Planch.	Verleysen et al. 2005
Rubus idaeus L	Wang et al. 2005
Rubus spp.	Gupta and Reed 2006
Saccharum spp.	Gonzalez-Arnao et al. 1993a, b
Saintpaulia ionantha Wendl.	Moges et al. 2004
Salix	Blakesley et al. 1996
Solanum spp.	Fabre and Dereuddre 1990
Solanum tuberosum	Grospietch et al. 1999
Sygysium francissi	Shatnawi et al. 2004
Theobroma cacao L. somatic embryos	Fang et al. 2004
Vaccinium pahalae (Ohelo) cells	Shibli et al. 1998
Vinca minor L. hairy root cultures	Hirata et al. 2002
Vitis spp.	Wang et al. 2000
Vitis vinifera L.	Plessis et al. 1991
Vitis vinifera L.	Zhao et al. 2001
Vitis vinifera L. embryogenic cell suspension	Wang et al. 2002b
Vitis vinifera L. somatic embryos	Miaja et al. 2004
Wasabia japonica	Matsumoto et al. 1995
Zoysia sp.	Chang et al. 2000

4.2 Successive Steps of the Protocol

An encapsulation–dehydration protocol comprises the successive steps listed below. Conditions for each step require optimization to achieve maximal recovery of explants after cryopreservation. The basic steps of preconditioning, preculture, encapsulation, osmoprotection and dehydration before exposure to LN apply in all cases.

4.2.1 Physiological Status of the Plant Material

The physiological status of the plant material is of paramount importance for the success of cryopreservation. Samples are generally excised from actively growing mother plants, thus ensuring that they are composed of actively dividing meristematic cells (Engelmann 1997; Escobar et al.

1997). For example, kiwi are excised 14 days and *Citrus* shoot tips 20 days after the last subculture (Bachiri et al. 2001; Gonzalez-Arnao et al. 1998), i.e. when *in vitro* plantlets are in full growth. On the opposite side, apple shoot tips were sampled on mother plants that were not subcultured for 70 days and grape for 3–4 months (Zhao et al. 1999, 2001). The hypothesis of the authors, to explain the higher recovery noted after cryopreservation of such explants in comparison with those sampled on more frequently subcultured mother plants, is that the moisture content of apices of non-subcultured plants is lower. In the case of somatic embryos, they are cryopreserved at a given developmental stage to optimize recovery. Torpedo stage carrot somatic embryos give the highest recovery (Dereuddre et al. 1991), while with *Citrus* globular, torpedo and heart stage embryos are the material of choice for cryopreservation (Gonzalez-Arnao et al. 2003).

4.2.2 Preconditioning

Preconditioning corresponds to the culture of the mother plants under conditions different from standard ones, which aim at conditioning explants to withstand cryopreservation protocols. Preconditioning can involve various manipulations of the culture conditions such as culturing mother plants at low temperature in the case of cold-tolerant species (Wu et al. 2001; Matsumoto and Sakai 1995; Hirata et al. 1996; Zhao et al. 1999; 2001; Sakai et al. 2000; Dereuddre et al. 1990), or on medium with high sucrose content (Decruse et al. 1999; Grospietch et al. 1999).

4.2.3 Preculture

Preculture corresponds to the culture of the explants for several hours or days after excision and before encapsulation. Sugarcane and coffee apices are cultured overnight on standard solid medium after excision (Paulet et al. 1993; Gonzalez-Arnao et al. 1993a; Mari et al. 1995). Kiwi, strawberry, chrysanthemum and wasabi shoot tips are cultured from one to several days on agar-based medium with a high sucrose concentration (Bachiri et al. 2001; Clavero-Ramirez et al. 2005; Sakai et al. 2000). *Citrus madurensis* shoot tips are first cultured on solid medium with 0.1 M sucrose, then on medium with 0.3 M sucrose and 0.5 M glycerol (Cho et al. 2002). Black currant shoot tips are either cold acclimated for 2 weeks or cultured for 7 days on 0.75 M sucrose solid medium before encapsulation (Reed et al. 2005).

4.2.4 Encapsulation

For preparation of alginate beads the plant material is suspended in calcium-free liquid basal medium with 3% sodium alginate (low viscosity, 250 cps) (Gonzalez-Arnao and Engelmann 2006). This solution generally contains sucrose at the level used in the normal culture medium. In some cases, explants may also be encapsulated in a medium with a slightly higher sucrose concentration. The mixture is dropped with a pipette into liquid culture medium containing a high concentration of calcium chloride (usually 100 mM $CaCl_2$). This induces the polymerization of alginate in presence of an elevated concentration of calcium, thereby producing beads around the explants. These spherical beads are usually 4 or 5 mm in diameter and contain one apex or more. They are held in the calcium solution for 20–30 min after the last bead is formed to guarantee a good polymerization. Bead polymerization can be controlled visually since beads are translucent immediately after their formation and become progressively opaque as polymerization progresses. Beads should be transferred to the sucrose preculture medium only when they are completely opaque, i.e. when polymerization seems to be complete.

4.2.5 Osmoprotection

Osmoprotection corresponds to the treatment of encapsulated explants in medium with sucrose for several hours or days immediately before desiccation and cryopreservation. Beads containing the explants are placed in Erlenmeyer flasks in agitated liquid medium with between 0.50 and 1.25 M sucrose. The sucrose concentration most commonly employed is 0.75 M. Sucrose treatment durations vary from 16 to 18 h as for sugarcane (Paulet et al. 1993; Gonzalez-Arnao et al. 1993a) to 7–10 days in the case of coffee (Mari et al. 1995) and yam (Malaurie et al. 1998). With some plant materials osmoprotection of samples directly in a medium with high sucrose concentration is toxic and produces very low regrowth. In such cases a progressive increase in sucrose concentration by daily transfers of the plant material in medium with higher concentration may result in reducing the toxic effect of the high osmotic stress and in increasing regrowth. Gradual increase of sucrose by 0.25 M incremental steps ensured over 80% survival for shoot tips of grape for 1 M final sucrose concentration and 70% for 1.5 M (Plessis et al. 1991), whereas direct exposure to these media led to very low recovery. Recovery of apple shoot tips was maximal after daily increase of the sugar concentration with the following sequence: 0.1 M/0.3 M/0.7 M/1.0 M (Zhao et al. 1999). Some plant materials are treated with a

mixture of osmoprotectants. Lily shoot tips were protected with a mixture of 0.8 M sucrose and 1 M glycerol (Matsumoto and Sakai 1995) and *Citrus madurensis* apices with 2 M glycerol and 0.6 M sucrose (Cho et al. 2002). In an alternative treatment for cryopreservation of encapsulated explants they are treated with highly concentrated vitrification solutions (Sakai 2000) and frozen directly in LN without air dehydration. This technique, termed encapsulation vitrification, is described in detail by Sakai (see Chap 3).

4.2.6 Dehydration

After osmoprotection beads are rapidly surface dried on sterile filter paper to remove any remaining liquid medium and are submitted to physical dehydration by evaporation at room temperature. Two desiccation methods can be employed: dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel. Desiccation under the laminar flow can produce variable desiccation rates depending on the airflow rate, air temperature, and relative humidity. By contrast desiccation in airtight containers with silica gel provides reproducible conditions from one experiment to the next and is, thus, highly recommended, especially in tropical countries where the relative humidity is usually very high (Gonzalez-Arnao and Engelmann 2006). In general, the bead water content that ensures highest regrowth after cooling in LN is around 20%, which corresponds to the amount of unfreezable water in the samples. At such water contents, only glass transitions are recorded by differential scanning calorimetry when samples are plunged in LN (Sherlock et al. 2005). This value may vary depending on the species and the type of samples. For example, bead water content was 27% for pear shoot tips encapsulated in larger alginate beads (Scottez et al. 1992), 33% for encapsulated meristems of apple and mulberry (Niino and Sakai 1992), and microspore embryos of oilseed rape survived best at 18–20% MC (Uragami 1993).

4.2.7 Cryopreservation

After dehydration, beads are placed in 1 or 2 ml polypropylene sterile cryotubes for cryopreservation. In most cases vitrification is employed by direct immersion of the cryotubes in LN. In some cases, including grape (Plessis et al. 1991; Zhao et al. 2001), potato (Fabre and Dereuddre 1990) and *Citrus* (Gonzalez-Arnao et al. 1998) shoot tips, controlled rate cooling using a programmable freezer followed by immersion of samples in LN was required to obtain higher regrowth after cryopreservation. For storage,

cryotubes are usually immersed in LN or held in LN vapors in storage tanks.

4.2.8 Rewarming

With the encapsulation–dehydration technique, rewarming is usually carried out slowly at room temperature since samples are sufficiently dehydrated before freezing and there is no risk of ice recrystallization upon warming (Gonzalez-Arnao and Engelmann 2006). Thus regrowth becomes independent of the rewarming method. For slow rewarming, beads are taken out of the cryotubes and placed in open Petri dishes in the laminar air flow cabinet for about 5 min or they are transferred directly onto the recovery medium. In cases where rapid warming is performed the cryotubes are stirred in a water bath for 2–3 min at 40°C as in the case of lily and wasabi (Matsumoto and Sakai 1995; Matsumoto et al. 1995), 1 min at 45°C for blackberry (Gupta and Reed 2006) or at 25°C as for mulberry and chicory (Niino et al. 1992; Vandenbussche et al. 1993). In some cases the beads are rehydrated with the addition of liquid culture medium for 5–10 minutes (Chang et al 2000; Gupta and Reed 2005; Reed et al. 2006).

4.2.9 Recovery

Regrowth generally takes place on standard semi-solid culture medium. Explants are transferred under standard conditions as in the case of citrus, apple and almond shoot tips (Gonzalez-Arnao et al. 1998; Zhao et al. 1999; Shatnawi et al. 1999). The composition of the recovery medium may be transitorily modified to eliminate the phenolic compounds produced by dead cells either by adding activated charcoal to the medium as in the case of sugarcane apices (Paulet et al. 1993; Gonzalez-Arnao 1996) or to stimulate proliferation of frozen explants by modifying the growth regulator content of the medium as with sugarcane and yam apices (Paulet et al. 1993; Malaurie et al. 1998). In some cases it is necessary to extract the explants from the beads and to place them directly on the recovery medium to ensure their regrowth, as in the case of microspore embryos of oilseed rape (Uragami 1993), apices of grape (Plessis et al. 1991) and mulberry (Niino et al. 1992). A softer medium is used in some cases to facilitate shoot regrowth (Gupta and Reed 2005; Reed et al. 2006). The environmental conditions are also important for regrowth. It is beneficial to perform the post-warming recovery in the dark for a short period (around 1 week) for organized structures such as meristems (Gonzalez-Arnao and

Engelmann 2006), to prevent or decrease detrimental photo oxidation of cryopreserved samples (Benson 1990).

4.2.10 Viability Assessment

The ultimate viability assessment after a cryopreservation experiment is the direct production of new tissues from the cryopreserved explants. Direct regrowth without intervening callus formation is required to maintain genetic stability. Most plants recovered from encapsulation dehydration grow without the production of callus.

4.3 Current Development and Use of Encapsulation Dehydration

Encapsulation dehydration was successful with over 70 plant species (Table 4.1). However there are few cases where this technique has been tested on a large number of accessions of the same species or species of the same genus. Sugarcane apices of 15 commercial varieties representing a broad genetic diversity were successfully cryopreserved with recovery ranging between 24 and 91% (Gonzalez-Arnao 1996). Apple shoot tips from over 20 commercial cultivars were frozen using the encapsulation–dehydration technique (Wu et al. 1999; Zhao et al. 1999). Shoot tips of 25 genotypes in nine *Rubus* species and nine *Rubus* hybrids representative of the diversity in the genus *Rubus* were successfully cryopreserved with recovery of 60–100% using the encapsulation–dehydration protocol (Gupta and Reed 2006). A wide range of *Ribes* germplasm, including 9 species and 19 cultivars, was tested with encapsulation dehydration with a mean regrowth of 58% (Reed et al. 2005). More than 35 accessions of Bermudagrass (*Cynodon*) were successfully stored by encapsulation dehydration (Reed et al. 2006). The most advanced development and application by far of encapsulation dehydration is with cassava apices. CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia) is responsible for the maintenance of the world germplasm collection of cassava (which includes 5941 accessions). The core collection, which represents 630 clones, was cryopreserved using encapsulation dehydration (Manrique 2000; Escobar Pérez 2005). Around 75% of these clones display recovery above 30%. A safety cryopreserved duplicate of this collection is being established.

4.4 Genetic Stability

The effect of encapsulation–dehydration cryopreservation on the stability of plants was assessed after regrowth for several species. No modifications were revealed at the morphological, agronomical, chromosomal, biochemical and/or molecular levels for sugarcane (Gonzalez-Arnao 1996), apple (Hao et al. 2002), yam (Sangeeta et al. 2002; Sonali-Dixit et al. 2005), kiwi and grape (Zhai et al. 2003).

4.5 Conclusions

Encapsulation dehydration is a very efficient cryopreservation technique which is simple to implement. One of its advantages from a practical point of view is its user-friendliness. Encapsulated explants are very easy to manipulate throughout the freezing protocol. Encapsulation dehydration was successfully implemented with a large number of species, both from temperate and tropical origin. It was applied to a broad number of genotypes within the same species in several cases, thereby demonstrating its efficiency and practicality. Cryopreservation represents the only safe, efficient and cost-effective long-term storage option to facilitate the conservation of genetic resources of plant species. Encapsulation dehydration is one more technique to place in the hands of researchers or genebank curators to meet germplasm conservation goals.

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Chapter 5
Controlled Rate Cooling

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5.1 Basic Concepts of Controlled Rate Cooling

Controlled rate cooling is based on osmotic regulation of cell contents and freeze-induced dehydration. The samples are pretreated in cryoprotectant solutions and cooled at a standard rate to an intermediate temperature such as –35°C or –40°C, with ice nucleation initiated at about –9°C. At the freezing point of the cryoprotectant solution, ice nucleation is initiated, and ice forms in the cryoprotectant solution and the intercellular spaces. The cytoplasm remains unfrozen due to solute concentration and the cell wall protects the cell membrane from damaging ice crystals. As the temperature is further decreased to –35°C or –40°C, the extracellular solution becomes increasingly icy and the intracellular solutes become highly concentrated. The plant cells lose water to the exterior ice and the cytoplasm is further concentrated. The intracellular freezable water is safely reduced before samples are plunged into liquid nitrogen (LN). If the cells are optimally dehydrated, the cytoplasm vitrifies on contact with LN. If the samples are under dehydrated, leaving freezable water in the cytoplasm, ice will form. If over dehydrated, the cells may die from desiccation.

Controlled rate cooling is very efficient for storing suspension and callus cultures, embryogenic cultures, and *in-vitro* shoot tips from temperate and subtropical plants. The advantages of controlled rate cooling include the use of standardized procedures, programmed cooling rates, and large batch sizes, and the effective use of technician time.

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

Several conditioning strategies are used to prepare plant materials for controlled rate cooling. They include osmotic adjustments, cold acclimation and other *in-vitro* culture treatments that last for weeks or entire culture periods (Dumet et al. 1993; Gonzalez-Benito et al. 1998; Meryman and Williams 1985; Thierry et al. 1997). Preconditioning is usually applied as part of a cryoprotection strategy and works best in combination with other factors (Reed 1996). The health of the plant is very important, and standardizing the culture conditions, interval between subcultures, and the size of the shoot tips or cell culture samples can make a difference in the success of the protocol. For organized tissues, skill at meristem dissection is also highly important. Control tissues should grow nearly as well as the untreated cultures (~90%).

5.2.1 Cold Acclimation

The freezing tolerance (cold hardiness) of most temperate and some subtropical species is increased when the plants are exposed to low non-freezing temperatures. This process is termed cold acclimation (CA) or cold hardening when done artificially (Weiser 1970). When naturally acclimated, fully dormant twigs of temperate plants can survive liquid nitrogen temperatures (Sakai 1960; Sakai and Nishiyama 1978).

CA is also very effective in improving regrowth of cryopreserved temperate and subtropical plants. Some of the first efforts to cold harden shoot tips were for only a few days and were not successful. However later studies showed that longer acclimation periods were necessary. Cold acclimation was successfully applied to actively-growing *in-vitro* plants with greatly improved regrowth after cryopreservation (Reed 1988; Reed and Lagerstedt 1987). A 1-week CA with alternating cold and warm temperatures (22°C and 8-h light (10 µE m^{-2} s^{-1}) / –1°C 16-h dark) more than doubled recovery of blackberry shoot tips cryopreserved by controlled rate cooling. Improvement was also seen in the recovery of shoot tips of four pear species where 5% of shoot tips grew after controlled rate cooling when grown at 25°C, but 95% of shoots developed from plants cold-acclimated for 1 week (Reed 1990). A key parameter was the length of the cold acclimation period. CA periods of 4–12 weeks greatly improved the recovery of some pear and blackberry cultivars that had little regrowth with 1–2 weeks acclimation (Chang and Reed 1999; 2000a, b; 2001). This link was genotype dependent. Alternating temperatures were important for the acquisition of cold tolerance by pear shoot tips but the effect of

photoperiod was not significant (Chang and Reed 2000b). Pear plantlets cold acclimated with short warm days and long cold nights became significantly more cold hardy than those acclimated at a constant 4°C, with corresponding high recovery from cryopreservation. Plantlets acclimated with alternating temperatures in the dark had the same cold hardiness and regrowth as those with light (Fig. 5.1). Brison et al. (1995) reported a significant difference in recovery between non-acclimated *Prunus* shoot tips and those cold acclimated for only 24 h prior to cryopreservation. Plants lose CA within 48 h at warmer temperatures, so once dissected, shoot tips should remain in CA conditions until cryopreserved. CA is now widely used for cryopreservation of shoot tips and is effective for temperate and some subtropical plants (Reed 2001).

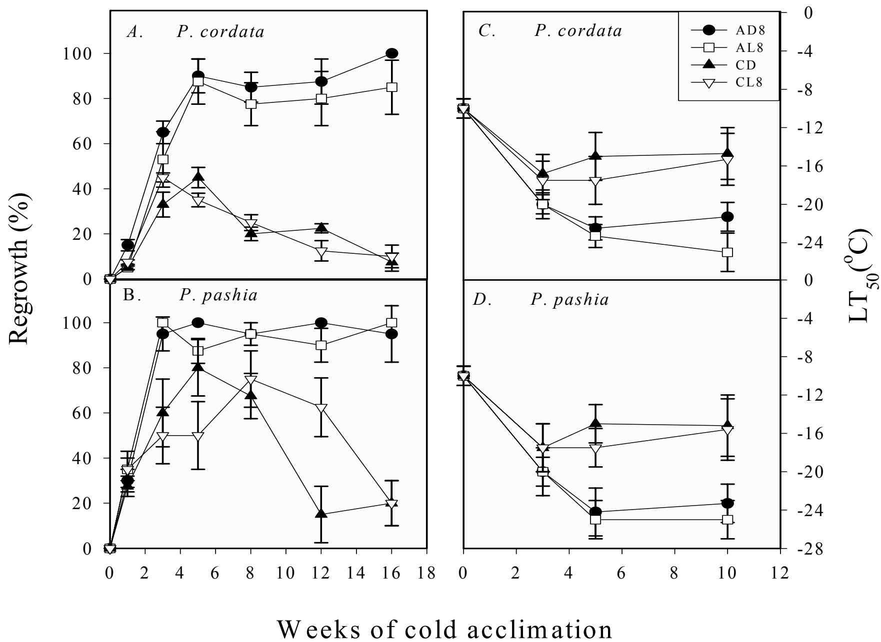

Fig. 5.1 A and B: regrowth of pear shoots after cold acclimation and cryopreservation. C and D: cold hardiness of pear shoots after cold acclimation (CA). AD8, Alternating temperature-8 h warm, dark. AL8 Alternating temperature-8 h warm with photoperiod. CD Constant temperature, darkness. CL8 Constant temperature with 8 h photoperiod (From Chang and Reed, 2000b)

5.2.2 In Vitro *Culture*

The length of the *in vitro* culture period directly influences the physiological status of plants before and after cryopreservation. For undifferentiated cultures such as cell suspensions, specific harvest times are required during the growth cycle (Withers and King 1979a). The age of the cultures is important in algae and plant cell cultures as well as for shoot tips of higher plants. Most micropropagated plants are transferred on a 3 to 4 week cycle before cryopreservation, but in some cases extended culture is advantageous. Culture durations may be as long as 12 weeks without transfer. These long transfer periods impart desiccation tolerance in some cultures.

Sucrose or other osmotically active substances are often incorporated into the medium. Sucrose can improve the tolerance of plants to cold temperatures by accumulating in the cells and increasing the osmotic tolerance. This was shown repeatedly in preconditioning for the encapsulation-dehydration technique (Dumet et al. 2000; Dumet et al. 1993; Malaurie et al. 1998). Sucrose preconditioning used with controlled rate cooling did not show any benefits for pear shoot cultures. Osmotic conditioning with 5–7% sucrose in the growth medium for a total of 5 weeks, with the final 2-weeks in CA, increased regrowth of shoot tips of *Pyrus cordata* from 0–75% over low concentrations (2%), but 3% sucrose as in normal medium was not significantly different from higher concentrations (Chang and Reed 2001) (Table 5.1).

Table 5.1 Percent recovery of cryopreserved *P. cordata* shoot tips excised from shoots grown for 3 weeks on conditioning medium with sucrose and benzyladenine and an additional 2 week without transfer in combination with low temperature cold-acclimation (CA) treatments (From Chang and Reed 2001)

BA concentration	**Sucrose concentration (%)**			
(μM)	**2**	**3**	**5**	**7**
0.89	35 ± 11.4^{b}	60 ± 15.8^{a}	70 ± 7.8^{a}	58 ± 12.3^{a}
2.22	0^{c}	69 ± 10.7^{a}	68 ± 9.5^{a}	72 ± 6.1^{a}
4.44	0^{c}	25 ± 8.9^{b}	67 ± 6.7^{a}	75 ± 5.0^{a}

Shoots were precultured on medium with sucrose and BA for 3 week followed by CA treatment for 2 week [22°C with 8-h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and –1°C 16-h darkness] ($n = 60$). Means followed by different letters are significantly different at $P \leq 0.05$

5.2.3 Abscisic Acid

Abscisic acid (ABA) is an important stress hormone that increases plant tolerance to low temperature (Bravo et al. 1998; Heino et al. 1990). It can

be an important factor in the adaptation of some species to cryopreservation (Ryynanen 1998). ABA's mode of action includes maintenance of water balance in cells (Tanino et al. 1990) and triggering specific genes that initiate the production of antifreeze products (Lee et al. 1991). ABA preconditioning treatments are sometimes effective in improving regrowth following subsequent cryopreservation. Suspension cultures of winter wheat grown on 7.5×10^{-5} M ABA for 5 days at 20°C had higher recovery compared to the control (Chen et al. 1985). ABA preconditioning (0.5 mg/l) improved the recovery of cryopreserved zygotic embryos of spring wheat (*Triticum aestivum* L.) (Kendall et al. 1993). The somatic embryos of horse-chestnut (*Aesculus hippocastanum* L.) cultured on 75 µM ABA medium for 4 days had better recovery than controls without ABA (Jekkel et al. 1998). A 7-day pretreatment with 50 µM ABA alone did not signifcantly improve recovery of the blackberry and raspberry genotypes but was effective when combined with 1-week CA (Reed 1993). Gentian axillary buds precultured with 1–10 mg^{-1} ABA for 11 days at 25°C following 1 day preculture on MS medium containing 0.4 or 0.7 M sucrose, without ABA had 81–86% recovery (Suzuki et al. 2006). ABA (50 µM) combined with CA significantly improved cold hardiness of pear shoots as determined by LT50, the temperature lethal to 50% of the *in-vitro* shoots tested, but ABA alone did not improve cold hardiness enough to produce regrowth following cryopreservation (Table 5.2; Fig. 5.2) (Chang and Reed 2000b).

Table 5.2 Cold hardiness of *in-vitro* grown *P. cordata* shoot tissues determined by LT_{50} following 3-week growth on 75 µM abscisic acid with or without 2-week cold acclimation treatments. (From Chang and Reed 2000b)

Type of tissues	LT_{50} (°C)			
	Control ^{z}	+ABA^{y}	+CA^{x}	+ABA + CA^{w}
Shoot tips	-10.8^{aD}	-12.4^{aC}	-16.9^{aB}	-22.5^{aA}
Lateral buds	-11.1^{aD}	-12.5^{aC}	-15.2^{bB}	-20.1^{bA}
Leaves	-10.1^{aD}	-12.1^{aC}	-14.1^{bB}	-17.9^{cA}
Means	-10.7^{D}	-12.3^{C}	-15.4^{B}	-20.2^{A}

Means in a row with different capital letters (A–D) are significantly different at $P \leq 0.05$. Means in a column with different lowercase letters (a–c) are significantly different at $P \leq 0.05$

^{z}Control: 3-week growth at 25°C with no ABA

^{y}+ABA: 3-week growth at 25°C with 75 µM ABA

^{x}+CA: 2-week CA at 22°C with 8-h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and –1°C 16-h darkness

^{w}+ABA + CA: Growth at 25°C for 3 weeks with 75 µM ABA followed by 2-week CA (alternating temperature) treatment.

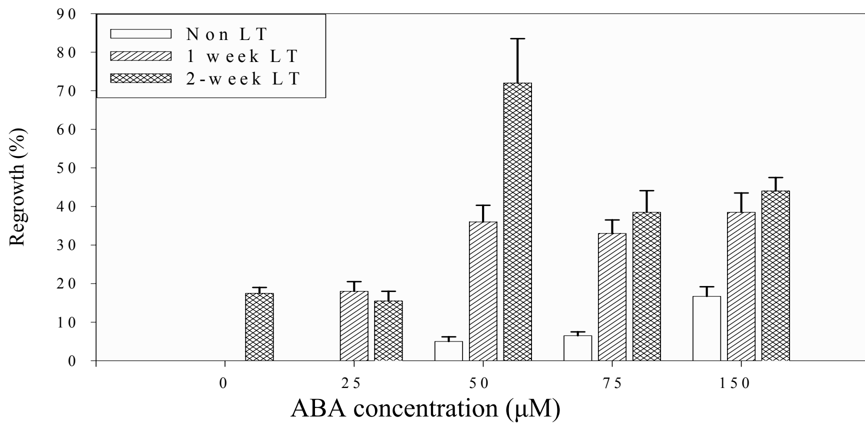

Fig. 5.2 Regrowth of *in vitro*-grown *Pyrus cordata* shoot tips after 3-week culture with 0 to 150 µM ABA treatments followed by 0, 1, or 2-week low temperature (LT) treatment (cold acclimation (CA) of 22°C with 8-h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and –1°C 16-h darkness) on the same medium. $N = 60$; means ± standard deviation (From Chang and Reed 2000b)

5.3 Preculture

Culture of plants on specialized media immediately prior to cryopreservation is very effective in improving recovery. Early studies by Withers and King (1979b) found that maize cells precultured in medium containing 10% proline for 3–4 days before cryopreservation significantly improved recovery even without the additional use of proline as a cryoprotectant. Growth of shoot tips on culture medium with 5% dimethyl sulphoxide (DMSO) for 48 h prior to controlled rate cooling is commonly used. This preculture was first demonstrated with a 2-day preculture of pea meristems on 10% DMSO at 26°C prior to controlled rate cooling (Kartha et al. 1979). Kartha et al. (1980) reported a 95% recovery of strawberry meristems treated with 5% DMSO for 2 days before LN exposure. *Arabidopsis* and tobacco cells precultured for 2 days in 0.5 M sorbitol before cryoprotection in 7.5% DMSO recovered at 90% (Menges and Murray 2004).

A 3 to 4 day preculture on 0.3 M mannitol was critical for successful freezing of maize suspension cultures (Withers 1985). Addition of 0.2 M sorbitol to a 24 h preculture of pine embryonic cells resulted in high (83.6%) viability following exposure to freezing for the three genotypes studied (Malabadi and Nataraja 2006). Preculture with one chemical may considerably reduce the toxic effect of another. Ford et al. (2000) found that preculture with sorbitol reversed the toxicity of DMSO on cryopreserved somatic embryos of *Pinus patula*.

5.4 Cryoprotection

Chemical cryoprotectants are an important factor in controlled rate cooling. Protective additives reduce the problems associated with ice crystal formation or dehydration injury. Most cryoprotectants are mixed in standard plant growth medium.

5.4.1 Single Chemicals

Dimethyl sulphoxide (DMSO) is one of the commonly applied cryoprotectants for controlled rate cooling cryopreservation. It was originally introduced by animal biologists who used it as a cryoprotectant for red blood cells (Lovelock and Bishop 1959). The first application of DMSO on plant cells was reported by Quatrano (1968) who recovered 14% viability from suspension cultures of *Linum usitatissimum* exposed to –50°C. DMSO acts by lowering the initial freezing temperature and also alters the characteristics of ice crystals at the point of formation (Lovelock and Bishop 1959). DMSO is used as a sole cryoprotectant for suspension and callus cultures. The optimal concentration of DMSO varies according to species but usually is applied at 10–15% in culture medium. DMSO was successfully used with *Zea mays* cells (Withers 1979). Suspension cultures of *Puccinellia distans* had 95% viability using 12.5 and 15% (v/v) DMSO (Heszky et al. 1990). The first animal cells were protected with glycerol. Protection with 15% glycerol was effective for *P. distans* cultures. Some studies suggest a direct correlation between increased survival and higher DMSO content (Dereuddre et al. 1988; Heszky et al. 1990). L-proline was also effective as a cryoprotectant for cultures of *Zea mays* at 2.5 – 24% in culture medium (Withers and King 1979b).

5.4.2 Chemical Combinations

Some of the first successful cryoprotectants for slow cooling were combinations of cryoprotective chemicals. The addition of 10% polyethylene glycol (PEG) to a solution of 8% glucose and 10% DMSO (w/v) reduced the toxicity of the solution (PGD) and increased the viability of callus cultures of *Saccharum* sp (Ulrich et al. 1979). DMSO and sorbitol combined were important for achieving improved recovery of cryopreserved *Catharanthus roseus* (L.) Don. cells. In combining cryoprotectants the toxic effects of individual compounds are reduced (Chen et al. 1984). Smith (1983) and Tao and Li (1986) reported good results when using combinations of DMSO, glucose and PEG. Hubalek (2003) demonstrated that the effect of one compound may dominate another or that both may combine to produce additive effects. The study found that a combination of rapidly penetrating and slowly penetrating cryoprotectants had greater effects than when used singly. A combination of 0.2 M sorbitol plus 5% DMSO was optimal for cryoprotection of somatic embryos of *Pinus roxburghii* Sarg. (Malabadi and Nataraja 2006). PGD is widely used in the controlled rate cooling of temperate fruit crops (Chang et al. 2000; Chang and Reed 1995; 2000b; Reed 1990; Reed and Hummer 1995; Reed and Hummer 2002; Reed and Lagerstedt 1987; Reed et al. 2003). A modification of the plant vitrification solution number 2 (PVS2) developed by Sakai et al. (1990; 1991) was useful for controlled rate cooling involving the cryopreservation of *Prunus* rootstocks (Brison et al. 1995). Several additional cryoprotectant combinations are used for suspension and callus cultures (Chap. 8).

5.5 Cooling

Controlled rate cooling involves the use of either a programmable freezer or a standard freezer to cool the cells or tissues at a specified cooling rate before plunging the samples into LN (–196°C). Samples in cryovials may be placed in styrofoam boxes in a freezer at –80°C. Specifically designed cooling containers with isopropanol are commercially available (e.g. Nalgene) and cool at about –1°C when placed in a low temperature freezer. Domestic and laboratory freezers can be used for this technique (Kartha and Engelmann 1994).

Cooling rate affects the level of cell dehydration and is critical to survival. A too slow cooling rate can cause excessive dehydration and concentration of electrolytes, while a very rapid cooling rate may result in insufficient dehydration. If cytoplasmic contents are adequately dehydrated,

water in the cells vitrifies (becomes a glass) on contact with LN. Hence, cooling rate and the temperature reached before plunging both require careful attention. Controlled rate cooling usually begins at 0–4°C and cools to the freezing point of the cryoprotectant. Initiation of ice at the freezing point of the cryoprotectant is required to avoid supercooling of the sample. Normally the exothermic reaction is initiated at –8 to –10°C, either by touching the sample with cold forceps or by automatic initiation by the programmed freezer. The cooling resumes to a terminal temperature (usually to –35°C or –40°C) where the sample may be held for a period of equilibration or directly transferred to LN.

For cell suspension cultures 1°C min^{-1} to –30°C is commonly used (Withers 1978) (Chap. 3). Heszky et al. (1990) compared cooling rates ranging from 0.5°C to 2°C min^{-1} and 1000–2000°C min^{-1} on cell suspension cultures of *Puccinellia distans* derived from callus and found 1°C min^{-1} with a –40°C transfer temperature to yield 45% survival without a cryoprotectant. Recovery increased to 78% with addition of 12.5% (w/v) proline and a –30°C transfer temperature. The optimum cooling rate for survival of wheat suspension cultures was 0.5°C min^{-1}, with a –35°C prefreezing temperature (Chen et al. 1985). A cooling rate of 0.1°C min^{-1} to –40°C was used in studies on pears (Reed 1990), currants (Reed and Yu 1995), hops (Reed et al. 2003) and mint (Uchendu and Reed 2007) while 0.3°C was most effective for *Rubus* (Chang and Reed 1999; Reed and Lagerstedt 1987). Towill (1988) used a rate of 0.25°C min^{-1} to –37°C for mint shoot tips.

Combining other techniques with controlled rate cooling can be very effective as well. Encapsulated grape axillary-shoot tips cooled at 0.1°C min^{-1} to –100°C prior to LN exposure had significant improvement in recovery over encapsulation alone (Plessis et al. 1993). Controlled cooling to –40°C was found optimal for successful cryopreservation of shoot tips from dormant buds of Japanese pear (Moriguchi et al. 1985). Controlled rate cooling for dormant buds is usually about 5°C day^{-1} with hold times of 12–24 hr between each drop in temperature (Forsline et al. 1993) (Chap. 16).

5.6 Warming

Warming is a critical factor for successful cryopreservation. Rapid warming prevents ice recrystallization within plant cells (Sakai and Nishiyama 1978; Tyler and Stushnoff 1988; Tyler et al. 1988). Carrot cells warmed at 20°C produced the best recovery in early studies (Withers 1979). Many cell cultures are warmed at 20–40°C for 1–2 min (Chap. 8). Menges and

Murray (2004) used 40°C for 3 min and achieved 90% viability of cryopreserved *Arabidopsis* cell suspension cultures. Embryonic cells of pine were successfully warmed at 35°C for 5 min (Malabadi and Nataraja 2006). Towill (1988) found that rapid warming at 40°C for 3–5 sec promoted recovery of mint shoot tips whereas samples warmed at 2°C or 22°C had no regrowth. Warming of shoot tips is commonly done at 37°C to 45°C for 1–2 min.

5.7 Recovery and Viability Assessment

Recovery from cryopreservation begins immediately upon rewarming. Shoot tips are normally rinsed with liquid medium to remove cryoprotectants. Removal of the cryoprotectant is important in most cases, however, the cryoprotectants used in controlled rate cooling are not highly toxic with relatively short exposure. Significant recovery was reported for maize cell cultures rinsed with sterile water, fresh liquid medium, or fresh medium with 10% proline (Withers and King 1979b). Some cell cultures exhibit better regrowth if they are not rinsed, but instead plated on a filter paper over semi solid growth medium or directly on the growth medium. A standard liquid growth medium was used to rinse embryonic cell cultures of *Pinus roxburghii* Sarg. following cryopreservation (Malabadi and Nataraja 2006). Standard liquid culture medium is commonly used to dilute and remove cryoprotectant chemicals.

Special recovery media may improve regrowth of cryopreserved cultures. Mannitol (6% w/v) in the regrowth medium improved recovery of maize cell cultures following cryopreservation (Withers and King 1980). Rice cell suspension lines were cultured in liquid medium at 28°C under dark conditions for 3 days before transferring to semi-solid medium (Benson et al. 1995). *Prunus* rootstocks were recovered on auxin enriched medium for 48 h in darkness followed by low illumination (~19 $\mu E \cdot m^2 \cdot s^1$) with 69–74% average shoot formation (Brison et al. 1995). In other cases, shoot cultures were recovered on medium without auxins, to decrease callus and increase shoot production (Chang and Reed 1999).

The term “viability” is often erroneously used interchangeably with survival even when initial signs of life (green color) do not further develop or when cell division has ceased in cryopreserved tissues. Such tissues should normally be considered dead. Regrowth should be the term used to describe actual growth patterns with observable cell divisions or elongation of shoots. If unorganized growth is seen, it should be described separately from organized growth.

Staining with a solution of triphenyl tetrazolium chloride (TTC) can provide rapid assessment of viability of cell or callus cultures (Finkle and Ulrich 1979; Jain et al. 1996). Several studies of cell cultures analyzed cell viability using 0.05% fluorescein diacetate (FDA), acetocarmine and Evans blue (Heszky et al. 1990; Malabadi and Nataraja 2006). In general, chemical viability tests are unsuitable for organized tissues and instead evaluation should be based on regrowth of plantlets. A comparison of viability tests for shoot tips found that electrolyte leakage and visually analyzed TTC staining were both effective for shoots and dormant buds (Verleysen et al. 2004).

5.8 Summary of the Basic Protocol

The basic protocol for controlled rate cooling is similar to that listed below for shoot tips of temperate plants. A specific type of controlled rate cooling apparatus may require slightly different steps.

1. In vitro cultured plants (3 weeks from last transfer)
2. Cold-acclimate plantlets for 2–4 weeks with alternating temperatures (8 h, 22°C and 16 h, –1°C).
3. Dissect 1 mm shoot tips and plate on 5% DMSO, 8% agar preculture medium for 48 h in cold acclimation conditions.
4. Add cryoprotectant (PGD or DMSO) to shoot tips in a cryo tube over 30 min (on ice).
5. Hold at 0°C for 30 min.
6. Cool at a rate of 0.1–0.5°C min^{-1} to –40°C with initiation of freezing at the freezing temperature of the cryoprotectant solution (about –9°C). Plunge in LN.
7. Rewarm in 45°C water for 1 min then move to 25°C water for 2 min.
8. Rinse with liquid MS medium and plate on recovery medium with no auxins.

Controlled rate cooling technology is convenient for handling large plant collections in a standardized fashion. The health of the plant cultures, preculture conditions, skill of dissection, and the cooling and rewarming rates are key elements for successful cryopreservation. An outstanding advantage of controlled rate cooling over other techniques can be the valuable time saved in handling a large number of samples. This ease of handling makes conservation of many plant species manageable and convenient for scientists especially where labor is a major constraint. The investment in

a programmable freezer is considered cost effective because it is offset by the reduction in technician time required by this technique.

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Section II. Cryopreservation Protocols for Working Laboratories

Chapter 6
Cryopreservation of Algae

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6.1 Algae

Only microalgae (algae with cell/filament/thallus size <2 mm) are considered in this chapter. However, the methods described are applicable to a wide variety of prokaryotic cyanobacteria and eukaryotic microalgae. Most culturable cyanobacteria and soil microalgae that have been examined can be cryopreserved with relatively high viability. Furthermore, many freshwater and marine eukaryotic algae can also be cryopreserved, but usually with lower viability. Marine diatoms can be cryopreserved, and often have high viability (Day and Brand 2005), although there has been limited

[1] The authors acknowledge the invaluable input and financial support provided by the EU funded COBRA project (The conservation of a vital European scientific and biotechnological resource: microalgae and cyanobacteria), contract No. QLRI-CT-2001-01645.

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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success preserving freshwater diatoms (McLellan 1989). Large numbers of strains have been examined, most notably at six of the major protistan collections:

ATCC (www.atcc.org)
CCAP (www.ccap.ac.uk)
CCMP (www.ccmp.bigelow.org)
NIES (www.nies.go.jp/biology/mcc/home.htm)
SAG (www.epsag.uni-goettingen.de)
UTEX (http://www.bio.utexas.edu/research/utex)

It has been observed that chlorarachniophytes, eustigmatophytes, pelagophytes, phaeothamniophytes and ulvophytes often have very high success rates, comparable to the other green algae and cyanobacteria (Day and Brand 2005). However, to date, most morphologically complex taxa including members of the dinoflagellates, cryptophytes, synurophytes and raphidophytes cannot be successfully cryopreserved. It is anticipated that further research on the basic mechanisms of freezing damage and the empirical development of improved protocols will continue to expand the number and diversity of algal taxa responsive to cryopreservation techniques.

6.2 Physiological Status

Most protocols use late log or early stationary phase cultures (Morris 1978; Day and Brand 2005). However, the key factor to successful cryopreservation is cell viability shown in the "vigor" of the culture prior to preservation. Cultures that are actively growing generally survive cryopreservation with higher viability than do those that are in stationary or declining phase, or cultures growing under other stress conditions (Day and Brand 2005). Cryopreserved cultures are not required to be axenic, but non-axenic cultures often have a greater risk of major contamination after rewarming, since many kinds of bacteria survive cryopreservation and will grow on the medium nutrients and those released by algae that do not survive the cryopreservation procedures.

6.3 Preconditioning

Cultures of algae growing either in liquid or on agar solidified medium may be successfully cryopreserved; however, cultures grown on agar are generally resuspended in the appropriate liquid medium prior to cryopreservation. There were a number of studies to optimize viability related to the

physiological status of the cryopreserved strains. Parameters investigated include age of culture (Morris 1978; Day and Fenwick 1993), light intensity (Beaty and Parker 1992), incubation temperature (Morris 1976b; Cañavate and Lubian 1997), osmotic potential of the medium (Cañavate and Lubian 1995), nutrient limitation (Ben-Amotz and Gilboa 1980; McLellan 1989) and mode of nutrition (Morris et al. 1977; Fenwick and Day 1992). However, other reports show high post-cryopreservation viability without any special preconditioning conditions (Day and Fenwick 1993; Crutchfield et al. 1999). It is noteworthy, that the vast majority of microalgae cryopreserved strains tested during the EU funded COBRA project, and other large-scale programmes undertaken elsewhere have been successfully cryopreserved without any special pre-adaptation conditions (Morris 1978; Watanabe et al. 1992; Day et al. 2005; Day and Brand 2005).

6.4 Preculture

It is an important prerequisite to success that algal cultures should not be subjected to severe physical or environmental stress immediately prior to their cryopreservation. However, to facilitate easy handling, some filamentous and/or thalloid strains may be cut into smaller pieces before cryopreservation. As a result of this stressful treatment, it is advisable to allow the damaged cells to recover for at least 24 h under non-stressful conditions (Fleck et al. 1997). Also, centrifugation is frequently used to harvest algae, to replace the culture medium with cryoprotective solutions, or to adjust the culture density. Viabilities of surviving rewarmed cultures are sometimes significantly diminished in cultures that are subjected to centrifugation immediately prior to their cryopreservation. Thus, it is preferable to avoid centrifugation or to use the minimum required centrifugal force prior to cryopreservation.

Algae are generally cryopreserved in their normal culture medium, usually with an appropriate cryoprotective additive (CPA) at an appropriate concentration. Cultures growing on agar may be first suspended in liquid medium so that a uniform and controlled concentration of CPA can be added. At the CCAP, cultures grown on agar are normally transferred to liquid medium and incubated under standard conditions for at least 2 weeks prior to preservation.

The effect of culture density of the frozen culture on viability subsequent to rewarming has not been extensively examined, although Brand and Diller (2004) reported that *Chlamydomonas reinhardtii* does not survive cryopreservation with high viability unless the culture density at the time of freezing

is approximately 2.5×10^6 cells ml^{-1} or less. It has been speculated that the proportional number of dead cells after cryopreservation is higher at high cell concentrations, and these dead cells release a substance(s) that is lethal to viable cells.

6.5 Cryoprotection

With the exception of a handful of algal taxa that can survive direct immersion into liquid nitrogen (LN) (Morris 1978; Day 1998), successful cryopreservation of an algal culture almost always depends upon the addition of appropriate exogenous water-soluble cryoprotective agents (CPA) prior to freezing the culture. These cryoprotectants generally must be added at high concentrations (e.g. 2–12%) to afford protection during freezing and rewarming. As detailed elsewhere (Chap. 2) two classes of CPAs may be distinguished, those that passively move through the plasma membrane to equilibrate between the extracellular solution and the cell interior (penetrating CPAs) and those that do not pass through the plasma membrane and remain in the extracellular solution (non-penetrating CPAs). In general, penetrating cryoprotectants are used for algae and methanol (MeOH), dimethylsulphoxide (DMSO=Me_2SO) and glycerol are most frequently employed (Taylor and Fletcher 1998). Most freshwater and terrestrial strains tested have higher viability when MeOH or DMSO is employed, rather than glycerol, with MeOH often being the CPA of choice (Morris 1976a, 1978; Day and Brand 2005). Conversely, many marine microalgae are more effectively cryopreserved with DMSO compared to MeOH (Day and Brand 2005), while glycerol is effective for members of the genus *Tetraselmis* (Day and Fenwick 1993).

Penetrating CPAs are often toxic at high concentrations, and prolonged exposure to methanol at concentrations used as a cryoprotectant (typically 5–10% v/v) is toxic to *Euglena gracilis*, and even short-term (20 min) exposure to concentrations greater than 15% (v/v) can be damaging (Fleck 1998). To reduce this problem they may be added after the culture has been cooled to 0°C or a lower temperature to minimize the effects of intracellular toxicity (Fleck 1998).

Non-penetrating CPAs are not extensively utilized for cryopreserving algae, although Morris (1976a) used 10% (w/v) PVP as a CPA to cryopreserve *Chlorella* spp., but achieved higher viability using the penetrating CPA DMSO. The authors have examined the cryoprotective effects of non-penetrating CPAs including HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA and 250 mM sucrose), PEG (polyethylene glycol) and PVP (poly[N-

vinyl-2-pyrrolidone]) on a variety of microalgae that are recalcitrant to cryopreservation and these compounds did not provide effective cryoprotection in any of the strains examined.

6.6 Cryopreservation

Conventional controlled rate cooling (two-step) cryopreservation remains the method of choice for preserving microalgae and cyanobacteria (Day and Brand 2005). As with other biological materials there is an optimal cooling rate (step 1) that minimizes the formation of lethal intracellular ice (fast cooling) and excessive osmotic shrinkage of cells that may cause chemical damage (toxic increases of intracellular solutes) and/or physical damage (disruption or crushing of intracellular organelles) generally resulting from excessively slow cooling rates (see Chap. 2). For many algal taxa, an intermediate rate of cooling (e.g. 1°C min^{-1}) is a compromise that results in high viability. A variety of approaches were developed to achieve the desired cooling regime and these may be categorized as passive freezing systems or controlled-rate freezers. The most commonly employed of passive freezing system involves the use of an insulated container (e.g. Mr. Frosty, Nalge Nunc, Rochester, New York, USA) which holds the algal samples. The container is then exposed to a very low temperature (typically by placing Mr. Frosty in a –80°C freezer). When the cryopreserved samples reach a sufficiently low temperature (e.g. –30°C to –80°C), they are removed from the insulated container and transferred directly to a permanent ultra-cold storage vessel (< –150°C) (step 2 of this two-step cooling procedure). Cultures cryopreserved utilizing MeOH as the CPA are cooled slowly to a temperature of –45°C or lower before they are transferred to their permanent storage. For protocols utilizing other cryoprotectants, a higher terminal temperature for step 1 may suffice; –40°C is routinely used at the CCAP when DMSO is the CPA. An alternative approach employed extensively at the CCAP (Morris 1978; Day 1998; Day and Brand 2005) involves the use of a denatured ethanol bath pre-cooled to –40°C.

Some algae require slower or more carefully controlled cooling rates than those normally achieved using passive cooling systems. A variety of instruments are commercially available (e.g. Biotronics, Leominster, UK; Planer Products, Sunbury, Thames; Cryomed, Marietta, Georgia; Gordinier Electronics, Roseville, Michigan; CryoLogic, Musgrave, Victoria, Canada) that allow much more accurate manipulation of the cooling regime. New models provide data outputs to enable each cryopreservation run to be carefully documented, which may be important for protocol development,

reproducibility and quality control. These instruments deliver user-controlled cooling rates over a broad range of temperatures. Ultra-cold vapor-phase nitrogen is fed into the chamber in a feedback system that accurately regulates the temperature sensed by a probe inserted into a control cryogenic vial. In some cases, it is necessary to allow additional time for a sample to cryo-dehydrate, so controlled rate protocols may incorporate a "dwell period," e.g. cooling at 1°C min^{-1} from +20°C to –40°C with a further 15 min "dwell" at –40°C prior to transfer to liquid nitrogen. As with the passive freezing protocols, it is important to transfer the cryogenic vial quickly from the cooling chamber to the storage vessel to prevent excessive warming of the sample.

6.6.1 Rewarming

The standard rewarming method employed is to transfer the cryogenic vial from the storage container to a water bath held at 35–40°C and frequently agitate the sample. The cryogenic vial is quickly removed from the water bath as soon as its contents are fully re-warmed (typically 1.5–4.0 min for a filled 2 ml cryogenic vial). When MeOH is the cryoprotectant, some workers conduct the entire rewarming procedure under reduced light conditions (Day and Brand 2005). For encapsulated-dehydrated samples, cryogenic vials containing the alginate beads are re-warmed at room temperature (20–25°C) for approximately 30 min. Beads are then aseptically transferred into 5 ml of the appropriate medium for up to 1 h (alginate re-constitution, or pre-swelling) and then transferred to fresh medium and cultured under standard light and temperature conditions.

6.6.2 Recovery

Many strains of algae survive cryopreservation with an acceptably high viability when incubated under normal growth conditions immediately after the CPA has been removed by centrifugation/membrane filtration, or diluted to a non-toxic level. However, higher viabilities are sometimes achieved when the rewarmed cultures are left in darkness, or in subdued light, for 12–24 h after rewarming and the CPA has been removed/diluted. Alternatively, the addition of small amounts of yeast extract (0.1 g l^{-1}), proteose peptone (0.1 g l^{-1}) or soil extract (10 ml l^{-1}) has been noted to enhance viability and recovery for some axenic strains (Day and Brand 2005).

6.6.3 Viability Assessment

The most direct way to determine viability is to observe the actively growing cell fraction of algae that reproduce normally after rewarming, expressed as a percentage relative to the total cell number prior to cryopreservation. This generally involves the use of a standard agar plating method. An aliquot of the algal culture to be cryopreserved is diluted to a defined culture cell density, and a specified volume of culture is replicated on at least three identical agar plates containing a suitable algal growth medium. The number of algal units spread on 100 mm plates should be such that between 50 and 200 colonies will be produced. The plates are placed under favorable growth conditions. When all viable algal units have grown into visible colonies, the total number of colonies on each plate is counted. The same culture for which control cell count was determined is used for cryopreservation. The culture is cryopreserved, rewarmed and diluted as appropriate. Aliquots of the revived culture are placed on replicate agar plates identical to the plates described for the determination of the control cell count in the pre-cryopreserved culture. Percent viability of the cryopreserved culture is determined as:

$$\% \text{ viability} = (\text{post-treatment cell count})/(\text{control cell count}) \times 100\%.$$

This approach can be used for a wider range of algae by using pour plates, where molten agar at 40°C is added to 1 ml aliquots of exponential dilutions of algae in Petri dishes. For temperature-sensitive strains low-melting point agarose and ultra-low temperature agarose may be used as gelling agents.

An alternative approach involves the use of vital staining with fluorescein diacetate (FDA). As described above it is necessary to dilute out cryoprotectant by transferring the rewarmed culture into 9 ml of appropriate sterile medium and incubating for up to 24 h prior to staining. This incubation period allows repair of sub-lethal damage but is too short for cell division to occur and will give a more accurate index of viability when using vital staining rather than staining immediately after rewarming (Day and De Ville 1995). The procedure involves the addition of 50 µl of FDA stain stock solution to 1 ml of culture, incubation at room temperature for 5 min. and observation of the cells by blue-light fluorescence microscopy. Viable cells fluoresce green (FDA positive) and non-viable cells appear red or colorless. Viability is expressed as a percentage of non-treated unfrozen control culture.

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6.7 Protocols

6.7.1 Passive Cooling Two-Step Methods

Prepare in advance

1. Healthy cultures of algae grown under standard optimal nutrient and environmental conditions
2. 10% (w/v) DMSO in BBM+V (Starr and Zeikus 1993), or alternative appropriate medium [on 1: 1 dilution with culture results in a final cryoprotectant concentration of 5% (w/v)]
3. Pre-sterilize (autoclaved) 90 ml of BBM+V
4. After cooling to room temperature, mix thoroughly with 10 ml of DMSO. Filter sterilizes 10–20 ml aliquots into sterile universal bottles.

6.7.1.1 Mr. Frosty

This method is derived from the COBRA validation protocol exercise (www.cobra.ac.uk).

Materials and equipment

1. A Mr. Frosty or freezer unit capable of cooling at 1°C min^{-1}
2. Isopropanol (IPA) 250 ml (needed for placing in Mr. Frosty's chamber)
3. Small bench top LN Dewar of ~1-l capacity
4. Cryovials NUNC 377267 CryoTube 1.8 ml or equivalent

The procedure

This is a 1 day procedure. All culture manipulations should be done with good microbiological practice/aseptic technique in a laminar flow cabinet if possible. From each flask decant 10 ml aliquots into sterile universal bottles:

1. To each universal, add 10 ml of cryoprotectant solution.
2. Seal universal and invert several times in ensure thorough mixing.
3. Aseptically decant 1 ml aliquots into the cryovials and incubate for 10 min at room temperature.
4. Transfer the cryovials to the cooling chamber of a Mr. Frosty or equivalent freezing container/unit. These should be set up in accordance with the manufacturers' instructions as follows:
   (a) Fill the freezing container/Mr. Frosty unit with clean IPA (C_3H_8O) to the 250 ml lines on the freezing unit. Alcohol

must be replaced after every fifth use. It is recommended that between each new freezing run the IPA is decanted from the container, which is washed and dried and stored at room temperature.

(b) Insert cryovials into the unit and place in a –70°C or –80°C freezer.

5. Cool at –1°C min^{-1} to –70°C and hold for 1.5 h; transfer into LN.
6. Using long forceps rapidly, transfer/plunge the vials into LN.
7. Transfer vials to the standard cryostorage facility.
8. Vials are warmed in a 40°C water bath and agitated until the ice has melted. The liquid can be gently decanted and fresh culture medium added to the cryogenic vial.

6.7.1.2 Alcohol Bath

This procedure is modified from Day and De Ville (1995).

Materials and equipment

1. Refrigerated alcohol bath
2. Small bench top LN Dewar of ~1-l capacity
3. Cryovials NUNC 377267 CryoTube 1.8 ml or equivalent

The procedure

This procedure may take a whole day. From each flask decant 10 ml aliquots into sterile universals

1. To each universal add 10 ml of cryoprotectant solution.
2. Seal universal and invert several times in ensure thorough mixing.
3. Aseptically decant 1 ml aliquots into cryovials and incubate for 10 min at room temperature.
4. Transfer the filled cryovials to a pre-cooled refrigerated bath (–40°C) and incubate for 15 min. Make sure the bath is uniformly cool.
5. Cooling: transfer the vials rapidly, using forceps, to a wide-necked Dewar containing LN. Then transport the vials into the Dewar to the LN storage system.
6. Vials are warmed in a 40°C water bath and agitated until the ice has melted. The liquid can be gently decanted and fresh culture medium added to the cryogenic vial.
7. Controls should include untreated, non-cooled samples. Some algae are very sensitive to cryoprotectants. Care should be taken to ensure that the refrigerated bath is uniformly cool and has been cooled to –40°C.

6.7.1.3 Algae on Solid Agar

Original method by Bodas et al. (1995) later modified by Day and Brand (2005).

Materials and equipment
Day 1

1. A Mr. Frosty or any equivalent passive freezer. Alternatively, a controlled rate unit capable of cooling at 1°C min^{-1} may be used.
2. If using a passive freezer unit: IPA 250 ml (needed for placing in Mr. Frosty chamber, see instructions on chamber).
3. Small bench top LN Dewar of ~1-l capacity
4. Cryovials NUNC 377267 CryoTube 1.8 ml or equivalent
5. Growth chamber, incubator or controlled environment room suitable for growing algae

Prepare in advance
Many strains of algae grown on semi-solid media can be cryopreserved directly on an agar slope.

1. A small volume (0.3–0.5 ml) of melted agar in appropriate culture medium is transferred aseptically to a sterile 2 ml cryogenic vial and allowed to solidify as a slope. A fresh algal culture is spread onto the surface of the solidified agar.
2. The cap is placed on the cryogenic vial, and the vial is placed under normal growth conditions. The culture is ready for cryopreservation after it has grown into a heavy streak or a lawn on the agar surface.
3. Many cyanobacteria and unicellular chlorophytes can be cryopreserved after 2–3 weeks on the agar slope, even when the lid of the cryogenic vial is securely tightened.
4. Cryoprotectant: 10% (w/v) methanol in BBM+V (Starr and Zeikus 1993), or alternative appropriate medium [on 1: 1 dilution with the culture to give a final cryoprotectant concentration of 5% (w/v)].

The procedure
This is may be a whole-day procedure once the algal lawn has grown to an appropriate cell density.

1. Normal growth medium containing an appropriate CPA is added carefully to fill the cryogenic vial with minimum disturbance of the algae on the agar surface.

2. Incubate for 10 min. at room temperature.
3. Transfer the cryovials to the cooling chamber of a Mr. Frosty or equivalent freezing container/unit. These should be set up in accordance with the instructions detailed on the Mr. Frosty chamber and as follows:
   (a) Fill the Freezing Container/Mr. Frosty unit with clean isopropyl alcohol (IPA) (C_3H_8O) to the 250 ml lines on the freezing unit. Alcohol must be replaced after every fifth use. Between each new freezing run the IPA is decanted from the container which is washed and dried and stored at room temperature.
   (b) Insert cryovials into the unit and place in a –70°C or –80°C freezer.
4. Cool at –1°C min^{-1} to –70°C, hold 1.5 h.
5. Using long forceps rapidly transfer/plunge the vials into LN.
6. Transfer to the LN cryostorage facility.
7. Vials are warmed in a 40°C water bath and agitated until the ice has melted. The liquid can be gently decanted and fresh culture medium added to the cryogenic vial. If the algal cells adhere to the agar surface then the liquid medium can again be decanted and the cryogenic vial placed under normal culture conditions.
8. Algae remaining on the agar surface will grow into a lawn, typically within 2–3 weeks. This method is especially convenient when a culture must be kept axenic, since it requires minimum handling of cultures exposed to the atmosphere. It also is convenient for cultures that are highly sensitive to mechanical stress since it avoids centrifugation. However, it is not convenient for quantifying viability of cryopreserved cultures and cannot be used for strains that are difficult to grow on solid medium.
9. Controls should include untreated, non-cooled samples. Loss of viability may occur as some algae are very sensitive to cryoprotectants.

Note: vials can be stored in either liquid phase or vapor phase LN.

6.7.2 Controlled Rate Cooling Protocols

Materials and equipment

1. A controlled rate cooler: Planer or equivalent freezer unit capable of cooling at 1°C min^{-1} and LN
2. Small bench top LN Dewar of ~1-l capacity
3. Cryovials NUNC 377267 CryoTube 1.8 ml or equivalent

Prepare in advance

1. Healthy cultures of algae grown under standard conditions
2. 10% (w/v) DMSO in BBM+V (Starr and Zeikus 1993), or alternative appropriate medium [on 1: 1 dilution with culture gives a final cryoprotectant concentration of 5% (w/v)].
3. Pre-sterilize 90 ml of BBM+V. After cooling to room temperature, mix thoroughly with 10 ml of DMSO.
4. Sterilize 10–20 ml aliquots by filter sterilization into sterile universal bottles.

Controls should include untreated non-cooled samples. Loss of viability may occur as some algae are very sensitive to cryoprotectants.

6.7.2.1 Simple Protocol

This method is based on COBRA validation protocol exercise (www.cobra.ac.uk).

The procedure

This may take a single day to perform. From each flask decant 10 ml aliquots into sterile universals:

1. To each universal add 10 ml of cryoprotectant solution. Seal universal and mix by inverting several times.
2. Aseptically decant 1 ml aliquots into cryovials and incubate for 10 min. at room temperature.
3. Transfer the cryovials to the cooling chamber of the programmable freezer and cool at –1°C min^{-1} to –40°C.
4. Hold at –40°C for 10 min, then rapidly transfer/plunge the vials into LN. Vials can be stored in either liquid phase or vapor phase.
5. Vials are warmed in a 40°C water bath and agitated until the ice has melted. The liquid can be gently decanted and fresh culture medium added to the cryogenic vial.

6.7.2.2 Complex Protocol

Based on method used at CCAP (Andersen et al. unpublished) adapted by Day and Brand (2005).

The procedure

This procedure may take a whole day. From each flask decant 10 ml aliquots into sterile universals:

1. To each universal add 10 ml of cryoprotectant solution. Seal universal and invert several times in ensure thorough mixing.
2. Aseptically decant 1 ml aliquots into cryovials and incubate for 10 min. at room temperature.
3. Transfer the cryovials to the cooling chamber of the programmable freezer. Cool the chamber from ambient temperature to –4°C at –1°C min^{-1}. Hold at –4°C for up to 5 min (This is good for instantces where one adds cryoprotectant to cold polar strains and some time is required for the CPA to penetrate).
4. Cool at –1°C min^{-1} to –9°C at which temperature, for salt-based solutions, it is still a super cooled liquid. The chamber is rapidly cooled to –45°C until the sample reaches –12°C. This causes/ induces ice nucleation, the eutectic point. This may be detected as a heat spike, in the cooling profile.
5. The sample is then cooled at 1°C min^{-1} until it reaches –45°C.
6. The sample is then cooled very rapidly (~100°C min^{-1}) to –90°C.
7. Rapidly transfer the vials to LN using long forceps. Note: vials can be stored in either liquid phase or vapor phase LN.
8. Vials are warmed in a 40°C water bath as above.

6.7.2.3 Encapsulation

A method from the COBRA project (www.cobra.ac.uk) derived from Fabre and Dereuddre (1990) following modifications by Day et al. (2000) and Harding et al. (2004).

Additional equipment

1. Pipettes (3 ml sterile)
2. Sterile 9 cm filter papers, sterile 9 cm Petri dishes
3. Sterile strainer, sterile forceps
4. Sterile 250 ml beaker
5. Heated magnetic stirrer
6. Water bath set at 40°C

Prepare in advance

1. Healthy cultures of algae grown under standard conditions
2. BBM+V culture medium for re-suspending the beads (10–25 ml)
3. BBM+V culture medium without calcium ions containing 3% (w/v) low viscosity sodium alginate and 0.75 M sucrose (in a flask). This is very difficult to dissolve, so heat the medium and slowly add the alginate then boil to dissolve.
4. 100 mM calcium chloride medium (100 ml in a flask) in BBM+V
5. Sterile 100–250 ml beakers for forming beads in calcium chloride
6. Liquid BBM+V culture medium for re-suspending the beads
7. Sterile pipettes for forming beads
8. Sterile sieves or tea strainers for removing beads from solutions
9. Petri dishes with sterile filter paper for draining beads
10. Filter-sterilized cryoprotectant—5% (v/v) methanol or 5% (v/v) DMSO in culture medium
11. Multi well recovery plates (10 mm^2 Petri dish)

You will need a separate (sterile) 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile Petri dish for each algal strain.

The procedure

This normally is a 1 day procedure.

1. Centrifuge liquid cell culture (1,000 rpm for 1–2 min or longer for smaller algae) to concentrate dilute culture or add medium to dilute high cell density cultures.
2. Remove the supernatant and add ~10 ml of alginate solution to achieve a cell density of 10^5–10^6 ml^{-1} for larger algae e.g. euglenoids, or ~10^7 ml^{-1} for smaller algae.
3. Gently swirl the mixture of cells and alginate to evenly distribute the cells, but ensure that air bubbles are not formed.
4. Using a 3 ml plastic Pasteur pipette and holding it in a vertical position slowly dispense drop-wise the alginate/cell solution into 150 ml of 100 mM $CaCl_2$ and allow the alginate beads to equilibrate and polymerize for 60 min.

5. Carefully decant off the calcium solution and transfer the beads to BBM+V.
6. Carefully decant the suspending medium and transfer the beads to cryovials.
7. To each cryovial, add 1.0 ml of cryoprotectant solution.
8. Place the vials in the cooling chamber of a controlled rate freezer and cool to –60°C at –0.5°C min^{-1}; then hold at this temperature for a 30 min and plunge directly into LN.
9. Rewarming: Remove cryocanes from the storage Dewar, rapidly remove the cryovials and place them in a 40°C water bath for 2–3 min (do not completely cover the cryovials in water). Carefully wipe the cap and tube with a cloth containing 70% (v/v) ethanol to ensure sterile conditions.
10. Transfer the (now very sticky) beads from the cryovial (with a small spoon spatula) to 30 ml of BBM+V culture medium for 5 min, to dilute/wash out the cryoprotectant. Transfer each bead (5 beads per cryovial) into one square of the (5 × 5 squares) Petri dish (Biddy Sterilin, Staffordshire, UK), each square containing 1 ml fresh BBM+V medium and culture the beads under standard light and temperature conditions.

Possible Problems

Control treatments should examine the following during initial testing as some algae are sensitive to certain osmotic dehydration or cryoprotective steps. Loss of cell viability during any of the four steps (see below) requires investigation before further development of the technique.

1. encapsulation
2. cryoprotectant treatment
3. controlled cooling
4. LN exposed beads

6.7.3 Encapsulation Dehydration Method

A modification of the alginate encapsulation procedure (Fabre and Dereuddre 1990; Harding et al. 2004) based on method employed for COBRA protocol validation exercise (www.cobra.ac.uk)

Materials and equipment

1. Small bench top LN Dewar of 1-l capacity
2. CryoTube 1.8 ml Nunc internal Starfoot round or equivalent
3. Pasteur pipettes (3 ml sterile)
4. Sterile 9 cm filter papers, sterile 9 cm Petri dishes
5. Sterile strainer
6. Sterile 250 ml beaker
7. Liquid BBM+V (Starr and Zeikus 1993) culture medium for rehydrating beads
8. Multi well recovery plates (100 mm^2 Petri dish)
9. Sterile forceps
10. Heated magnetic stirrer
11. Water bath set at 40°C
12. Oven for determining dry weights (set at 105°C)
13. Thermometer and humidity meter (optional)

Prepare in advance

1. Healthy algal cell cultures grown under standard conditions
2. Standard medium for re-hydrating the alginate beads (10-25 ml)
3. Standard medium with 0.5 M and 0.75 M sucrose for osmotic pretreatment (75 ml in 125 ml flasks)
4. Standard medium without calcium containing 3% (w/v) low viscosity alginate and 0.75 M sucrose (in a flask). This is very difficult to dissolve, so heat medium and add alginate slowly, boil to dissolve.
5. Calcium chloride 100 mM for forming beads (in a flask)
6. Sterile 250 ml beakers for forming beads in calcium chloride and for draining beads
7. Sterile Pasteur pipettes for forming beads
8. Sterile Petri dishes for holding beads during dehydration
9. Sterile sieves or tea strainers for removing beads from solutions
10. Petri dishes with sterile filter paper for draining beads

You will need separate flasks of 0.5 M and 0.75 M sucrose containing medium, 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile Petri dish and pipette per treatment (or algal strain).

The procedure

This is usually a 3 day procedure:

1. Centrifuge liquid cell cultures (1,000 rpm for 1–2 min or longer for smaller algae) to concentrate dilute culture or add BBM+V to dilute high cell density cultures.
2. Remove the supernatant and add ~10 ml of alginate medium to achieve a cell density of 10^5–10^6 ml^{-1} for larger algae e.g. euglenoids, or ~10^7 ml^{-1} for smaller algae.
3. Gently swirl the mixture of cells and alginate to evenly distribute the cells, but ensure that air bubbles are not formed.
4. Using a 3 ml plastic Pasteur pipette and holding it in a vertical position slowly dispense the alginate/cell solution drop-wise into 150 ml of 100 mM $CaCl_2$; allow the beads to equilibrate for 60 min.
5. Carefully decant off the calcium solution and transfer the beads to 0.5 M sucrose medium for 24 h.
6. Decant this medium and replace with 0.75 M sucrose medium for a further 24 h (under standard culture conditions)
7. Remove the beads from the sucrose solution and remove excess medium by blotting the surface of the beads on sterile filter (7-8 cm) papers placed on Petri dishes.
8. Transfer the beads to sterile Petri dishes (9 cm) ensuring that they are not touching each other and that they are evenly distributed throughout the dish.
9. Place the open Petri dishes with the beads (near to the air source) in a horizontal flow laminar air-flow bench. Note and monitor the temperature of the bench (25°C optimum) and laboratory relative humidity. Air dry the beads in the air-flow $(1 m \cdot sec)^{-1}$ for 3–4 h. This results in beads with a residual moisture content of ~ 25–30% (w/w). Check that the beads have ~25% moisture content.
10. Transfer the desiccated beads into cryovials (10 beads per cryovial) and plunge into a 1-l LN Dewar.
11. Rewarming: Remove cryocanes from the storage LN Dewar, rapidly remove the cryovials and place them in a 40°C water bath for 2–3 min (do not completely cover the cryovials in water). Wipe the cap and tube with 100% ethanol to ensure sterile conditions.
12. Transfer the (now very sticky) beads from the cryovial to 5 ml of culture medium for 1 h, then transfer each bead into one square of the (5 × 5 squares) Petri dish into 1 ml fresh medium and culture the beads under standard conditions.

6.7.4 Viability Assessments used for Microalgae and Cyanobacteria

Method based on COBRA protocol validation exercise (www.cobra.ac.uk)

Materials and equipment

1. Small bench top LN Dewar of ~1-l capacity
2. Water bath at 40°C
3. Petri dishes
4. Parafilm
5. Sterile pipettes
6. Cryovials NUNC 377267 CryoTube 1.8 ml or equivalent.
7. Haemocytometer
8. FDA stain

Prepare in advance

1. Suitable logarithmic dilution series (9 ml medium per tube)
2. Molten BBM+V medium (Starr and Zeikus 1993) (1% agar w/v) held at 40°C
3. Prepare a standard FDA stock solution. FDA (Sigma, F 7378). FDA [0.001% (w/v)] is prepared by first dissolving 25 mg of FDA crystals in a few drops of acetone and making up to the final volume (25 ml) with methanol.

The procedures

This is a 2 day procedure with control viability ascertained prior to the cryopreservation procedure. Viability is assessed the next day, or after a longer period of storage.

6.7.4.1 Plating and Cell Colony Counts

Assessment of control (100%) viability

1. Perform haemocytometer counts to obtain the cell density of the culture to be cryopreserved.
2. Using 1 ml aliquots and logarithmic dilutions in sterile medium. Dilute to a defined culture cell density that would result in 50–500 cells in 1 ml. (e.g. for a culture with a cell density of 5×10^7 dilute the culture by 10^5, i.e. through five logarithmic dilutions).

3. Transfer 1 ml aliquots of the final dilution obtained in step 2, and place in replicate (three) identical Petri dishes (50 mm diameter).
4. Pour approximately 2.5 ml molten medium into the Petri dish and agitate gently to insure uniform mixing. (Note: you need to hold the medium at 40°C prior to dispensing. It is optimal to restrict to small flasks/bottles containing ~50 ml agar).
5. The number of algal units in the agar should be such that between 50 and 200 colonies will be produced.
6. After gel solidification, seal the plates with Parafilm or Clingfilm to prevent excessive dehydration.
7. For sensitive strains incubate in the dark/very low light for 8–24 h prior to transfer to standard culture conditions.
8. Incubate the plates, inverted, under standard culture conditions.
9. Calculate the number of colony forming units (CFU) by counting the number of colonies present on each plate after 7–14 days (counting once). Note: periodically check plates for growth, if there is a risk that colonies will merge, and then count as soon colonies are distinguishable. Calculate the mean control viability.

Percent viability = (post-treatment colony count)/(control colony count) × 100.

Assessment of frozen/rewarmed material

1. Stored vials should be transferred from the LN cryostorage to a small LN Dewar containing LN and then transferred to the laboratory for warming.
2. Vials are warmed in a 40°C water bath and agitated until the ice has melted. Rapidly transfer to a laminar flow cabinet and wipe the outside of the vial with 70% (v/v) ethanol.
3. Transfer 1 ml of each of three samples to separate logarithmic dilutions that would result in 50–500 cells in 1 ml.
4. Follow the remaining steps 2–9 above.

6.7.4.2 Fluorescein Diacetate (FDA) Vital Staining

1. Transfer 1 ml of each of three rewarmed samples to 9 ml of fresh sterile BBM+V. Incubate under standard conditions for 24 h.
2. Decant 3 × 1 ml aliquots from step 4 into test tubes and add 50 μL of FDA stock solution to each tube.
3. Incubate at room temperature for 1–5 min.
4. Observe under excitation-fluorescence (cells with functioning esterase activity cleave this stain, which then fluoresces intensely yellow/green under UV illumination; non-viable cells appear colorless or red due to the auto fluorescence of chlorophyll).
5. As a control use non-cryopreserved cells to test stain efficiency.
6. Count 50–100 cells and record FDA positive as bright green fluorescent cells.
7. Viability is expressed by number of FDA positives (under fluorescence) against the total number of cells observed within the field(s) viewed.

   Percent viability = (No. post-treatment FDA positives)/(Total No. of cells observed in field(s) of view assayed) × 100.

Notes

1. Frozen/rewarmed material was initially diluted 1: 1 with cryoprotectant prior to logarithmic dilution.
2. If control (step 9) had less than 100% viability then this should be factored into the absolute viability e.g. if control samples had viability of 80% and cryopreserved viability was 80% then in reality 100% of the cells capable of surviving have survived. This approach will allow direct comparison with the CFU data.

Chapter 7
Cryopreservation of Bryophytes and Ferns

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7.1 Introduction

Although not as widely studied, bryophytes (mosses and liverworts) and ferns[1] have requirements for *ex situ* conservation and germplasm storage similar to those of vascular seed plants. Cryostorage of tissues or spores of endangered nonseed plants can provide a back up to threatened wild populations, while genetically valuable research lines can be maintained in a stable and cost-effective fashion using liquid nitrogen (LN) storage. Spore banks and *in vitro* collections provide methods for economically maintaining a large number of genotypes of both groups of taxa, and cryostorage can greatly increase the potential longevity and stability of these collections.

Many of the same methods applicable to tissues of seed plants are adaptable for the cryopreservation of tissues of bryophytes and ferns. Nonseed plants, however, have several characteristics that make them particularly good candidates for cryopreservation and which provide more flexibility in approaches than with seed plants. First, both bryophytes and ferns have pronounced gametophyte and sporophyte stages. In the case of ferns, this provides two very different tissues that can be adaptable to cryopreservation: pieces of gametophytes and shoot tips of

[1] In this review, the term "fern" will be used to include all pteridophytes (ferns and "fern allies").

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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sporophytes. Second, in both ferns and bryophytes, the gametophyte stages are highly regenerative and can reproduce vegetatively from fragments. Finally, some bryophytes and ferns have vegetative tissues that are naturally desiccation tolerant. This adaptation can be useful in providing tissues that can more easily survive the reduction in water that is needed in many cryopreservation protocols. In addition, spores of both bryophytes and ferns can be used for cryostorage. This chapter will provide a brief review of the various types of tissues that can be used for cryopreservation of bryophytes and ferns and the different methods that were used to successfully recover living tissues after exposure to low temperatures.

7.2 Bryophyte Gametophytes

Bryophyte gametophytes exist either as filamentous protonemata, which emerge from the spore at germination, or as gametophores, the thalloid or leafy tissue that develops from a protonema and which is the characteristic life form of bryophytes. Cryopreservation protocols are available for *in vitro* grown tissues of both of these stages. In some cases, however, protonemata are preferred, and growth media were adjusted to favor protonemal growth over gametophores in preparation for cryopreservation (Schulte and Reski 2004).

One of the earliest reports of bryophyte cryopreservation describes survival of tissues of moss on agar frozen to –20°C (Longton 1981). A different approach was the use of cryoprotectants and slow cooling which allowed survival of protoplasts of *Marchantia polymorpha* and protonemata of *Physcomitrella patens* through LN exposure (Takeuchi et al. 1980; Grimsley and Withers 1983). More recently several procedures were published and applied to gametophyte tissue of a variety of species of bryophytes. Christianson (1998) described a method for protonemata or gametophores involving preculture with proline and abscisic acid (ABA) and freezing in a simple alcohol bath in a –20°C freezer. This procedure was used to cryopreserve over 40 species of an *in vitro* bryophyte collection, in order to reduce maintenance time and costs. Pence (1998) cryopreserved four species of moss and liverwort gametophores using a preculture of ABA with or without encapsulation dehydration (Fabre and Dereuddre 1990) with rapid freezing for LN storage. A variation on encapsulation dehydration was developed for the *ex situ* Conservation Project for UK Bryophytes at the Royal Botanic Gardens, Kew, in which moss protonemata are encapsulated in alginate strips to facilitate handling, with ABA and sucrose used as a pretreatment (Burch and Wilkinson 2002). This method was applied to at least five

species of endangered UK bryophytes for long-term storage. Finally, Schulte and Reski (2004) developed a procedure for high-throughput freezing of protonemata of multiple mutants generated in *Physcomitrella patens*, using rapid freezing after preculture on ABA and proline followed by cryoprotection with DMSO and glucose. Tissues are precultured and cryoprotected in the same cryovial used for freezing, thereby reducing the time involved in moving tissues through the prefreezing protocol. With this method 1100 mutants can be cryopreserved in a 5-day work week.

Most procedures for bryophyte gametophyte freezing involve a preculture step; incubating tissues for 3 days to 3 weeks on a preculture medium, including 10 µM ABA, 100 mM proline, 5% sucrose or some combination of these. These procedures likely induce any natural desiccation tolerance mechanisms of the species. Some mosses have constitutive desiccation tolerance, while tolerance can be induced in many other moss and liverwort species (Proctor and Pence 2002). Pence (1998) found that gametophytes of *Riccia fluitans* and *Helicondontium cappelare* were able to survive drying and cryopreservation with a preculture of 1 week on ABA, but that this procedure was not adequate for two other species. *Plagiochila* sp. survived freezing after encapsulation dehydration, but *Marchantia polymorpha* did not survive unless both preculture on ABA and encapsulation dehydration were used. Thus, various levels of protection appear to be needed, depending on the species, and this may be related to natural tolerances to desiccation.

Based on natural desiccation tolerance, it was noted that some species are able to survive cryopreservation without pretreatment with ABA or with cryoprotective chemicals (Burch 2003). *In vitro* grown protonemata from *Ditrichum cornubicum* and *Bryum rubens*, which possess some natural desiccation tolerance, survived drying and cryopreservation after slow drying with or without encapsulation, but *Cyclodictyon laetevirens*, a desiccation intolerant species, did not survive either treatment. Similarly, 13 species of *in vitro* grown temperate bryophytes survived drying and LN exposure with or without preculture on ABA (Leverone and Pence 1993).

Two basic approaches to cryoprotection are used for bryophyte gametophyte freezing. First, tissues can be incubated for a given length of time with cryoprotectant solutions prior to freezing. Examples are the methods of Christianson (1998), who used 1 h in 5% v/v DMSO and 10% w/v glucose, and Schulte and Reski (2004) who used 20% v/v DMSO and 25% (w/v) glucose for 1–3 h before freezing. Others have used an encapsulation–dehydration procedure in which tissues are encapsulated in an alginate gel and exposed to sucrose, ABA, or both prior to drying and cryopreservation (Pence 1998; Burch and Wilkinson 2002).

Cryoprotectant solutions are generally used with slow freezing procedures, either using a controlled cooling apparatus or an alcohol bath in a freezer of –20°C or below. When encapsulation dehydration-based procedures are used, the dried tissues are frozen rapidly by direct immersion in LN. Rewarming procedures also vary, from rewarming slowly at ambient air temperature, used with dried tissues, to rapid rewarming using a 30°C or 40°C water bath, used with both dried and nondried tissues. In all cases, recovery takes place on semisolid culture medium.

Bryophyte gametophytes possess a remarkable ability to regenerate from small bits of tissue, and are sometimes deliberately cut or treated in a blender in order to stimulate growth (Schulte and Reski 2004). When recovering from cryopreservation, generally regrowth of new tissues from old tissues is observed. This is scored as green growth from each individual piece of tissue, 1–2 weeks after warming and transfer to recovery medium.

7.3 Fern Gametophytes

In vitro grown fern gametophytes possess a regenerative capacity similar to that of bryophyte gametophytes. Although their natural desiccation tolerance as a group is more limited compared with bryophytes, tolerance was reported for some species (Mottier 1914; Quirk and Chambers 1981). As with bryophytes, this is likely correlated with the extremes of their natural environment.

There are fewer reports of cryopreservation of fern gametophytes, compared with bryophytes, but it is likely that many of the same procedures used for bryophytes will be applicable to ferns. Encapsulation dehydration, using both beads and strips, was successfully used for cryopreserving gametophytes of several fern species (Pence 2000a; Wilkinson 2002). In some cases slightly higher survival was obtained when tissues were precultured on 10 μM ABA for one week, while ABA preculture also provided some desiccation tolerance for gametophytes that were dried and cryopreserved without encapsulation (Pence 2000a). Encapsulated and unencapsulated tissues were rewarmed at ambient laboratory temperature and transferred to culture medium for recovery and growth. As with moss gametophytes, growth was measured as green, growing tissue emerging from the original piece of cryopreserved tissue.

7.4 Fern Spores

Cryopreserving spores is a fairly straightforward procedure, which, in most cases, requires drying spores and freezing them in LN. There are several reports on the freezing of fern spores, as they are produced in large quantities and are readily available for experimentation (Agrawal et al. 1993; Whittier 1996; Simabukuro et al. 1998; Pangua et al. 1999; Rogge et al. 2000; Pence 2000b). Bryophyte sporophytes are much smaller and less conspicuous than those of pteridophytes, producing fewer spores. As a result, there are fewer reports on bryophyte spore cryopreservation, although the same procedures appear to be applicable to mosses as well as ferns (Pence unpublished results).

Brown (nonchlorophyllous) spores of ferns appear to be analogous to orthodox seeds in higher plants, in that they are desiccation tolerant and often quite long lived (Lloyd and Klekowski 1970; Windham et al. 1986). Successful cryopreservation of air dried brown spores was reported for a number of species. Survival of frozen wet spores was also recorded (Hill 1971), but in other cases wet freezing reduced survival (Pangua et al. 1999; Quintanilla et al. 2002).

Green (chlorophyllous) spores lose viability more quickly than brown spores. However, in tested species green spores appear to be desiccation tolerant and can survive air drying and subsequent storage in LN if frozen while fresh and still viable (Agrawal et al. 1993; Pence 2000b). Green spores of *Onoclea regalis* germinated after silica gel drying or encapsulation dehydration prior to cryopreservation (Pence 2000b).

Recovery of spores after cryopreservation can begin with either rapid warming in a 40°C water bath, direct sowing onto recovery medium, or slower rewarming at room temperature. Agrawal et al. (1993) compared rapid and slower rewarming with dried spores of *Cyathea spinulosa* and found that slow warming provided much better survival. Viability is assessed by observing spores after a given interval of time (e.g. 30 days) and counting germinated and ungerminated spores. Further assessment of the production of two-dimensional growth would also be of interest in cryopreserved spores (Ashcroft and Sheffield 2000). The medium used for germination can also affect results and may need to be optimized for a species. The study with *C. spinulosa* spores indicated that the Knudson's medium (Steeves et al. 1955) gave better germination than Murashige and Skoog's (1962) medium, with the addition of 2% sucrose being inhibitory in both media (Agrawal et al. 1993). Sucrose stimulated fern spore germination in other studies of noncryopreserved spores (Sheffield et al. 2001).

7.5 Fern Sporophytes and Fern Allies

There are few reports of cryopreservation of fern or fern ally sporophyte tissues, although one procedure was reported for *Selaginella uncinata* (Pence 2001). Preculture was for 1 week on 10 µM ABA and freezing was done using the encapsulation dehydration procedure. However, this procedure was not successful with similar, *in vitro* grown shoot tips of *Selaginella silvestris,* a species native to the moist tropical forests of Trinidad (Pence unpublished results) and one which likely possesses much less natural desiccation tolerance than *S. uncinata.* Preliminary tests in this laboratory have also recovered surviving shoot tips from *Adiantum tenerum* after cryopreservation.

There are a number of procedures available for the cryopreservation of shoot tips of seed plants, and fern sporophytes possess tissues that could be adapted to those, e.g. runner tips, meristems. More work is needed in this area to confirm the applicability of shoot tip freezing to a wide range of pteridophytes.

7.6 The Future

As with seeds, spore cryopreservation should prove to be an efficient and stable method for storing a large amount of genetic diversity of ferns within a small amount of space. The spore collections being stored in LN in the Germplasm Bank of the Botanic Garden of the University of Valencia and in the Pteridophyte Bank at the Cincinnati Zoo and Botanical Garden are two examples of what is likely to be a growing trend in the ex situ conservation of endangered ferns. Whereas bryophyte spores are likely also adaptable to cryostorage, this approach still awaits further exploration for endangered mosses and liverworts.

Apart from freezing spores, methods for cryopreserving bryophytes and ferns have focused almost entirely on *in vitro* grown cultures. When cultures are initiated for research, cryostorage is a relatively straightforward step for preserving those lines. For conservation purposes, however, initiating *in vitro* culture lines of rare species in order to provide tissues for cryopreservation may prove to be a significant hurdle. If spores are available, they are the most-efficient choice for cryostorage, but they can also be germinated *in vitro* to initiate aseptic culture lines. Initiating sterile lines from gametophyte tissues directly, however, is more challenging. These

tissues are from one to only a few cell layers thick and do not possess the surface morphology found in many higher plants that can protect against damage from surface sterilants, such as sodium hypochlorite. Most surface sterilization procedures for gametophytes use lower concentrations of sterilant, making a fine balance between disinfesting and killing the tissues. When attempting to initiate an aseptic gametophyte culture, multiple pieces of tissue should be sterilized and cultured. Those that remain uncontaminated should be separated quickly from those pieces that do develop contamination, and any of these that remain green can be used to initiate the aseptic culture. Sodium dichloroisocyanurate (DCCA) (0.5%, 2–5 min) was used in some studies as a surface sterilant (Rowntree and Ramsay 2005), as it provides sterilization at a more moderate pH than sodium hypochlorite solutions, which generally range around a pH of 10.

Another option with some species of bryophytes and pteridophytes which deserves exploration is freezing tissues *ex vitro*. Many species of bryophytes and pteridophytes produce vegetative tissues that are desiccation tolerant either as a constitutive feature or one that can be induced, either with slow drying or the application of ABA. This characteristic is quite widespread in mosses, and is also found in some liverworts, as well as in the sporophytes of those pteridophytes classified as "resurrection plants". If spores are not available, using desiccation tolerant vegetative tissues might preclude the need to initiate aseptic cultures.

Because of the highly regenerative nature of gametophytic tissue, only a small proportion of surviving tissue is needed to regrow the tissue after cryopreservation. A preliminary study in which bryophytes from a tropical rainforest were slowly dried, frozen in LN and then transported by air in a dry shipper to a laboratory several days later resulted in regrowth of several of these bryophytes, when they were warmed and placed on sterile soil (Pence et al. unpublished results). Cryopreservation procedures that provide survival of even a small amount of tissue may be adequate in maintaining germplasm.

Bryophytes and pteridophytes offer a variety of possibilities for cryopreservation for long-term storage, both of genetically valuable research lines and of endangered species. Several procedures using different approaches were developed for these taxa. Further work with protocols for cryopreserving spores and tissues of nonseed plants should continue to improve their efficiency and to provide information on their applicability to a wider range of species.

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7.7 Protocols

7.7.1 Bryophyte Gametophyte Cryopreservation— Mr. Frosty

By ML Christianson based on Christianson, 1998

Day 1: Preconditioning of gametophytic tissues

Prepare in advance
Liquid preconditioning medium: Knop's medium (Brandes and Kende 1968) with 10 µM ABA and 100 mM proline.

Checklist

1. Tools, forceps
2. Preconditioning medium
3. 50 ml sterile disposable centrifuge tubes
4. Bryophyte gametophyte cultures (gametophores or protonemata)
5. Shaker (optional)

The procedure

1. If using gametophores, slice the shoots with a scalpel or damage in a blender for 30 – 90 seconds.
2. Add gametophyte tissue to a 50 ml sterile centrifuge tube.
3. Add preconditioning medium in a ratio of 20:1 (medium: fresh weight of packed cell volume of the tissue).
4. Incubate on a shaker (approximately 125 rpm) or let tubes lie flat.
5. Incubate under normal growth conditions for 3 or 4 days.

Day 5: Cryoprotection and freezing

Prepare in advance
Filter sterilized cryoprotectant solution: 10% w/v glucose and 5% v/v DMSO in water.

Checklist

1. Cryoprotectant solution
2. Tabletop centrifuge
3. Sterile transfer pipettes
4. 2 ml cryovials
5. Freezing container: e.g. "Mr. Frosty", or a Pyrex baking dish
6. –20°C freezer, –80°C freezer
7. Ethanol

The procedure

1. Centrifuge preconditioning tubes to pellet the gametophytic tissues.
2. Decant off the preconditioning solution.
3. Add an equal amount of cryoprotectant solution.
4. Resuspend the tissues and incubate at ambient temperature for 1 h.
5. During this hour, transfer 1 ml aliquots of solution with the tissues into labeled 2 ml cryovials.
6. After 1 h, place cryovials into the freezing container (e.g. Mr. Frosty) with ethanol.
7. Incubate at –20°C to cool at approximately 1°C min^{-1}.
8. Transfer to a –80°C freezer for storage.

Warming protocol

Prepare in advance

Plates of culture medium

Checklist

1. Plates of culture medium
2. Forceps
3. Large-bore sterile transfer pipette
4. Water bath at 40°C
5. 95% ethanol

The procedure

1. Transfer vials from freezer to 40°C water bath to warm quickly.
2. Dip the vials in 95% ethanol and let dry in the laminar flow hood.
3. Use pipette to move solution and tissues to culture plate.
4. Use forceps to transfer tissues to a second plate of culture medium.

Possible problems

Controls should include all of the following during initial testing as some plants are sensitive to certain steps. Loss of tissues at steps 1 or 2 requires investigation before further use of the technique.

1. Precultured
2. Cryoprotected
3. Frozen

7.7.2 Cryopreservation of Bryophyte Gametophytes—Three Drying Protocols

By V Pence based on Pence 1998

Three treatments were tested with different bryophytes (Pence 1998): (1) preculture with encapsulated drying; (2) preculture with open drying; (3) no preculture with encapsulated drying. The steps for treatment 1 are given below, and instructions for variations follow. The need for particular steps will likely depend on the natural desiccation tolerance of the species (Burch 2003).

7.7.2.1 Preculture with Encapsulated Drying

Day 1: Preculture of gametophytic tissues

Prepare in advance

Preculture medium: ½ strength Murashige and Skoog (MS 1962), 1.5% sucrose, 0.22% Gelrite, 10 µM ABA, or similar medium with ABA, appropriate for the species.

Checklist

1. Tools, forceps
2. Preculture medium
3. Bryophyte gametophyte cultures

The procedure

1. Cut or pull off tips of gametophytes.
2. Place gametophyte tips onto preculture medium.
3. Incubate in normal growth conditions for 1 week.

Day 8: Encapsulate tissues

Prepare in advance

1. Alginic acid solution: 3% alginic acid (sodium salt, low viscosity) in calcium-free standard MS medium with 0.75 M sucrose
2. 100 mM $CaCl_2$ solution in MS medium
3. MS medium with 0.75 M sucrose, 25 ml in 125 ml flasks (or similar container to fit on a gyratory shaker).

Checklist

1. Alginic acid solution
2. $CaCl_2$ solution, approximately 75 ml in 125 ml flasks
3. Standard MS medium with 0.75 M sucrose in flasks

4. Sterile plastic Petri dishes (60 × 15 mm) for the alginate
5. Sterile 1 ml pipette for picking up shoot tips in alginate
6. Pipetter
7. Larger sterile glass Petri plate into which to pour beads and $CaCl_2$
8. Long forceps for transferring beads into high sucrose medium

The procedure

1. Pour alginate solution into a sterile plastic Petri dish.
2. Transfer isolated tips into the alginate with forceps.
3. Use sterile 1 ml pipette with pipetter to pull up alginate medium containing a shoot tip in to the pipette.
4. Drip alginate solution into $CaCl_2$ solution to form beads.
5. Let beads stand in $CaCl_2$ for 20 min.
6. Pour beads and $CaCl_2$ solution in large Petri dish.
7. Transfer beads to 0.75 M sucrose incubation medium in flasks.
8. Place flasks on gyratory shaker, approximately 100 rpm, for 18 h.

Day 9: Drying of encapsulated tissues

Prepare in advance

Sterile filter paper (90 mm diameter)

Checklist

1. Sterile filter paper
2. Sterile Petri dishes (100 × 15 mm)
3. Sterile cryovials
4. LN
5. Forceps, long, blunt tipped
6. Laminar flow hood

The procedure

1. Aseptically transfer sterile filter paper into sterile Petri dishes, two layers in each dish, at least two dishes per 30 beads. One of these will be the blotting dish and one will be the drying dish.
2. Transfer beads to the filter paper in the drying dish, first blotting it on the filter paper in the blotting dish. Beads should be as evenly spaced throughout the dish as possible and should not be touching each other or the edge of the dish.
3. Leave the beads in the open dish under the air flow of the laminar flow hood for 4 h to approximately 0.25 g H_2O g^{-1} DW or less.
4. Transfer dried beads to cryovials, ten per vial, and seal caps.
5. Immerse in LN.

7.7.2.2 Preculture with Open Drying

Follow steps for preculture of tissues. On day 8, remove tissues from preculture medium and place them directly onto sterile filter paper in sterile Petri plates under the air flow of the laminar flow hood. Dry for 4 h. Transfer to cryovials and immerse directly into LN.

Encapsulated Drying with No Preculture

Do not preculture the tissues. On day 1 collect the tips of growing cultures as indicated. Follow the procedure for encapsulation, overnight treatment in 0.75 M sucrose, drying and LN exposure.

Warming protocol

Prepare in advance
Plates of ½-strength MS medium with 1.5% sucrose and 0.22% Phytagel.

Checklist

1. Plates of growth medium
2. Forceps, long and blunt-tipped

The procedure

1. Remove vials from liquid nitrogen and place on bench top for 20 min.
2. Aseptically transfer beads or tissues from the vial to growth medium for rehydration and the resumption of growth.

Possible problems
Controls should include all of the following during initial testing as some plants are sensitive to certain steps. Loss of any shoot tips at steps 1–4 requires investigation before further use of the technique.

1. Precultured
2. Encapsulated
3. Sucrose
4. Dried
5. LN exposed

7.7.3 Encapsulation Dehydration of Bryophyte Gametophytes

By J Rowntree based on Wilkinson et al. 1998

In Alginate Strips or Beads

Day 1: Encapsulating gametophytic tissues

Prepare in advance

1. Enough gametophyte material for the procedure
2. Filter paper (Whatman No. 1) cut into 2.0 cm × 0.5 cm strips and sterilized
3. Growth medium: Basal nutrient solution (1/2 or ¼ strength MS at pH 5.8, solidified with 3.5 or 4.0 g l^{-1} Gelrite)
4. 3% alginic acid (sodium salt, low viscosity) in basal nutrient solution without calcium (1 l)
5. Divide alginate solution into jars (1/2 lb honey jars), ca 100 ml in each.
6. 100 mM solution of $CaCl_2$ in basal nutrient medium
7. Divide $CaCl_2$ solution into jars (1/2 lb honey jars), ~100 ml in each.
8. Sterile deionized water
9. Sterile filter paper

Checklist

1. Tools, forceps
2. Alginate solution, sterile 50–100 ml beaker or Petri dish
3. $CaCl_2$ solution, sterile 250 ml beaker
4. Bryophyte cultures
5. Sterile water
6. Sterile filter paper
7. Basal nutrient medium

For alginate strip procedure

1. Filter paper strips 5 × 30 mm, autoclaved
2. 9 cm Petri dishes

For alginate bead procedure

1. Sterile forceps
2. Sterile plastic pipette

Encapsulation
Strips

1. Pour alginate solution into a 9 cm Petri dish.
2. Place double thickness filter paper strips into alginate solution.
3. Remove strips and place into $CaCl_2$ solution for 10 min.
4. Remove from $CaCl_2$ and rinse twice in sterile deionized water.
5. Separate the two strips of filter paper and place on sterile Petri dish with the alginate-coated side upwards.
6. Remove a piece of protonemata (1–3 mm) from stock culture and push into the alginate with fine forceps.
7. Place three protonemal plugs in each strip.
8. Place filter paper strip with protonemal sample into fresh alginate.
9. Remove strip and place into fresh $CaCl_2$ for 10 min.
10. Remove from $CaCl_2$ and rinse twice in sterile deionized water.
11. Remove excess water from strips by placing briefly on sterile filter paper.

Beads

1. Pour alginate into small vials or beakers about 10 ml per vial.
2. Pour $CaCl_2$ solution into a sterile beaker.
3. Remove gametophore shoot tips with fine forceps.
4. Place shoot tips into vials of alginate solution.
5. Cut the end off a sterile plastic pipette using a sterile scalpel.
6. Pick up single shoot tips with pipette and drop into $CaCl_2$ solution. Hold for 10 min.
7. Remove beads from $CaCl_2$ and rinse twice in deionized water.
8. Remove excess water from beads by placing on sterile filter paper.

Day 7: Pretreatment

Prepare in advance
Standard medium with 5% sucrose and 10 μM ABA.

Checklist

1. Forceps
2. Growth medium

The procedure
Transfer strips or beads into Petri dishes containing growth medium with 5% sucrose and 10 μM ABA for 14 days.

Day 21: Dehydration and cryopreservation

Checklist

1. Sterile Petri dishes
2. Laminar flow hood
3. Cryovials
4. LN

The procedure

1. Place strips or beads on the lid of a sterile Petri dish and place in laminar flow cabinet.
2. Dehydrate strips or beads under sterile air flow (0.45 $m \cdot s^{-1}$) for 6 h to 0.1 g H_2O g^{-1} DW.
3. Place the dehydrated alginate strip or bead in a cryovial.
4. Seal vial and immerse into LN.
5. Place frozen vial immediately into long-term storage Dewar.

Warming and recovery

Prepare in advance

Growth medium for that species

Checklist

1. Water bath at 40°C
2. Growth medium

The procedure

1. Remove cryovial from cryostorage.
2. Immerse into a 40°C water bath for 2 min.
3. For recovery place alginate beads or strips with shoot tips in Petri-dish with standard solidified basal medium for 1 week.
4. Place in normal growth conditions.

Possible problems

Controls should include all of the following during initial testing as some plants are sensitive to certain steps. Loss of tissues at steps 1, 2, or 3 requires investigation before further use of the technique.

1. Precultured
2. Pretreated
3. Dried
4. Frozen

7.7.4 Controlled Rate Cooling of Moss Protonemata

By Ralf Reski based on Schulte and Reski 2004

Day 1: Preculture of protonemata

Prepare in advance

Complete medium (Egener et al. 2002) with 87 g l^{-1} mannitol, 10 µM ABA, 100 mM proline (filter sterilized).

Checklist

1. Tools, forceps, and scalpels
2. Sterile Petri dishes
3. Sterile cryovials
4. Complete medium sterilized
5. Micropipette for aseptically transferring medium
6. Protonemata cultures

The procedure

1. Add 800 µl of complete medium to 2 ml cryovials.
2. Chop plant material into very small pieces in sterile Petri plates (if possible colony diameter 0.5 cm or more, but even smaller colonies can be prepared for cryoconservation).
3. Place several pieces of chopped plant material into each cryovial.
4. Cap cryovials and incubate at 25°C for 7 days.

Day 8: Cryopreserve tissues

Prepare in advance

Filter sterilized cryoprotectant solution: 20% (v/v) DMSO + 25% (w/v) glucose in H_2O

Checklist

1. Sterile cryoprotectant solution
2. Micropipette for aseptically transferring medium
3. Controlled freezing apparatus
4. Source of LN

The procedure

1. Add 300 µl of cryoprotectant solution to each cryovial (without removing preculture medium).
2. Incubate for 1–3 h with cryoprotectant solution at room temperature.
3. Controlled cooling: begin at 20°C and cool at –1°C min^{-1} to –35°C. Hold for 10 min. Transfer to vapor phase of LN for long-term storage.

Warming protocol

Prepare in advance

Plates of complete medium for regrowth

Checklist

1. Plates of regrowth medium
2. Water bath set at 30°C
3. Forceps for holding cryovials

Procedure

1. Warm rapidly in a 30°C water bath with agitation.
2. Pour plant material (without washing) onto regrowth medium.

Possible problems

Controls should include all of the following during initial testing as some plants are sensitive to certain steps. Loss of any shoot tips at steps 1–3 requires investigation before further use of the technique.

1. Chopping
2. Preculture
3. Cryoprotection
4. Exposure in the vapor phase of LN

7.7.5 Encapsulation Dehydration of Fern Gametophytes

By V Pence based on Pence 2000a

Day 1: Encapsulate fern gametophyte tissues

Prepare in advance

1. 3% alginic acid (sodium salt, low viscosity) in calcium-free Murashige and Skoog (1962) (MS) medium with 0.75 M sucrose
2. 100 mM $CaCl_2$ solution
3. MS medium with 0.75 M sucrose, 25 ml in 125 ml flasks (or similar container to fit on a gyratory shaker)

Checklist

1. Alginic acid solution
2. $CaCl_2$ solution
3. MS 0.75 M sucrose medium in flasks
4. Sterile plastic Petri dishes (60 × 15 mm) for the alginate
5. Sterile 1 ml pipette for picking up pieces of tissue in alginate
6. Pipetter
7. Larger sterile glass Petri plate into which to pour beads.
8. Long forceps to transfer beads into high sucrose medium

The procedure

1. Pour alginate solution into a sterile plastic Petri dish.
2. Transfer pieces of gametophyte tissues (1–3 mm in length) into the alginate with forceps.
3. Use sterile 1 ml pipette with pipetter to pull up alginate medium containing a piece of tissue and drip into the $CaCl_2$ solution to form a bead.
4. Let beads stand in $CaCl_2$ for 20 min.
5. Transfer beads to MS 0.75 M sucrose medium in flasks.
6. Place flasks on gyratory shaker, approximately 100 rpm, for 18 h.

Day 2: Drying of encapsulated tissues

Prepare in advance

Sterile filter paper (90 mm)

Checklist

1. Sterile filter paper
2. Sterile Petri dishes (100 × 15 mm)

3. Sterile cryovials
4. LN
5. Forceps, long, blunt tipped
6. Laminar flow hood

The procedure

1. Aseptically transfer sterile filter paper into sterile Petri dishes, two layers in each dish, at least two dishes per 30 beads. One of these will be the blotting dish and one will be the drying dish.
2. Transfer beads to the filter paper in the drying dish, first blotting it on the filter paper in the blotting dish. Beads should be as evenly spaced throughout the dish as possible and should not be touching each other or the edge of the dish.
3. Leave the beads in the open dish under the air flow of the laminar flow hood for 4 h to ~0.25 g H_2O g^{-1} DW or less.
4. Transfer dried beads to cryovials, ten per vial, and seal caps.
5. Immerse in LN.

Variation: Preculture

In the species tested, survival was high using this procedure. Survival may be increased further using preculture with ABA. For preculturing fern gametophytes, the following procedure can be used.

Day 1: Preculture of fern gametophyte tissues

Prepare in advance

Preculture medium: ½ MS (or species specific growth medium) with 1.5% sucrose, 0.22% Gelrite and 10 µM ABA

Checklist

1. Tools, forceps
2. Preculture medium
3. Fern gametophyte cultures

The procedure

1. Separate gametophytes from clumps with forceps or cut pieces with scalpel.
2. Place gametophyte pieces into preculture medium.
3. Incubate in normal growth conditions for 1 week.

Day 8: Encapsulate tissues

Proceed with the encapsulation procedure described above.

Warming protocol

Prepare in advance
Plates of growth medium, e.g. ½ MS with 1.5% sucrose and 0.22% Phytagel.

Checklist

1. Plates of growth medium
2. Forceps, long and blunt-tipped

The procedure

1. Remove vials from liquid nitrogen and place on bench top for 20 minutes.
2. Aseptically transfer beads or tissues from the vial to growth medium for rehydration and the resumption of growth.

Possible problems
Controls should include all of the following during initial testing as some plants are sensitive to certain steps. Loss of any shoot tips at steps 1–3 requires investigation before further use of the technique.

1. Precultured
2. Encapsulated
3. Sucrose solution
4. Dried
5. LN exposed

If preculture is not used, omit controls for step 1.

7.7.6 Cryopreservation of Fern Spores 1

By Ramón Arbesú, M. Angeles Revilla, and Helena Fernández (unpublished)

Prepare in advance

1. Collect fern fronds when they have mature sporangia, between January and July depending on the species.
2. Place the collected fronds between newspaper sheets for 2–3 days.
3. Sieve the released spores through sieves of 250 and 80 µm successively. Keep the spores in tubes.

Items needed

1. Spatula, sieves 250 µm and 80 µm, cryovials
2. Sterile solution of Tween in distilled water and sterile water
3. Sterile centrifuge tubes (10 ml)
4. Solid ½ MS medium with 20 g l^{-1} sucrose, pH 5.7 (9 cm Petri dishes)

The procedure

This is a 1-day procedure once the spores are collected.

1. Place about 5 mg of spores in a cryovial.
2. Plunge the cryovials into LN. Hold in LN for 30 min.
3. Warm at room temperature for 5 min.
4. Remove the spores from the cryovial and rehydrate them in 5 ml of Tween solution for 2 h (one drop Tween in 100 ml).
5. Transfer the solution with the spores to sterile centrifuge tubes with caps and spin at 2,500 rpm for 5 min.
6. Remove the supernatant and add 5 ml of commercial bleach diluted 10%, shake and leave for 3–4 min.
7. Spin 2,500 rpm for 3–4 min, remove supernatant and add 5 ml of sterile distilled water, shake and leave 3–4 min. Repeat this step twice.
8. Dispense 0.5 ml of the spores solution onto a Petri dish with solid ½ MS with 20 g l^{-1} sucrose, pH 5.7. Keep the plates with the spores in growth chamber at 25°C and 16 h photoperiod.
9. Depending on the species and on the year, after 1 or 3 weeks it is possible to observe the germination of the spores (ranges from 40 to 90%). After another 4 weeks, the gametophytes are regenerated and subsequently the sporophytes.
10. Spores of *Davallia canariensis, Ceterach officinarum, Dicksonia antarctica, Polypodium cambricum, Cyathea cooperi* and *Osmunda regalis* were stored and recovered with this protocol.

7.7.7 Cryopreservation of Fern Spores 2

By AM Ibars, D Ballesteros, and E Estrelles (unpublished)

Checklist

1. Fern spores collected from mature sporangia and sieved
2. Cryovials, freezer gloves, silica gel
3. –80°C freezer or/and LN

The procedure

1. Put spores in cryovials.
2. If the ambient relative humidity is higher than 60%, the spores should be desiccated over silica gel to a relative humidity of 20–55% over 48 h. Green spores can not be dried after sporangia dehiscence.
3. Label tubes with reference number; ideally, use Cryo Labels.
4. Put cryovials directly in –80°C or LN.

Thawing protocol

1. Remove the cryovials containing the spores from the freezer or LN and thaw at ambient temperature for 30 min. Alternatively, the spores can be thawed rapidly with a warm water bath at 37–40°C for 4 min.
2. Dry the outside of the cryovials before opening them. The cryovials can be wiped with alcohol, as an additional measure to prevent contamination.

Spore culture checklist

1. Plates of sterile liquid culture medium (Dyer, 1979)
2. Micropipette, 1 ml
3. Sterile micropipette tips, Eppendorfs
4. Sterile liquid medium
5. 70% ethanol

The procedure

1. Dispense medium into culture plates in a laminar flow hood.
2. Transfer 1 mg of spores to an Eppendorf.
3. Suspend spores in 1 ml of liquid medium.
4. Sow eight drops per plate and distribute them evenly around the plate.
5. Germinate spores in a culture chamber at 20–23°C and a photoperiod of 12 h of white light (daylight fluorescent tubes, photon irradiance 30±45 μmol m^{-2} s^{-1} in the 400–700 nm region).

Chapter 8
Cryopreservation of Dedifferentiated Cell Cultures

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8.1 The Use of Dedifferentiated Plant Cell Cultures

When Gottlieb Haberlandt made the first efforts to cultivate single isolated plant cells in salt solutions his goal was to prove the totipotency of single cells (Haberlandt 1902). The cultivation of isolated plant cells in a chemically defined culture medium became possible only after the discovery and application of auxins (Gautheret 1939). Today plant cells as well as tissues can be cultivated *in vitro* for many applications in plant breeding, plant propagation, germplasm preservation and molecular biology.

The idea to produce valuable plant metabolites by large-scale fermentation of cell cultures was soon born (Routien and Nickel 1952). Scientists were fascinated by the idea of using dedifferentiated cell cultures as a replacement for intact plants in research and biotechnology. Tulecke and Nickell (1959) wrote: "…in essence these cell cultures represent a new kind of microorganisms…" The formation of a number of secondary metabolites was detected in the new material and in some cases the concentration of these compounds in the cell cultures exceeded even that in intact plants (for review see Carew and Staba 1965). The concept to produce such valuable compounds by growing cell cultures in fermentation vessels offers many advantages: the production is independent from specific climatic conditions and it can be carried out under defined and

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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sterile conditions. Furthermore, the production of extractable raw material from plant cell cultures can be quicker than with intact plants, especially for plants like ginseng where harvest starts many years after planting and destroys the plants. Other candidates for cell mass production by cell culture are plants growing extremely slowly or where the content of a certain compound is extremely low like for the drug paclitaxel in *Taxus* plants.

Although in many cases high concentrations of certain compounds were obtained in plant cell cultures, like rosmarinic acid in *Coleus blumei* cell lines (see Berlin 1997), ginsenosides in *Panax ginseng* cell cultures (Thanh et al. 2005) or Raucaffricine in cell lines of *Rauvolfia serpentina* (Schuebel and Stoeckigt 1984), examples for the economic application of large scale fermentation of dedifferentiated plant cell cultures remain rare. The first process was established by the Mitsui Petrochemical Company for the production of Shikonin, a red-colored antimicrobial compound traditionally used in Japan (Fujita et al. 1982). Presently the best known example is the production of paclitaxel by Phyton in a large scale fermentation facility specific for plant cell cultures close to the city of Hamburg (Venkat 1998).

Cell lines were also investigated for their potential to carry out bio-transformations. An example is the transformation of hydroquinone to arbutine by cell lines of *Rauvolfia serpentina*. The fact that the gycolsyltransferase responsible for this biotransformation was successfully transferred to microorganisms shows that plant cell cultures may serve also as a source of model enzymes from secondary metabolism, even when they are not directly used for production (for review see Alfermann et al. 2003). More recently, molecular biology was used to influence or modify metabolic pathways of secondary metabolites and thereby solve the problem of product accumulation in cell cultures (Verpoorte et al. 1999).

Today not only the production of classical secondary plant metabolites but also of recombinant proteins is accomplished using dedifferentiated plant cell cultures. Microoorganisms are usually unable to complete the glycosylation of eucaryotic proteins so production is often impossible. Today transformed animal cells are used most often for the production of pharmaceutical proteins. In addition to costs, animal cell cultivation bears much higher risks concerning biosafety than that of plant cell lines. Animal cells can easily be contaminated with microorganisms or with viruses pathogenic for humans. Such contaminations may even occur from medium components needed for animal cell line cultivation. In contrast these risks are extremely low in the case of plant cell lines. A contamination of plant cell lines with human-pathogenic viruses is almost impossible. Compared to the production with transgenic plants, dedifferentiated plant

cell cultures provide the advantage of growth under controlled and strictly sterile conditions. This will possibly meet regulatory requirements more easily than plant cultivation in the field or even in glass houses. The use of transgenic plant cell lines will eliminate environmental risk of the release of transgenic plants. Recently recombinant proteins were successfully expressed in plant cell cultures. Examples are the production of thrombomodulin by tobacco cells (Schinkel et al. 2005), the production of human serum albumin by rice cell cultures (Huang et al. 2005) and hepatitis B surface antigen production by tobacco cell cultures (Sunil Kumar et al. 2005). The most important drawback is the comparatively low protein concentration in plant cells.

More important is that dedifferentiated plant cell cultures are widely used for a broad range of applications in basic and applied research. An early and still ongoing application is the search for novel bioactive compounds. The first big project was initiated by Nattermann & Co., a German pharmaceutical company (now a part of Sanofi-Aventis). Currently the approach is carried on using improved methods for isolation as well as for activity testing. Recent investigations were performed by Phytera Inc. (now Galileo Pharmaceuticals Inc.) (Stafford 2002).

In basic research it was mainly the investigation of biochemical pathways leading to secondary metabolites where dedifferentiated plant cell lines turned out to be powerful tools. Examples are the biosynthesis of indole alkaloids (Kutchan 1993), morphinan and benzophenanthridine alkaloids (Huang and Kutchan 2000) and anthraquinones (Han et al. 2001). Not only biosynthesis of compounds was investigated, but also their biodegradation, especially of xenobiotics (Bode et al. 2004).

Dedifferentiated plant cell cultures are important tools in plant stress research. Early investigations on phytoalexin production (Schumacher et al. 1987) were made with plant cell cultures. An important class of stress metabolites, the phytochelatins, was first discovered in dedifferentiated cell cultures (Grill et al. 1991).

8.2 The Need for Cryopreservation

It is obvious that the various applications of dedifferentiated plant cultures all need stability of traits. This aspect is most important in the case of cell lines used for production. Furthermore, if pharmaceutical compounds are concerned, any production system has to meet strict regulatory requirements including the characterization of the production source and process. Stability of traits and characters is of basic importance for other applications

as well. Experiments must be reproducible and in screenings, testing of herbicides, fungicides, growth regulators or stress conditions, the metabolic stability of the cell line is a pre-condition for the comparability of results over time. Even in the search for novel compounds stability must be provided, at least as long as other means to obtain the compound are not available.

The need for stability is contrasted by the lack of any alternative long-term preservation method other than cryopreservation for dedifferentiated plant cell lines. Laboratory maintenance may be affected by technical failure and infection. Sometimes fungal or bacterial contaminations do not much affect an intact plant, while a cell line is normally destroyed by any microbial contamination. Furthermore, the specific genetic situation exposes a dedifferentiated cell line to an increased risk for loss of metabolic traits by genetic changes. Often in the case of intact plants durable seeds provide a simple system that allows at least medium-term storage, sometimes for many years. For vegetatively propagated species differentiated growth *in vitro* provides stability, or specific organs like tubers can be stored for some time. In contrast dedifferentiated cell cultures grow continuously. Although slow-growth methods for callus and suspensions exist, they cannot stop genetic changes completely and furthermore expose the material to an undesired selection pressure. Therefore cryopreservation is the only way to solve all these problems.

8.3 Culture Stability

The most important demand on a preservation method is maintaining the material true to type. Concerning the stability of traits, specific aspects should be taken into consideration for dedifferentiated plant cell cultures. The most important threat for the valuable traits of a cell line results from the specific genetic and epigenetic situation of these materials. For many callus cultures heterogeneity of the initial material can be observed clearly from secondary metabolite formation. Often the colours of the cells are indicators of differences in metabolite content. Many of the callus cultures maintained in the cell culture collection of DSMZ show such heterogeneity of colour, which has been stable over many years. Although showing a higher degree of homogeneity, most suspension cultures consist of a wide range of cell cluster sizes and cell shapes.

The diversity among single cells or cell clusters of a cell culture was measured. The heterogeneous distribution of different enzyme activities was observed as well as a characteristic oscillation of enzyme activities

reflecting the growth cycle of the cell line (DeJong et al. 1967). Very often higher product content was measured in older cells, and cell cultures often contain higher metabolite concentrations in the late growth phase. For non-coloured substances like nicotine, Ogino (Ogino et al. 1978) showed a high variation of content in different cells of the same culture. Many researchers used the heterogeneity of dedifferentiated cell cultures to establish high yielding strains for production of metabolites. The first economically applied cell culture process, the production of shikonin, was already based on cell selection. Nevertheless a debate about the stability of these high yielding strains soon came up and is still continuing today. In some cases the strains seem to be stable (Sato and Yamada 1984), whereas in other cases high yielding strains reverted to normal yields when the selection pressure was no longer maintained (Deus-Neumann and Zenk 1984). Often it remained unclear whether high yielding strains were genetically or "only" metabolically different from low yielding strains.

Variation among single cells of a cell culture is also reflected by plants regenerated from embryogenic cell cultures for propagation or breeding purposes. This phenomenon termed somaclonal variation was defined by Larkin and Scowcroft in 1981. Often these variations were attributed to chromosomal aberrations (Bayliss 1980), point mutations or even transposon activation (Alves et al. 2005). In these cases, the variation is based on stable genetic changes. Nevertheless in many cases somaclonal variants revert to the normal phenotype. This indicates that also epigenetic effects seem to be involved (Kaeppler et al. 2000). In early experiments it was evident that certain traits of cell cultures were not always genetically fixed. Plants regenerated from callus selected for salt tolerance did not retain the same salt tolerance (Chandler and Vasil 1984). Similar evidence came also from work done in the field of secondary metabolites. Chaprin and Ellis (1984) showed differences of the rosmarinic acid production among cells of a culture of *Anchusa officinalis*. Nevertheless they demonstrated that no correlation occurred between the production rate of a suspension culture and the production rate of the corresponding mother cells. Interesting work was also done by Dougall and coworkers. They performed simple cooling experiments with cell lines selected for high and low anthocyanin production and could show that the production capacity was not affected by the freezing process. Nevertheless they also demonstrated that during prolonged propagation high-producing cells were occurring again in the low-producing cell lines and—vice versa—low producing cells occurred again in the high producing lines (Dougall and Whitten 1980).

The effects described above should be taken into account if the stability of a cell culture after cryopreservation is to be judged. With undifferentiated cell lines it may be even more difficult to define as well as to measure stability. Certain cell types in a cell culture, callus or suspension may withstand the stresses of a cryopreservation procedure better than others. Product yield, cell shape, water content, or metabolic capacity may favour specific cells during cryopreservation. All this may result in a population of cells which may differ from the initial population subjected to freezing in many ways, but nevertheless resemble the initial cell line completely after a number of passages. On the other hand a cell line recovered from cryostorage may reflect the character of the cell line subjected to freezing completely, but after some passages may differ from the initial cell line due to a general instability of the original cell culture during sub-culturing.

8.4 Cryopreservation

The breakthrough for cryopreservation of animal cells was the application of DMSO as a cryoprotectant (Lovelock and Bishop 1959). Plant cells are more difficult to freeze than all other cells due to their sub-cellular structure. High water content and the sub-cellular vacuoles are specific features of plant cells. Many suspension cultures consist of elongated and highly vacuolated cells. Furthermore many cell types are present in a suspension culture. This has to be taken into account when developing a cooling procedure. Dedifferentiated cell cultures were the first plant cells to be cryopreserved. The first successful *in vitro* cryopreservation experiment was carried out by Quatrano in 1968. In 1985 Withers listed in a review 22 cell lines of different plant species frozen to liquid nitrogen temperatures. Several secondary metabolite containing cell lines were cryopreserved. In almost all cases product formation turned out to be stable (Schumacher 1999).

Initial studies were done with the classical approach, controlled rate or equilibrium freezing, combining preculture for dehydration, exposure of cells to a cryoprotectant solution and slow cooling of cells either stepwise or by defined cooling rates (Chap. 5). These were achieved by more or less sophisticated equipment (Withers and King 1980) (Protocol 8.10.1). More recently the vitrification method, initially applied to differentiated plant material, was used for dedifferentiated cell lines (Chap. 3). Similar is true for the encapsulation-dehydration method (Chap. 4). Nevertheless not much work was done using these newer methods with dedifferentiated cell lines. Therefore, it is difficult to compare the potential of these methods

for the cryopreservation of dedifferentiated cell lines with that of controlled rate cooling. Recently a combination of encapsulation and vitrification was used for suspension culture freezing. In this method, cells are not dehydrated by air desiccation after encapsulation but by incubation in PVS2 (see Chap. 3). Since there is not much experience with this new approach it is not further mentioned in this chapter.

8.5 Cultivation Aspects

Several general aspects of plant cell line cultivation have importance for cryopreservation. Dedifferentiated plant cell lines are either cultivated as callus cultures on solid medium or as suspension cultures in liquid medium. Normally, it is possible to shift from one to the other cultivation method. Callus cultures grow more slowly than suspensions and what is more important they are normally more heterogeneous. The higher degree of homogeneity may be one reason why suspensions in general perform better in cryopreservation experiments than callus. Another reason may be that callus is always injured when small pieces are taken for cryopreservation or that individual cells are not as evenly exposed to cryoprotectants. Both types of material show a growth curve that is typical for each specific cell line. The physiological state of cells differs in different stages of the growth curve. Very often cells show more secondary metabolites in late stages. Often, but not always, the water content and the size of vacuoles in the cytoplasm increase in the late growth phase. The starting material for cryopreservation experiments is best taken from the logarithmic growth phase of a cell culture.

All important aspects and all possible details about the growth curve of a cell line should be known before starting a cryopreservation experiment. The easiest and most practical method to determine that a suspension is in a certain growth stage is the measurement of the packed cell volume (pcv). Pcv is measured by pouring a defined volume of a homogeneous suspension in a graduated tube, let the cells settle down for a defined time (for example 15 min) and measure the ratio of volume of cells settled down to total volume of suspension.

The standardization of a cryopreservation experiment requires that the initial material is always taken from the same physiological state. For the reproduction of a once measured growth curve it is therefore important to develop and apply a standardized method for the sub-culture procedure of the cell line. This can be achieved by pouring a defined volume of homogeneous suspension into a defined volume of fresh medium (see

Swan et al. 1999). For this purpose either a sterilized pipette or a sterilized measuring cylinder may be used. Another method is to harvest the cells by filtration through nylon net cut to fit into a Buchner funnel. Such woven polyamid screens are available with different pore sizes from laboratory equipment dealers (for example neoLab, Heidelberg, www.neolab.de) (Fig. 8.1). Let the medium drain off and weigh a defined amount of cells into a flask containing fresh medium under sterile conditions.

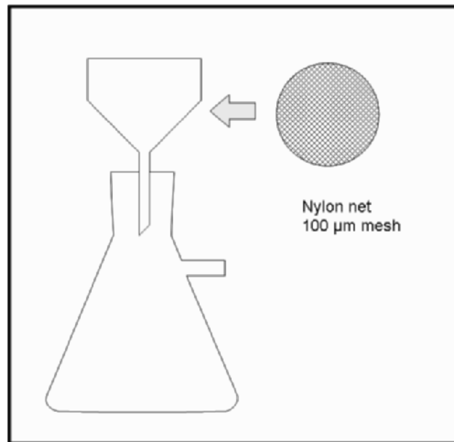

Fig. 8.1 For harvesting suspension cells we use an autoclaved Buchner funnel on top of a sterilized suction bottle. A sterilized nylon net is placed in the Buchner funnel. Nylon tissue is available with different pore sizes (we usually use a pore size of 100 µm) and can be cut out to fit for a specific Buchner funnel

Often the first method, transferring some of the used medium into the new flasks, yields better results. Nevertheless the second method is more exact. For both methods dedifferentiated cell cultures often stop growth if the ratio of cells to medium is too small. If for routine maintenance a standardized transfer method is not applied it is recommended to apply such a method for at least three passages before cryopreservation experiments are started.

During the steps of a cryopreservation procedure the cell density is often a critical parameter for success. This may differ from cell line to cell line. It is therefore not possible to give a general recommendation for how dense a specific cell line should be in a specific step of a procedure. For any cell line, cell density should always be standardized in a certain step of a procedure. Here again the measurement of pcv values is the most simple approach to standardize cell density. After determination of the pcv, fresh

medium can be added or used medium removed to standardize the cell density in a specific step of the whole cryopreservation procedure.

8.6 Controlled Rate Cooling

Most of the controlled rate cooling approaches are based on the method published by Withers and King (1980). This classical approach consists of several steps. The first step is a dehydration of cells achieved by a preculture period in medium supplied with osmotically active substances. After the preculture procedure cells are exposed to a cryoprotectant solution. A variety of cryoprotective mixtures are available (Table 8.1). Almost all mixtures contain DMSO and DMSO alone can be used as a cryoprotectant. The most frequently applied solutions are DGS and DGP, although they are highly viscous and somewhat difficult to handle. Further dehydration of cells is then achieved by slowly cooling the cells applying controlled cooling rates ranging around –1°C min.

For the preculture solutions, the osmoticum used is simply dissolved in normal culture medium. Sorbitol and sucrose can be used at higher concentrations than mannitol which dissolves only up to approximately 1M. An adjustment of the pH is important. For the final concentration a dilution factor has to be taken into account if not only cells but a part of the old medium is transferred into the fresh medium during subculture. Preculture medium can be sterilized by autoclaving.

The cryoprotectant solutions DGS and DGP are prepared by dissolving the components in normal culture medium. Be aware that a comparatively low volume of additional medium will be necessary. The resulting solution is highly viscous. It may be heated moderately to dissolve the sugar. The solutions should be filter sterilized. To facilitate filter sterilization compounds may be warmed up moderately and vacuum may be applied.

To achieve survival of a cell line or to optimize the results for the cell line, the basic procedure offers the possibility for modifications (see Protocols). Depending on the cell line, mannitol, sorbitol or sucrose may yield better results for preculture. The concentrations used for preculture range from 0.1 to 1.2 M. The time span varies from 24 h to up to 6 days. Incubation time in the cryoprotectant solution can be modified. It depends on the viscosity of the mixture used and the size of the cell clusters which occur in the suspension. If a fine suspension culture is to be frozen and only DMSO is used as cryoprotectant, then the incubation time can be short (15 min). For a suspension culture forming bigger cell clusters and if DGS or DGP is used, longer incubation times are recommended (30–60 min). Finally the cooling rate has a great influence.

Although in the literature cooling rates of 1°C/min are most often recommended, we achieved good results for some cell lines with much slower cooling rates down to 0.1°C/min. The optimum end temperature for the two step cooling process and the hold time at that temperature can also differ. The range of hold temperatures applied lies between –30°C and –40°C and times between 15 and 40 min. The various parameters cannot always be optimized independently of each other. Combinations should be empirically tested. An expensive controlled rate freezer is not always necessary to achieve slow cooling rates. Cryovials may be simply placed in styrofoam boxes placed in a freezer at –70°C or –80°C. Cooling containers filled with isopropanol are commercially available from Nalgene Inc. Placed in a freezer they achieve cooling rates of approx. 1°C/min. An example of a typical controlled rate cooling procedure (after Swan et al. 1999) is listed in the protocols section (Protocol 8.10.2).

Table 8.1 Cryoprotectant solutions used for controlled rate cooling of dedifferentiated cell lines

Name	Composition	Reference
DGS	1 M DMSO 1 M Glycerol 2 M Sucrose	Withers and King 1980
DGP	1 M DMSO 1 M Glycerol 2 M Proline	Withers and King 1980
DGSP	1 M DMSO 1 M Glycerol 1 M Sucrose 1 M Proline	Meijer et al. 1991
DGlu	2.5 M DMSO 1.1 M D-Glucose	Watanabe et al. 1983

Rewarming of cells is normally done in a water bath at 30–40°C. Contamination is especially a problem for dedifferentiated suspension cultures. Disinfecting the cryovials from the outside using a disinfectant like 70% ethanol avoids microbial infections when cryovials are opened to remove the cells. Care should be taken to not over tighten the caps of cryovials as contaminations may also occur from mechanical distortion of the vials when they are rewarmed in the water bath. Rewarming of cryovials in a water bath is therefore probably the most critical step for

contamination. It is important to use a small water bath or a beaker filled with sterile water.

For most of the suspension cultures a recovery phase on solid medium seems to be important. After warming, removal of the cryoprotectant solution is important. Washing of cells turned out to be disadvantageous. Draining off the cryoprotectant can be achieved by placing the cells onto a filter paper on top of the normal medium. After diffusion of the cryoprotective solution into the growth medium the filter paper can be carefully moved to a new dish without touching and damaging the cells. If the cryoprotectant turns out to be more toxic, the transfer can be done after a shorter time or even for several times. A stepwise reduction of the high osmotic value of the cryoprotectant solution down to the osmotic value of the normal growth medium may be needed for some cultures. Cells can be transferred stepwise onto medium containing decreasing concentrations of the preculture medium.

Dedifferentiated plant cells normally regrow better in the neighbourhood of other cells. Therefore clustering of cells in the middle of the filter paper is recommended. This can be achieved by first removing the cryoprotectant solution with a pipette or by decanting it from the cells and manipulating the cells out of the cryovials with a sterile spatula. Initially cells should be cultivated for some days in the dark. A new suspension can be initiated by transfer of the cells to liquid medium; keeping in mind the critical number of cells needed to initiate a culture.

Controlled rate cooling has many advantages for dedifferentiated cell lines. It offers the potential to adjust parameters to the needs of each specific cell line. The major advantage may be that, once a method is worked out and optimized, it allows the preservation of large numbers of cryovials in one batch and in a standardized manner.

8.6.1 Minitest Method for Evaluation of Parameters for Controlled Rate Cooling

Many parameters may require adjustment before a particular cell line can be successfully cryopreserved. To speed up the experimental work to find the right conditions for a specific line, we developed a miniaturized system, where steps are carried out in 24-multiwell titerplates. Each well has a total volume of 3 ml. Preculture procedures as well as cryoprotection can be carried out without removing the cells from the plates. Also viability tests are performed in these plates. The minitest system is reproducible and allows the optimization of several cryopreservation parameters at the same time under identical cultivation conditions using only small amounts

of cell material. The solutions and media needed are essentially the same as used for conventional controlled rate cooling. The equipment for the cultivation of cells requires some explanation. This minitest system follows the classical approach of Withers and King (1980).

Cooling

1. Apply a standard subculture procedure for routine maintenance.
2. Determine the specific wet weight of the cells for each specific cell line (= ratio wet weight of cell mass/volume of cell mass). Determine the weight of an empty costar tube (15 ml) having a scale. Pour 100 ml of cell suspension into a Buchner funnel containing nylon net (100 µm pore size), placed on a collecting flask and let the cells drain off until medium stops dripping out of the Buchner funnel. Fill the costar tube with cell material up to a volume of 9 ml (even meniscus). Determine the weight of the filled tube, and by subtraction of the tube's empty weight calculate the wet weight of the cells.
3. Harvest cells in the logarithmic growth phase by filtering off the medium through a nylon net (100 µm pore size) in a Buchner funnel and let the cells settle down (use the same conditions as for determination of specific cell wet weight).
4. For testing at 30% pcv, put 1 ml preculture medium in each well (testing different preculture conditions we usually apply media with six different osmotic values (including controls) with four replicates making up 24 wells in total; if different cryoprotectants should be tested in combination with one specific preculture procedure all 24 wells are filled with the same preculture medium; remember to check the controls).
5. Place the multiwell plate on a balance in a laminar flow bench and add an appropriate cell mass by weighing into each of the 24 wells (Fig. 8.2) Example for 30% pcv: if the specific wet weight of cells is 1.1 g/ml, to reach 30% pvc in each well at a total volume of 1.5 ml the cell volume should be 0.45 ml corresponding to 0.5 g cell weight; we use flamed little spoons; keep the plate covered by the lid as much as possible to avoid evaporation of the medium; the recommended pcv value in the wells is 30–40%.

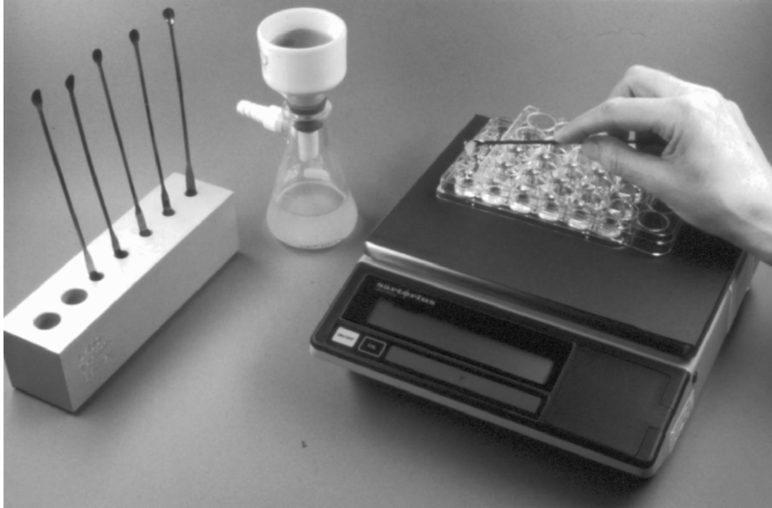

Fig. 8.2 For inoculation, the wells of a multiwell plate are first filled with medium of the respective preculture solutions. Then the plate is placed on a balance in a laminar flow bench. For transferring the cells into the wells we use flamed little spoons

6. After inoculation seal the plate twice with Parafilm to minimize evaporation. Place the plate on a shaker under normal growth conditions. Cultivation can usually be done for up to 7 days (for shaking: avoid mechanical damage of the cells but prevent cells from settling down. Normal microplate shakers cannot be used because of their short stroke length requiring too high rpm values).
7. To minimize toxic effects of the cryoprotectant, first precool the titerplates with the desiccated cells on a cool pack in a 4°C cold room on a shaker.
8. Add the cryoprotectant to the plate on ice in the laminar flow bench.
9. Add the cryoprotectant
    - Compounds with low viscosity like pure DMSO can be added in small amounts directly to the cell suspension.
    - For treatment with highly viscous cryoprotectants, e.g. DGS, draw off the preculture medium with autoclaved and dried cigarette filters under sterile conditions. We use filter cartridges (cellulose acetate 17 mm long, 8 mm in diameter) commercially available as smoking supplies (see Fig. 8.3).

Fig. 8.3 To expose the cells to different solutions without removing them from the multiwell plate commercially available sterilized and dried filters for homemade cigarettes are used. To draw off the old solution a cigarette filter is placed gently in an upright position into each well of the titerplate using a forceps. To regain those cells adsorbed to the bottom of the filter scrape off cells on the edge of the wells

10. Continue work with a double concentrated suspension (in our example 60% pcv) and add the same volume of DGS. (To determine the volumes needed first estimate the volume of the residual cells in the wells (usually ~0.4 ml); calculate a volume of fresh medium to make up 60% pcv (~0.26 ml); mix DGS and fresh medium at a ratio of 1:0.4 (~70% DGS) and add 0.92 ml of this previously chilled mixture to each well).
11. After addition of the cryoprotectant, incubate the plate on ice in a cold room at 4°C on a shaker for a defined period of time (usually 20–40 min).
12. Fill the suspensions of one well into a cryovial and immediately place it on ice. In the case of non-viscous cryoprotectants and fine suspensions, pipette tips are cut to achieve a wider opening. Cells which are not easily transferred with a pipette are transferred with flamed spoons followed by transfer of the residual liquid with a pipette. Fix cryovials to aluminium holders and cover them with ice until the total incubation time of the cryoprotectant has passed (for DMSO and DGS 1h of total incubation time is often favorable).
13. Continue cooling as described for conventional controlled rate cooling.
14. Rewarming and recovery is carried out as described for conventional controlled rate cooling.

8.6.2 Determination of Cell Viability

To assess the influence of different cryoparameters and their interaction for the final development of an optimal cryopreservation procedure it is necessary to evaluate the cell viability also after each step of the procedure. To test viability in intermediate states of the procedure we usually use a modified triphenyl tetrazolium chloride (TTC) (2,3,5-triphenyl tetrazolium chloride) test performed directly in the multiwell plates. Although other procedures might be more exact, the test in the multiwell dishes allows a comparison of parameters tested and helps to facilitate handling and avoid mixing of samples.

Reagents: Tris buffer 50 mM pH 7.5; TTC (2,3,5-triphenyltetrazolium chloride) 2% solution (w/v) in 50 mM Tris buffer pH 7.5. TTC is not stable in the light; store the TTC stock solution and the samples in the dark.

Procedure

1. First the supernatant in each well is drawn off with cigarette filters: Place a cigarette filter in an upright position into each well. To reduce loss of cells to a minimum, regain those cells which are adsorbed to the bottom of the filter by scraping cells on to the wall of the wells. Discard the cigarette filters. If the cells are still wet, repeat using another cigarette filter.
2. Add 1250 µl of Tris buffer (50 mM, pH 7.5) and 300 µl TTC (2%, w/v) to each well.
3. Seal titerplates twice with Parafilm strips covering the gap between top and bottom of the titerplate and briefly mix the titerplates on a titerplate shaker.
4. Incubate overnight at room temperature in the dark.
5. The supernatant is removed in two steps: Remove about 1 ml using an automatic pipette, then draw off the residual liquid with one cigarette filter. Move scraped cells with a small spoon).
6. Add 1.5 ml ethanol to each well (placing the tip of the pipette above the middle of each well helps to mix the cells). When all wells of one column are filled immediately seal them with one strip of self-adhesive titerplate film to minimize evaporation. When all columns are filled with ethanol seal them all with one large sheet of the same self-adhesive titerplate film and close the titerplate with its lid.
7. Seal the titerplate twice with Parafilm strips.
8. Incubate overnight at room temperature in the dark for extraction.

9. For spectrophotometric quantification cut with a scalpel the self-adhesive titerplate film into strips along the gaps, between the columns of the titerplate. Then cut every strip into pieces covering one well each.
10. Open one well at a time by removing the film. Transfer 1 ml or less of the supernatant into an Eppendorf vial and centrifuge at 14.000 rpm for 5 min to remove residual cells and measure absorption at 500 nm after appropriate dilution.

Although the simplified version of the TTC test does not take into account the amount of cells in each well, which may differ due to growth, it is reproducible and exact enough to allow a comparison between the different treatments tested in almost all cases. The results are reproducible for up to 7 days of growth in the wells.

The basic advantage of the system is that many parameters can be evaluated with a minimum amount of cell material. The approach allows us to test the effect of osmotic compounds on the dehydration of the respective plant cells, the influence of concentrations of one osmotic agent and incubation times in the presence of an osmotic agent. Since the method allows changing the incubation solution without transferring the cells, it allows testing parameters in combination. Sometimes these tests do not show a distinct optimum and scale up is required to find the optimum.

8.7 Vitrification

The vitrification method is very successfully applied to many differentiated plant tissues. Fewer efforts are made to preserve dedifferentiated cell lines using this approach. The composition of some of the solutions used for vitrification of dedifferentiated cell lines is given in Table 8.2. In most cases PVS2 created by Sakai (Sakai et al. 1990) is the most successful vitrification solution. Several procedures are published, but only Reinhoud et al. 1995 describe a single method which was used for suspension cultures from different plants.

In many simple procedures the osmotic loading and the dehydration process is carried out in one step with the same solution (Protocols 8.10.3 and 8.10.4). Sometimes the loading solution is a diluted version of the solution used for dehydration Chen Y, Wang JH (2002). For the recovery of the cells exposed to the highly concentrated vitrification solutions a rinsing step is necessary before cells can be placed in the normal growth medium again. In almost all procedures for rinsing cells, a solution consisting of

Table 8.2 Combinations of loading and dehydration-vitrification solutions used for the cryopreservation of dedifferentiated cell lines. The best vitrification solution for plants is PVS2. A 1.2 M sucrose solution in culture medium is used for rinsing

Loading Solution	Dehydration/Vitrification Solution	Reference
20% PVS2 and 0.4 M sucrose	100% PVS2 composed of 3.26 M glycerol, 1.92 M DMSO, 2.42 M ethylene glycol, 0.4 M sucrose	Reinhoud et al. 1995
25% PVS2	100% PVS2	Chen and Wang 2002
2 M glycrol and 0.4 M sucrose	100% PVS2	Tsukatzaki et al. 2000
1.5 M ethylene glycol	7 M ethylene glycol 0.88 M sorbitol 6% BSA	Langis et al. 1989

1.2 M sucrose dissolved in normal growth medium was used. Vitrification is largely facilitated by high cooling rates which can be better achieved by using straws instead of cryovials. Fine suspension cultures offer the chance to use very thin straws available from companies providing veterinary equipment for artificial insemination. Cooling rates can be increased using cryovials with thin walls and small volumes of suspension. The first plant cell suspension culture was successfully frozen by this approach in 1989 (Langis et al. 1989). Although often successfully applied to differentiated structures like embryos and shoot tips, the method was used for cell lines only rarely (Reinhoud et al. 1995; Chen et al. 2002; Huang et al. 1995). One disadvantage is that the short incubation time requires the use of small batches of samples at one time. This produces a considerable disadvantage compared to controlled rate cooling for dedifferentiated cell cultures.

8.8 Encapsulation Dehydration

The encapsulation-dehydration method was originally developed for shoot tips (Fabre and Dereuddre 1990) (See Chap. 4). The group of Jean Dereuddre was also the first to apply the method for cryopreservation of dedifferentiated cells (Bachiri et al. 1995) of *Catharanthus roseus*. Several suspension cell cultures, both embryogenic and dedifferentiated, were cryopreserved by this approach (Gazeau et al. 1998, Shibli et al. 1999, Swan et al. 1998, Wang et al. 2002). Most of the work followed the basic

procedure worked out by Bachiri (Bachiri et al. 1995). The most practical approach was published by Swan et al. (1998). Two approaches are described in the protocols section (8.10.5 and 8.10.6).

For the preculture medium, no special recommendations are necessary. To achieve nicely shaped homogeneous beads, calcium ions should be removed from the cells and adherent medium as completely as possible before the cells are placed in the alginate solution. Wash the cells in a calcium free medium. This medium is used not only for washing the cells but also for preparing the sodium alginate solution for the immobilization procedure. Sodium alginate does not dissolve easily in the culture medium. It should be dissolved as completely as possible and then autoclaved even if small amounts of the alginate are not yet dissolved.

For a homogeneous, drying process it is important to obtain spherical and homogeneous beads. Use an automatic pipette with a disposable tip with the opening cut wider to allow the cells to pass through better. The size of the opening at the end of the tip depends on the size of the cell clusters formed by the suspension. The suspension should pass easily through the hole and should not get stuck. If cells are dropped into the calcium solution too quickly, unevenly shaped beads are the results. If the cells are dropped too slowly into the solution cells settle down in the bottom part of the tip and then different beads contain different amounts of cells resulting in a different drying behaviour. The opening of the pipette tip does not determine the size of the beads, which is more influenced by the surface tension of the solution. Even for a very fine suspension smaller beads will not be obtained by using a finer tip. Sodium alginate concentrations used vary from 3% to 1.5% or 2%.

Several procedures are used for drying. Swan and coworkers (Swan et al. 1998) exposed the beads to a defined amount of silica gel in glass jars; others (Fabre and Dereuddre 1990, Bachiri et al. 1995) dried beads in a laminar air flow bench. The drying time for the beads depends on the percentage of cells in the beads as well as on the tolerance of the specific cells to drying. The best final moisture content usually ranges from 20% to 30% of the initial wet weight of the beads.

Since many beads can be easily produced from suspended cells, monitoring the drying process by measuring wet weight and dry weight of the beads and survival is very simple. For measurement of survival by TTC test, beads should be rehydrated before initiating the viability test procedure. Partially dehydrated beads take up the vital stain more quickly than beads that are not dehydrated. The TTC test is normally carried out in phosphate buffer which tends to dissolve the alginate beads. Tris buffer can be used to perform the TTC test, which keeps the beads unaffected and facilitates extraction of the resulting formazan product.

The encapsulation-dehydration procedure consists of many manipulations. Nevertheless the approach of Swan et al. (1998) to immobilize the cells prior to exposure to the various preculture procedures makes handling of cell cultures a lot easier. Encapsulation dehydration offers the advantage of simplifying handling of dedifferentiated cell cultures. Normally, transfer of cells from one medium or solution to another includes centrifugation and resuspension procedures that stress the cells. Immobilization of cells allows manipulation of cells, avoiding further stress. Another advantage may be that solutions containing high concentrations of osmotics enter slowly into the beads leading to gradually increased osmotic values.

8.9 Viability Testing

For characterization of the final success of a cryopreservation experiment, even for dedifferentiated cell lines, it is strongly recommended to rely only on regrowth tests. Regrowth often is achieved only after a long period of time, especially if a procedure is still in a sub-optimum state. In this case, application of viability tests cannot be avoided. We recommend three different methods for dedifferentiated cells because of the specific information they provide.

8.9.1 Tetrazolium (TTC) Test

The most widely applied method is the TTC test (Steponkus and Lanphaer 1967). Cells are incubated in a colorless solution of a tetrazolium salt. The compound acquires red color by reduction to a formazan derivative by mitochondrial dehydrogenases, and becomes water insoluble. It can be extracted with ethanol and gives a measure of the activity of these dehydrogenases. The major advantage is that the test provides a quantitative result without counting cells. Counting cells is difficult even for dedifferentiated cell cultures because of the cell clusters formed. On the other hand the small cell clusters of a dedifferentiated cell line do not hinder the tetrazolium salt from entering into the cells compared to organized plant tissues. Therefore, dedifferentiated cell lines are better targets for this test than differentiated structures. Nevertheless we observed in several cases that cell lines which showed red colour later appeared to be unstained initially (unpublished results). No explanation is given for this phenomenon. In addition it has to be noticed that the TTC test is influenced by the osmotic strength of the medium (Duncan and Widholm 2004).

Procedure

1. Weigh 50 mg of callus material or settled or filtered suspension cells (fresh weight) into a 2 ml test tube.
2. Add 300 µl of TTC solution (2% (w/v) of 2,3,5 triphenyl tetrazolium chloride (TTC) in TRIS-HCl buffer 0.05 M, pH 7.5) and 1.2 ml TRIS-HCl buffer (0.05 M, pH 7.5) to the cells.
3. Incubate the suspension for 6 h in the dark.
4. After gentle centrifugation remove the reaction mixture from the cells.
5. Add 3 ml of ethanol to the cells and incubate the suspension at room temperature overnight.
6. After gentle centrifugation pipette 2 ml of the cell-free supernatant into a glass tube with a screw cap.
7. Measure the absorbance of the supernatant at 500 nm in a spectrophotometer and compare to control readings.

8.9.2 Fluorescein Diacetate (FDA) Test

Another simple test is the FDA test. Fluorescein diacetate is cleaved by cell wall esterases to a derivative showing a green fluorescence. The green fluorescence indicates living cells (Widholm 1972). When FDA-stained suspension cells are observed under the microscope illuminated by fluorescent light, and in addition normal light at a very low level, both fluorescent and non-fluorescent cells can be seen. It can now be observed how dead and living cells differ. Sometimes it can be guessed whether the dead cells were dehydrated too much or not enough.

Procedure

1. To prepare an FDA stock solution, dissolve 5 mg of fluorescein diacetate in 1 ml acetone.
2. Cool 2.5 ml cell culture medium on ice.
3. Add 0.5 ml of the FDA stock solution and store this diluted FDA solution on ice.
4. Mix 1 ml of the diluted FDA solution with 1 ml cell suspension and incubate for 5 min at room temperature. Or mix 1.5 ml of the diluted FDA solution with 100–200 mg callus (fresh weight) and incubate for 5 min.
5. Put a small amount of the solution containing FDA-stained cells (1 or 2 drops) onto a microscope slide and cover with a cover slip.
6. Observe the greenish fluorescence of the cells at ×100 to ×400 magnification under a microscope with fluorescence equipment (filter set 09 ZEISS Co., l ex = 450–490 nm, l em = 520 nm).

8.9.3 Evans Blue Test

Finally, the Evans blue test should be mentioned. Evans blue is a dye which penetrates into cells only after membrane damage. For testing viability with Evans blue, cells are incubated in the dye for some time. Cells are washed afterwards and Evans blue is finally extracted from the cells and measured spectrophotometrically. This test may complete the picture if the TTC test does not yield satisfying results. The Evans blue test gives quantitative results on the basis of damaged cells, whereas the TTC test measures surviving cells. Since the test is based on diffusion of the dye into damaged cells it is not useful for differentiated structures like shoot tips but can be easily applied to dedifferentiated cells. The method described below follows the procedure of Baker and Mock (1994) and Ikegawa et al. (1998).

Procedure

1. Let cells settle down or centrifuge mildly at 100 g.
2. Weigh 100 mg of cells in a 2 ml test tube.
3. Add 1.5 ml Evans blue solution [0.25% (v/v) in medium] and resuspend the cells. Incubate for 5–15 min (depending on the sensitivity of the cells).
4. Let cells settle down or centrifuge mildly at 100 g.
5. Remove Evans blue solution and wash cells five times with distilled water. All blue color should be gone.
6. Add 1.5 ml of 2% (w/v) SDS solution and grind cells with a micro pestle.
7. Centrifuge cells at 14,000 rpm in an Eppendorf centrifuge for 15 min.
8. Measure absorption of 1 ml of the supernatant at 600 nm and compare to the control measurement.

Investigations were published which compared the results of viability tests. However their conclusions are contradictory. Ishikawa et al. (1995) found that the TTC test was most convenient to test survival after several stresses.

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8.10 Protocols

8.10.1 Controlled Rate Cooling: Simple

After Withers and King 1980 (modified by H.M. Schumacher).

The method of Withers and King represents the most classical approach to controlled rate cooling. It is an early and very simple approach but worked sufficiently well for several cell lines. The method was often combined with a preculture procedure. It influenced the evaluation of many modified approaches.

Equipment

1. Erlenmeyer flasks for cultivation
2. Rotary shaker
3. Ice bath
4. Automatic pipettes
5. Cryovials of 2 ml
6. Controlled rate freezer
7. Liquid nitrogen Dewar for storage
8. Water bath (2 l beaker with 40°C water)

Solutions

1. Normal liquid growth medium
2. Cryoprotectant solutions:
   DGS: 1 M DMSO, 1 M glycerol, 2 M sucrose in growth medium
   DGP: 1 M DMSO, 1 M glycerol, 2 M proline in growth medium
3. Semisolid growth medium with 6.5% agar

Cell material

Use a suspension culture in the logarithmic growth phase as starting material.

Cooling

1. A 100 ml Erlenmeyer culture flask containing 25 ml suspension culture in the logarithmic growth phase is chilled on ice (the packed cell volume may vary from cell culture to cell culture but should not exceed 20%).
2. When the cells have settled down, add an equal volume of cold DPG or DGP. Mix thoroughly.
3. Incubate the suspension for 1 h in the cryoprotectant.
4. Assure a homogenous dispersion of the cells by gentle mixing and dispense 1 ml portions of the suspension into 2 ml cryovials.
5. Cool cells with a controlled cooling rate of –1°C/min to –35°C.
6. Hold that temperature for 30 min.
7. Immerse cryovials quickly into liquid nitrogen.
8. Transfer the vials to final storage system after 1 h.

Rewarming and recovery

1. For rewarming remove cryovials from liquid nitrogen.
2. Immerse vials in a container of 40°C sterile water.
3. Agitate samples in the warm water until the contents have just rewarmed, then remove vials.
4. Let cells settle and remove excess cryoprotectant with a pipette.
5. Transfer the rest of the cells (pour or use sterile spatula) onto semisolid medium (containing 6.5 g/l agar).
6. Cultivate for 2 days in the dark, then under normal growth conditions.

8.10.2 Controlled Rate Cooling: Preculture

After Swan et al. 1999 (modified by H.M. Schumacher)

Swan et al. present one of the modified approaches including a preculture procedure and slightly different handling.

Equipment

1. Erlenmeyer flasks for cultivation and preculture
2. Rotary shaker
3. Ice bath
4. Automatic pipettes
5. Cryovials of 2 ml
6. Controlled rate freezer
7. Liquid nitrogen Dewar for storage
8. Water bath (2 l beaker with water at 40°C)

Solutions

1. Normal liquid growth medium
2. Cryoprotectant solution: normal growth medium with 0.5 M glycerol, 0.5 M DMSO, 1 M sucrose and 0.086 M proline
3. Preculture medium with 0.75 M sucrose
4. Semisolid growth medium with 6.5% agar

Cell material

Use a suspension culture in the logarithmic growth phase as starting material.

The following represents a typical controlled rate cooling protocol.

1. Apply a standard subculture procedure for routine maintenance. We recommend using a maximum of 100 ml suspension in a 250 ml flask for most cell lines; repeat the standardized transfer for three times before starting the experiments. Use one part old suspension to two parts new medium.
2. Let the cells grow into the logarithmic growth phase and transfer into preculture medium (Pour 50 ml of the old suspension into 100 ml medium containing 0.75 M sucrose to achieve a final sucrose concentration of 0.5 M).

3. Cultivate cells in the preculture medium (from 1 to 6 days).
4. Harvest cells by filtering them off the medium (We use a Buchner funnel containing a Nylon net of a pore size of 100 µm (Fig. 8.1); a mild vacuum may be applied).
5. Use a sterile spatula or spoon to transfer 0.75 ml of cells into a graduated 2 ml cryovial under sterile conditions.
6. Add 0.5 ml of pre-cooled cryoprotectant solution and mix gently.
7. Incubate the cells for 1 h at 0°C in the cryoprotectant solution.
8. Transfer cryovials into the controlled rate freezer and cool them at 0.5°C/min to a hold temperature of –35°C.
9. Leave the vials for 35 min at the hold temperature.
10. Transfer cryovials to liquid nitrogen.

Rewarming and recovery

1. Rewarm samples quickly by transfer of cryovials into a 45°C water bath for approximately 2 minutes. To assure that rewarming takes place as quickly as possible; swirl them around and do not expose them to 45°C longer than necessary.
2. When the suspension is rewarmed, remove the vials from the water bath and disinfect the outside of the vials with 70% ethanol.
3. Transfer the suspension onto a filter paper on top of two other filter papers placed on the surface of normal growth medium (0.8% agar) in a Petri dish. Try to concentrate the cells in the centre of the filter paper.
4. Transfer the growing cells after 14 days by moving the filter paper to fresh growth medium.

8.10.3 PVS2 Vitrification Procedure 1

After Reinhoud et al. 1995 (modified by H.M. Schumacher)

The method was used to cryopreserve suspension cultures of several plant species. Reinhoud et al. present also a simplified version of this protocol.

Equipment

1. Conical 10–15 ml centrifuge tubes
2. Cryovials of 2 ml (straws can be used as alternative)
3. Wide mouth sterile Pasteur pipette
4. Sterile filter paper disks
5. Liquid nitrogen Dewar
6. Petri dishes

Solutions

1. Solid culture medium (7 g/l agar)
2. 20% PVS2 in liquid medium
3. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M sucrose in liquid medium
4. Rinsing solution (culture medium containing 1.2 M sucrose)

Cell material

Use a suspension culture in the logarithmic growth phase as starting material. The suspension culture should have not more than 10% pcv.

1. Apply a standard subculture procedure for routine maintenance.
2. Transfer a defined volume of suspension into a conical 10–15 ml centrifuge tube. Let cells settle or centrifuge gently at 100 g for 1 min. The pellet of cells should have a volume of 0.7 to 1 ml.
3. Discard the supernatant (decant or remove carefully with a pipette).
4. For osmotic loading, add 5 ml 20% PVS2 and incubate for 5 min.
5. Add an additional 1 ml of 20% PVS2 five times at 1 min intervals.
6. Let cells settle or centrifuge gently at 100 g for 1 min.
7. Discard the supernatant (decant or remove carefully with a pipette).
8. After 1 min add full-strength PVS2 up to a volume of 1 ml.
9. Add an additional 1 ml of PVS2 four times at 1 min intervals.
10. Pipette suspension quickly into cryovials (0.5–0.75 ml each) (or straws; close straws tightly).
11. Immerse directly into liquid nitrogen.
12. After liquid nitrogen temperature is reached, transfer to a long-term storage system.

Rewarming and recovery

1. Remove cryovials (or straws) from the storage container.
2. Rewarm cryovials (or straws) in a clean water bath at 40°C for 1 min.
3. Disinfect the outer surface with 70% ethanol.
4. Open cryovials (or straws) and immerse the contents of five cryovials (or straws) into 7 ml rinsing solution.
5. Incubate in rinsing solution for 20 min.
6. Let cells settle down or centrifuge for 1 min at 100 g and remove rinsing solution (If cells do not settle properly add 1 ml culture medium and centrifuge again).
7. Manipulate cells with a wide mouth Pasteur pipette or a spatula onto a recovery Petri dish containing normal growth medium overlaid with a filter paper (like for controlled rate cooling, cells should be concentrated in the centre of the paper and not spread evenly over the whole filter paper area).
8. Cultivate for 2 days.
9. Transfer the filter paper to a Petri dish with fresh medium.
10. When cells have regrown to form a solid cell layer transfer cells to a Petri dish containing fresh medium without filter paper.
11. When enough cell material has regrown re-establish a suspension culture.

8.10.4 PVS2 Vitrification Procedure 2

After Hao et al. 2003 (modified by H.M. Schumacher)

The method was used to cryopreserve normal and transformed strains of citrus callus disintegrated to a suspension before cooling. It represents a very simple version of the vitrification approach.

Equipment

1. Sterile 2 ml Eppendorf centrifuge tubes
2. Cryovials of 2 ml
3. Wide mouth sterile Pasteur pipette
4. Sterile filter paper disks
5. Liquid nitrogen Dewar

Solutions

1. Liquid and solid (7 g/l agar) culture medium
2. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M sucrose in liquid medium
3. Rinsing solution: culture medium with 1.2 M sucrose

Cell material

Use a suspension culture in the logarithmic growth phase as starting material. The suspension culture should have not more than 10% pcv.

Cooling

1. Transfer 1.5 ml suspension containing 0.1 ml pcv into a sterile 2 ml centrifuge tube. Centrifuge gently (100 g for 1 min) and discard the supernatant. Add 1.5 ml PVS2 and mix gently.
2. Centrifuge gently (100 g for 1 min) and discard the supernatant.
3. Add 1.5 ml PVS2; incubate for about 3 min at 25°C. It needs to determine timing based on the toxicity of PVS2.
4. Transfer 1 ml to each cryovial with a wide mouth Pasteur pipette.
5. Plunge cryovials directly into liquid nitrogen.

Rewarming and recovery

1. Rewarm cryovials in a water bath at 40°C for 1 min.
2. Centrifuge gently (100 g for 1 min) and discard the supernatant.
3. Add 1.5 ml rinsing solution. Incubate for 10 minutes.
4. Transfer 0.5 ml suspension onto a double layer filter paper disk on solid medium in a 90 mm Petri dish and incubate overnight.
5. Transfer the filter paper with cells to new medium for culture.

8.10.5 Encapsulation Dehydration: Laminar Flow

After Shibli and Al-Juboory. 2000 (modified by H.M. Schumacher)

The method represents a simple approach, with alginate beads dried in a laminar air flow bench.

Equipment

1. Erlenmeyer flasks, shaker
2. Disposable plastic 50 ml centrifuge tubes, centrifuge
3. Beaker 250 ml, stir plate
4. Wide mouth Pasteur pipette
5. Petri dishes (90 mm in diameter), sterile filter paper
6. Laminar flow bench for drying the beads
7. Cryovials of 2 ml
8. Dewar for liquid nitrogen storage
9. Water bath or container for rewarming

Solutions

1. Normal growth medium
2. Growth medium with 0.75 M sucrose and no calcium
3. Alginate solution: 3 % in growth medium with 0.4 M sucrose and no calcium
4. Growth medium with 100 mM calcium chloride
5. Growth medium with 0.5 M sucrose
6. Growth medium with 0.25 M sucrose

Cell material

Use a suspension culture in the logarithmic growth phase.

Cryopreservation

1. Transfer cells from the logarithmic growth phase into medium containing 0.75 M sucrose and cultivate for 4 days.
2. Pour suspension into a 50 ml disposable plastic centrifuge tube.
3. Centrifuge at 350 g for 5 min.
4. Remove supernatant and mix packed cells 1:1 (v/v) with alginate.
5. Mix cells and alginate solution gently with pipette tip. Draw up alginate with cells suspended and drip the suspension into a 250 ml beaker with 100 ml of 100 mM calcium chloride. (See Section 3.8 for explanation of this critical step).

6. Let beads solidify for 20 min in the calcium solution.
7. Pour off the calcium solution and wash beads twice in normal growth medium.
8. Transfer the beads without medium onto three layers of filter paper placed in a Petri dish (90 mm in diameter).
9. Remove water from the outer surface of the beads by rolling them over the filter paper.
11. Transfer the beads into another Petri dish (90 mm without filter paper). The beads should not touch each other and be equal distances from other beads.
12. Place the Petri dish in a laminar air flow bench and dry for 2, 4, 6, 8 h in the laminar air flow to determine a suitable time.
13. After defined times transfer 5–10 beads into each 2 ml cryovial and immerse directly in liquid nitrogen.
14. Transfer the beads to the final storage system after 30 min in liquid nitrogen.

Rewarming and recovery

1. Remove cryovials with beads from cryostorage and rewarm by immersion in a 40°C water bath for 1 min.
2. Transfer ten beads into 25 ml of 0.75 M sucrose medium for 24 h.
3. Transfer beads to 0.5 M sucrose medium for 24 h.
4. Transfer beads to 0.25 M sucrose medium for 24 h.
5. Transfer beads to normal medium and cultivate for 14 days.
6. After 14 days remove the beads and continue cultivating those cells outside of the beads and growing on the medium. For cultures grown in the light, let them grow for the first 3 days in the dark or dim light.

8.10.6 Encapsulation Dehydration: Silica Gel

After Swan et al. 1998 (modified by H.M. Schumacher)

The method uses jars containing silica gel for desiccation and is usually very reproducible.

Equipment

1. Erlenmeyer flasks, shaker
2. Disposable plastic centrifuge tubes, centrifuge
3. Beakers 50 ml, 250 ml
4. Wide mouth screw cap jar with silica gel: for the drying procedure fill 80 g silica gel in wide mouth jars. Dry and sterilize the jars at 160°C for 6 h. Plastic lids may have to be sterilized by autoclaving and drying.
5. Wide mouth Pasteur pipette
6. Petri dishes (69 and 90 mm diameter)
7. Sterile filter paper (90 mm diameter)
8. Cryovials (2 ml)
9. Dewar for liquid nitrogen storage
10. Water bath or container for rewarming

Solutions

1. Normal growth medium
2. Alginate solution: (1.5%) in growth medium without calcium
3. Growth medium with 100 mM calcium chloride
4. Growth medium with 0.3 M sucrose
5. Growth medium with 0.5 M sucrose
6. Growth medium with 0.75 M sucrose
7. Growth medium with 1 M sucrose

Cell material

Use a suspension culture in the logarithmic growth phase.

1. Apply a standard subculture procedure for routine maintenance.
2. Use cells from the logarithmic growth phase; harvest cells by filtration (use Buchner funnel equipped with nylon net (Fig. 8.1) and wash cells twice with Ca^{2+} free medium).

3. Mix cells and alginate solution gently with pipette tip. Draw up alginate with cells suspended and drip the suspension into a 250 ml beaker with 100 ml of 100 mM calcium chloride. (See Section 8.8 for explanation of this critical step).
4. Let the beads solidify for 20 min.
5. Filter the beads through a Buchner funnel (without Nylon net) and wash the beads in the funnel with normal growth medium to remove excess calcium.
6. Transfer the beads into 0.3 M sucrose medium (50 beads in 50 ml medium in 250 ml Erlenmeyer flask) cultivate for 6 h.
7. Transfer beads to 0.5 M sucrose medium and cultivate for 18 h.
8. Transfer beads to 0.75 M sucrose medium for 6 h.
9. Transfer the beads to 1 M sucrose medium for about 5 days.
10. Remove the beads from the medium and surface dry by rolling them over dry sterile filter paper.
11. Transfer 10 beads onto a 5.5 cm filter paper and place in the lid of a 6 cm Petri dish.
12. Place the lid of the Petri dish containing the filter paper and bea on top of dry silica gel in a wide mouth screw cap jar, close and wrap with Parafilm. Determine the optimum drying time by exposing the cells to the silica gel for various times (2, 4, 6, 8 h) (for routine preservation only the optimum time is used).
13. Place dried beads in cryovials (five beads per vial), mount on aluminium holders and immerse in liquid nitrogen.

Rewarming and recovery

1. Fill Petri dishes (6 cm) with 1 M sucrose medium.
2. Remove cryovials from liquid nitrogen. Shake the vials to separate the beads from each other and from the walls of the cryovials. Open vials under the clean bench and immerse the beads directly into the medium in the Petri dishes for rewarming.
3. After rewarming, place beads on normal growth medium with 0.8% agar. Press beads very gently onto the medium.
4. Transfer the beads to fresh culture medium after 3 days and again after 14 days of growth. For cultures grown in the light, let them grow for the first 3 days in the dark or dim light.

Chapter 9
Cryopreservation of Embryogenic Cultures

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9.1 Introduction

The first reports on somatic embryogenesis date back to 1958 (Reinert 1958; Steward et al. 1958) and represent one of the most important milestones in plant tissue culture, as they give clear evidence of the concept of 'totipotency' of vegetative cells, a peculiarity which makes possible the regeneration of a whole plant from a single somatic cell (Bajaj 1995). Indeed, thanks to the 'bipolar' nature of the somatic embryo, shoot and root formation are generally induced at the same time from a single regenerative act. This differentiates somatic embryogenesis from organogenesis, where *de-novo* formed 'unipolar' structures, mainly shoots, need to be stimulated with growth regulators for the induction of adventitious roots, thus producing a complete plant in two steps. Somatic embryos can be induced directly from cells of the explant cultured *in vitro* ('direct' or 'adventitious' somatic embryogenesis), or more often, from the dedifferentiated cells of a proliferative callus (indirect or induced somatic embryogenesis) after the explant tissue is artificially stimulated (i.e., with growth regulators) to develop embryogenic competence (Hartmann et al. 1990;

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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Krikorian 2000). Embryogenic callus, established *in vitro* from the original explant, are periodically subcultured to maintain their embryogenic potential. These constitute 'embryogenic cultures' or 'embryogenic callus lines'.

The bipolar structure of the somatic embryo, as well as the developmental stages it passes through, makes it similar to the zygotic embryo (Dodeman et al. 1997; Benelli et al. 2001). However, the former evolves into a clonal plant, as it originates directly from a somatic cell without gametic fusion, while the latter produces a new genotype as a result of gametic reproduction. In addition, differently from zygotic embryos, somatic embryos are not protected by a seed coat and they are dependent on the culture medium for nutrition. The synthetic seed technology (i.e., the inclusion of a somatic embryo inside a nutrient-containing alginate bead) was developed to resemble as closely as possible the natural condition of the zygotic embryo.

Effective protocols for the induction and the establishment *in vitro* of embryogenic cultures were developed for a large number of plant species: conifers (Attree and Fowke 1991), temperate and tropical fruit trees (Akhtar et al. 2000; Jain and Gupta 2005), crops, and ornamental plants (Bajaj 1995). Advances in tissue culture technology opened the way to various and important applications of somatic embryogenesis, such as (i) the production of synthetic seeds (Redenbaugh et al. 1991; Lambardi et al. 2006) which allow easy storage, transportation and sowing of encapsulated somatic embryos, (ii) the scale-up production of plants in bioreactors (Denchev et al. 1992), (iii) the utilization in genetic transformation studies (Ellis 1993; Jackson and Linskens 2003), thus enabling the production of 'transgenic' lines carrying genes of specific interest, (iv) the production of somatic hybrids (Ozawa et al. 1996) and polyploid plants (Ezura and Oozawa 1994), (v) the elimination of viruses (Goussard and Wiid 1992), and, as reported here, (vi) the cryopreservation of plant germplasm.

Once established, embryogenic callus cultures require periodic subculturing to maintain both a high proliferative potential and the capacity of cells to develop into somatic embryo primordia. Repeated subculturing is not only labor intensive and time consuming, but also increases the risk of losing the embryogenic cultures through contamination, human errors or technical failures. The loss of embryogenic potential of culture lines and the occurrence of genetic alterations due to long-term subculturing is frequently reported (Harding 1996; Bhatti et al. 1997). Periodic re-initiation of embryogenic cultures can provide a solution to this drawback. However,

this practice is again time consuming, expensive and particularly difficult for species characterized by a specific ‘window’ of embryogenic competence, i.e., having explants suitable for embryogenic callus induction during a very brief period of the year (Becwar et al. 1990; David et al. 1995; Lambardi 2000). Particularly when working with woody species, once a good culture line is lost, there is never the certainty of getting another of equivalent embryogenic potential within a short time. Thus, it is of strategic importance to develop and optimize effective protocols for the maintenance of embryogenic callus cultures in semi-solid or in liquid media, with the aim of reducing the frequency of manipulations and, as a consequence, the risks of decay, loss or genetic alterations. Recent advances in the cryostorage of plant tissues and organs in liquid nitrogen (LN) (at –196°C) opened the door to safe, low-cost, long-term maintenance of embryogenic cultures. Both the controlled rate cooling method (two-step freezing), and procedures allowing the direct immersion of specimens in LN (vitrification or one-step freezing) were successfully tested with embryogenic callus lines from various plant species (Engelmann 2004; Panis and Lambardi 2005).

9.1.1 Controlled Rate Cooling

The traditional controlled rate cooling method is still the most common approach for the cryopreservation of clumps from embryogenic callus cultures. The main step in the protocols is the slow cooling of embryogenic clumps at 0.5–1°C min^{-1} to an intermediate temperature of –40°C, after which the clumps are immersed in LN. In recent years this approach allowed for the development of effective protocols for various conifer species (Table 9.1), finding practical application in Canada in the long-term storage of thousands of selected embryogenic callus lines (Cyr 2000). The technique was successfully applied to embryogenic clumps of hardwood and herbaceous species (Table 9.2 and 9.3). One disadvantage of the slow-cooling approach is that it requires a controlled-rate freezer. The major advantage is the ease of use for storing large numbers of cultures in a standardized manner. The Nalgene Freezing Container (Sigma-Aldrich) which cools at a rate of 1°C min^{-1} through the use of cooled (to –80°C) isopropyl alcohol is also successful (Martinez-Montero et al. 1998; Ford et al. 2000).

Table 9.1. Cryopreservation of embryogenic cultures from woody plants: conifers

Species	Explant[1]	Preculture	Cryoprotectant	Cooling rate	Maximum recovery (%)	Reference
Abies cephalonica	CC	5°C (14d), darkness+ sucrose, 0.2M (1d) + 0.4M (1d)	10% PEG6000+ 10% glucose+ 10% DMSO 30 min, 0°C	10°C h^{-1} to -38°C	75	Aronen et al. 1999
Picea abies	CC	sorbitol, 0.4M (2d)	5% DMSO, 30 min	0.3°C min^{-1} to -35°C	NR	Nørgaard et al. 1993
Picea abies	SC	sorbitol, 0.2M (1d)+ 0.4M (1d), darkness	5% DMSO, 0°C	0.5°C min^{-1} to -40°C	NR	Find et al. 1998
Picea abies	CC	sorbitol, 0.4M (2d)	5% DMSO, 30 min	0.3°C min^{-1} to -35°C	NR	Högberg et al. 1998
Picea glauca engelmanni	SC	none	0.4 M sorbitol + 5% DMSO	multistep cooling[2]	100	Cyr et al. 1994
Picea glauca	SC	sorbitol, 0.4M (1d)	0.4 M sorbitol + 5% DMSO, 30 min	0.3°C min^{-1} to -35°C	94	Kartha et al. 1988
Picea sitchensis	SC	sorbitol, 0.2M (1d)+ 0.4M (1d)	5% DMSO, 0°C	0.5°C min^{-1} to -40°C	NR	Kristensen et al. 1994
Picea sitchensis	SC	sorbitol, 0.2M (1d)+ 0.4M (1d), darkness	5% DMSO, 0°C	0.5°C min^{-1} to -40°C	NR	Find et al. 1998
Pinus caribaea 'Hondurensis'	SC	sucrose, 0.4M	0.4 M sucrose + 5% DMSO	0.5°C min^{-1} to -35°C	100	Lainé et al. 1992
Pinus patula	CC	sorbitol, 0.3M (1d)	0.3 M sorbitol+5% DMSO, 20 min, 0°C	-70°C, 2h[3]	60	Ford et al. 2000
Pinus pinaster	CC	maltose, 0.2M (1d)+ 0.4M (1d), darkness	10% PEG4000 + 10% sucrose + 10% DMSO 0°C	-80°C, 1d[3]	97	Marum et al. 2004
Pinus radiata	CC	sorbitol, 0.4M (1d) 20°C	0.4 M sorbitol +10% DMSO	-80°C, 75-90 min[3]	100	Hargreaves et al. 2002
Pinus roxburghii	CC	sorbitol, 0.3M (1d)	0.3 M sorbitol + 5% DMSO	multistep cooling[4]	70	Mathur et al. 2003
Pinus sylvestris	CC	5°C (14d) + sucrose, 0.2M (1d)+0.4M (1d)	10% PEG6000+ 10% glucose+10% DMSO, 30 min, 0°C	10°C h^{-1} to -38°C	78	Häggman et al. 1998
Picea mariana	CC	sorbitol, 0.8M (2d)	PVS2, 30 min, 0°C	Direct immersion in LN	67	Touchell et al. 2002

LN, liquid nitrogen; NR, not reported; [1] CC, clumps of callus (developmental stage not reported); SC, cells from suspension cultures. [2] Slow cooling was achieved as follows: 0.3°C min^{-1} to -3°C → 15°C min^{-1} to -8°C → 25°C min^{-1} to -32°C → 0.3°C min^{-1} to -35°C. [3] Slow cooling was achieved by incubating the samples in a freezer at -70 or -80°C before plunging them into LN. [4] Slow cooling was achieved as follows: 0.3°C min^{-1} to -35°C → 25°C min^{-1} to -50°C.

Table 9.2. Cryopreservation of embryogenic cultures from woody plants: hardwoods, shrubs and palm trees

Species	Explant[1]	Preculture	Cryoprotectant	Cooling rate	Maximum recovery (%)	Reference
Slow cooling						
Citrus deliciosa	CC		10% DMSO, 30 min, 4 °C	0.5°C min^{-1}	NR	Pérez et al.1999
Citrus sinensis	SE			0.5°C min^{-1}to -42 °C	5	Marin and Duran-Vila 1988
Citrus sinensis	HSE		10% DMSO	0.5°C min^{-1} to -42 °C	31	Marin et al. 1993
Citrus ssp.	CC		10% DMSO, 30 min, 0 °C	0.5°C min^{-1} to -40 °C	100	Pérez et al. 1997
Elaeis guineensis	SC	4°C (1h)	1M glucose + 10% DMSO, 1h, 4 °C	0.5°C min^{-1}to -40 °C	70	Chabrillange et al. 2000
Hevea brasiliensis	CC		1M sucrose + 10% DMSO, 1h, 0 °C	0.2°C min^{-1} to -40 °C	70	Engelmann and Etienne 2000
Musa spp.	SC		180 g l^{-1} sucrose + 7.5% DMSO	1°C min^{-1} to -40 °C	NR	Panis et al. 1990
Direct immersion in LN						
Aesculus hippocastanum	TSE	4°C (5d), darkness	2M glycerol + 0.4M sucrose, 30 min, 25°C + PVS2, 90 min, 0 °C		94	Lambardi et al. 2005
Citrus sinensis	SC		60% PVS2, 5 min, 25°C + PVS2, 3 min, 0 °C		84	Sakai et al. 1990
Citrus ssp.	SE	sucrose, 0.7M (1d)	encapsulation + dehydration, 5h, LF		100	Gonzàlez-Arnao et al. 2003
Elaeis guineensis	CC	sucrose, 0.75M (7d)	dehydration, 16h, SG		93	Dumet et al. 2000
Fraxinus angustifolia	CSE	4°C (10h) + sucrose, 0.5M (1d)	encapsulation + dehydration, 1h, SG		31	Tonon et al. 2001
Magnifera Indica 'Zihua'	SC	sucrose, 0.5M (1d) 25°C	PVS3[2], 20 min, 25 °C		94	Wu et al. 2003
Manihot esculenta	CC	sucrose, 0.3M (21d)	dehydration, 16h, LF		95	Danso and Ford-Lloyd 2004
Olea europaea 'Canino'	CC	4°C (4d), darkness	2M glycerol + 0.4M sucrose, 30 min, 25°C + PVS2, 90 min, 0 °C		38	Lambardi et al. 2002
Olea europaea 'Nabali'	CC	30°C (1d)	encapsulation + 0.4M sucrose + 2M glycerol, 60 min PVS2, 3h, 0 °C		64	Shibli and Al-Juboory 2000
Prunus avium	CC	multistep sucrose preculture [3] (until 20% MC)	dehydration, LF		89	Grenier-de March et al 2005
Quercus robur	GSE-HSE	sucrose, 0.3M (3d)	PVS2, 60 min, 25 °C		70	Martínez et al. 2003
Quercus robur	CC	multistep sucrose preculture [3]	dehydration, 4-5h, LF		NR	Chmielarz et al. 2005
Quercus suber	GSE	sucrose, 0.3M (3d)	PVS2, 60 min, 0 °C		93	Valladares et al. 2004

(LF, laminar flow; MC, moisture content; NR, not reported; SG, silica gel); [1] CC, clumps of embryogenic callus (developmental stage not reported); CSE, clumps or isolated somatic embryos at the cotyledonary stage; GSE, clumps or isolated somatic embryos at the globular stage; HSE, clumps or isolated somatic embryos at the heart stage; SC, cells from suspension cultures; SE, isolated somatic embryos; TSE, clumps or isolated somatic embryos at the torpedo stage. [2] PVS3: 50% sucrose (w/v) + 50% glycerol (w/v) in standard culture medium.[3] Multistep sucrose preculture was performed as follows: Sucrose, 0.25M (1d) + 0.5M (1d) + 0.75M (2d) + 1.0M (3d).

Table 9.3. Cryopreservation of embryogenic cultures from herbaceous species: crops and cut-flowers

Species	Explant[1]	Preculture	Cryoprotectant	Cooling rate	Maximum Recovery (%)	Reference
Slow cooling						
Asparagus officinalis	SC	sucrose, 0.8 M (2d), 24°C		0.5 °C min^{-1}	NR	Jitsuyama et al. 2002
Cyclamen persicum	SC	sucrose, 0.6 M (2d)	0.6 M sucrose + 10% DMSO, 1h	-20 °C, 2 h[2]	NR	Winkelmann et al. 2004
Festuca spp.	SC	sorbitol, 0.33 M (3d)	0.5 M sorbitol + 10% DMSO	multistep cooling [3]	71	Wang et al. 1994
Ipomoea batatas	CC	multistep sucrose preculture [4]	encapsulation + dehydration, 4h in LF	10°C min^{-1} to 0 °C + 0.5 °C min^{-1} to -40 °C	70	Bhatti et al. 1997
Ipomaea batatas 'Kokei-14'	CC	ABA, 3 mg l^{-1} (3d)	10% DMSO + 10% sucrose ± 5% glycerol	0.3°C min^{-1} to -30° C	80	Shimonishi et al. 2000a
Lolium spp.	SC	sorbitol, 0.33 M (3d)	0.5M sorbitol +10% DMSO	multistep cooling [3]	63	Wang et al. 1994
Oryza sativa	SC	mannitol, 60 g l^{-1}	1 M DMSO + 1 M glycerol + 2 M sucrose, 60 min, 0°C	-25 °C, 2 h	98	Jain et al. 1996
Saccharum spp.	SC	sorbitol, 0.33 M (3d)	0.5 M sorbitol + 0.64 M DMSO	0.5 °C min^{-1} to -40 °C	92	Gnanaparagasam and Vasil 1990
Saccharum officinarum	CC	sucrose, 0.5 M (1h), 0°C	10% DMSO	0.4-0.5 °C min^{-1}	90	Martinez-Montero et al. 1998
Direct immersion in LN						
Asparagus officinalis	SC	sorbitol, 0.3 M+ sucrose, 0.2M(16h), 22 °C	0.5 M sorbitol + 12% EG (5 min), 22°C + 85% PVS[5] (5 min), 0°C		65	Uragami et al. 1989
Cucumis melo	SE	ABA, 10 mg l^{-1} (3d)	dehydration, LF		65	Shimonishi et al. 2000b
Doritaenopsis (Orchid)	SC	Sucrose, 0.1 M + ABA, 1 mg l^{-1} (7d) 25 °C	2M glycerol + 0.4M sucrose, 15 min, 25°C + PVS2, 1h, 0°C		64	Tsukazaki et al. 2000
Ipomoea batatas	CC	multistep sucrose preculture [3]	dehydration, 2.5h, SG		89	Blakesley et al. 1996
Iris nigricans	CSE	sucrose, 0.75 M(3d), 22 °C +0.75 M (1d), 30 °C	encapsulation dehydration, 4h, in LF		60	Shibli 2000
Oryza sativa	SC	sucrose, 60 g l^{-1}(10d) + sorbitol, 0.4 M (1d)	25% PVS2, 10 min + 100% PVS2		45	Huang et al. 1995
Oryza sativa 'Taipei 309'	SC	mannitol, 60 g l^{-1} (3d)	CM[6], 1h, 0°C		NR	Lynch et al. 1995

(EG, ethylene glycol; LF, laminar flow; LN, liquid nitrogen; NR, not reported; RT,; SG, silica gel); [1] CC, clumps of embryogenic callus (developmental stage not reported); CSE, clumps or isolated somatic embryos at the cotyledonary stage; SC, cells from suspension cultures; SE, isolated somatic embryos. [2] Slow cooling was achieved by incubating the samples in a freezer at -70 or -80°C before plunging them into LN. [3] Slow cooling: 1°C min^{-1} to -10°C; -10°C for 15 min; 1°C min^{-1} to -40°C; -40°C for 60 min. [4] Multistep sucrose preculture: 0.1M (3d), 0.4M (3d), 0.7M (2d). [5] PVS: 22% (w/v) glycerol + 15% (w/v) ethylene glycol + 15% (w/v) propylene glycol + 7% DMSO. [6] CM, Cryoprotectant Mixture: 2M sucrose + 1M DMSO + 1M glycerol + 0.09M L-proline in H_2O.

9.1.2 Direct Immersion in Liquid Nitrogen

Recent advances in cryotechnology have opened the way to a simplified approach for the long-term storage of embryogenic callus clumps, allowing direct immersion in LN. Vitrification-based and encapsulation-dehydration protocols were developed for embryogenic cultures from various important plant species (*Citrus* spp., *Olea europaea*, *Fraxinus* spp., *Quercus* spp., *Oryza sativa*).

A summary of cryopreservation protocols developed for embryogenic cultures is reported in Table 9.1 (conifers), Table 9.2 (hardwoods, shrubs and palm trees) and Table 9.3 (crops and cut-flowers). Eleven successful protocols are also described in detail at the end of the chapter. Protocols were selected to cover embryogenic cultures from several groups of plants as well as the full range of techniques available, i.e., conifers (*Picea mariana*, *Pinus pinaster*), hardwoods (*Aesculus hippocastanum*, *Quercus suber*), fruit (*Prunus avium*, *Citrus* spp.) and palm (banana) trees, flower (*Cyclamen persicum*) and crop (*Saccharum officinarum*, *Ipomoea batatas*) species.

9.2 Application of Cryopreservation to Embryogenic Cultures

Cryopreservation of embryogenic cultures is a relatively recent application of cryogenic technology. Only in the early 1990s were the first examples of detailed and successful protocols published. They described the cryopreservation of embryogenic suspension cultures of *Musa* spp. (Panis et al. 1990) and *Citrus sinensis* (Sakai et al. 1990). Sakai's work on *C. sinensis* reported the development of Plant Vitrification Solution no. 2 (PVS2) to induce cell vitrification, a mixture of cryoprotectants which became a milestone in the history of plant cryopreservation. It was soon evident that the possibility of storing valuable embryogenic culture lines in LN could allow the long-term maintenance of their embryogenic potential, making them available only when necessary and avoiding the drawbacks resulting from repeated subculturing (contamination, genetic alteration, decrease of callus morphogenicity).

Cryopreservation of embryogenic cultures is a multi-step process, including preconditioning, preculturing, cryoprotectant treatments, storage in LN, rewarming and recovery of callus, all of which play an important role in achieving a successful protocol. An error in any of these steps, or the choice of an embryogenic culture line at an unsuitable stage of development,

may prevent the recovery of proliferative activity of the stored callus clumps. The technical challenge lies in optimizing each of these parameters for embryogenic cultures from a wide diversity of plant species.

9.2.1 Developmental Stage

The growth stage of embryogenic cells of suspension cultures can strongly influence survival after cryopreservation. In *Citrus* suspension cultures, embryogenic cells at the early lag phase and at the stationary phase are large and highly vacuolated, thus having high water content. On the contrary, rapidly growing cells in the late lag and early to mid-exponential phase are small and contain dense cytoplasm, with fewer vacuoles and relatively low water content (Pérez 2000). Cells characterized by dense cytoplasm and few vacuoles have low water content, hence tolerating cryopreservation treatments much better. Lainé et al. (1992) reported that, among the cryopreserved embryogenic cell suspensions of *Pinus caribaea*, only rapidly growing cultures could be recovered successfully after cryostorage. However, in contrast to the above general statement, Find et al. (1998) reported that embryogenic suspension cultures of *Picea abies* and *Picea sitchensis* showed maximum survival when cells were harvested at the stationary growth phase.

Embryogenic callus cultures of conifers are generally induced from the suspensor cells of immature zygotic embryos, cultured on 2,4-D-containing gelled medium. During proliferation the lines contain groups of densely cytoplasmic embryonic cells (the heads of the embryo primordia), subtended by elongated highly vacuolated suspensor cells (Stasolla and Yeung 2003). The suspensor cells have high water content and usually die during freezing. The embryonic head cells have a dense cytoplasm and, following appropriate cryoprotective treatments, they survive storage in LN (Häggman et al. 2000).

The developmental stage of embryogenic callus cultured in steady state is not well investigated for hardwood and herbaceous species, in terms of recovery from cryostorage, and the results are not consistent. Valladares et al. (2004) used a simple vitrification protocol to compare the post-thaw survival of globular, early-torpedo, and cotyledonary embryos of *Quercus suber*, and 90% of the embryogenic clumps of globular embryos could be successfully cryopreserved. However, contrasting results were obtained when isolated globular embryos were used. The recovery percentage achieved was the highest with one embryogenic line, and the lowest with the other, hence far from providing clear information on the aspect of better adaptability to cryostorage of somatic embryos at different

stages of development. Working with *Citrus sinensis*, Marin et al. (1993) chose only the smallest (0.5–1 mm) morphogenetic structures of the embryogenic callus culture, consisting of heart-shaped embryos which had the highest survival rate after cryostorage. Callus clumps of *Aesculus hippocastanum*, containing a prevalence of embryogenic masses at an advanced stage of somatic embryo maturation (i.e., the torpedo stage), gave optimum regrowth of healthy and proliferating embryogenic callus, in this performing better than callus clumps prevalently made up of globular, heart shaped and cotyledonary somatic embryos (Lambardi et al. 2005). In *Iris nigricans*, 2–4 mm somatic embryos had higher survival than smaller (1–2 mm) or larger (4–6 mm) ones (Shibli 2000). Medium and large somatic embryos of *Cucumis melo* showed higher survival than smaller ones (Shimonishi et al. 2000b).

9.2.2 Preconditioning of Embryogenic Callus

With few exceptions, cryopreservation protocols for embryogenic cultures report an initial preconditioning phase, during which the samples undergo moderate dehydration on media containing sugars (e.g., sucrose, glucose) or sugar alcohols (sorbitol). During preconditioning, callus cells are subjected to mild osmotic stress which induces metabolic changes and enhances chilling and desiccation tolerance. In addition the absorbed sugar stabilizes the membranes by replacing water and forming hydrogen bonds with the phospholipids (Turner et al. 2001). However, if applied at relatively high concentrations (i.e., over 1.0 M), sugar itself may become toxic to the mass of cells, particularly when the duration of the preconditioning period is prolonged (Wu et al. 2003). A progressive exposure of explants to increasing sugar concentrations, instead of a direct exposure to a large dose, plays a key role in avoiding toxic effects (Bhatti et al. 1997; Find et al. 1998; Chmielarz et al. 2005).

9.2.3 Preculture

Because of their high water content, cells of embryogenic cultures are extremely sensitive to cryoinjury. Often, sugar preconditioning alone does not dehydrate callus samples sufficiently to withstand the freezing process, and additional cryoprotection is required. Protocols include one or more additional preculture treatments which precede either slow cooling to –40°C, followed by storage in LN, or direct immersion in LN.

9.2.4 Cryoprotectants

Treatment with cryoprotectants is typical of controlled cooling procedures. Two kinds of cryoprotectants are used for this purpose, those that penetrate the plasma membrane (e.g., DMSO and glycerol), and the non-penetrating compounds (e.g. polyethylene glycol (PEG) and sucrose). DMSO alone, at a concentration of 5–10%, is often reported as the only cryoprotectant (Martinez-Montero et al. 1998; Find et al. 1998; Pérez et al. 1999). Other authors prefer combinations of cryoprotectants at lower concentrations, considering this approach more beneficial than a single cryoprotectant at high concentration. For instance, in *Picea abies* and *Pinus taeda* (Gupta et al. 1987), *Pinus sylvestris* (Häggman et al. 1998) and *Abies cephalonica* (Aronen et al. 1999) the replacement of DMSO with a mixture of PEG, glucose and DMSO resulted in better recovery of proliferating embryogenic callus after cryopreservation.

A variety of cryoprotectant treatments are used when the direct immersion of callus clumps in LN is pursued. The most common approach is the treatment of specimens with PVS2 for periods ranging from 30 to 90 min. Due to possible toxic effects of the PVS2 solution which can compromise embryogenic cell viability, the incubation time is a fundamental parameter which must always be optimized. Using the encapsulation-vitrification technique, longer incubation times with PVS2 are required. In *Olea europaea* (cv Nabali) encapsulated callus clumps were incubated in PVS2 at 0°C for 3 h before the beads were directly plunged into LN (Shibli and Al-Juboory 2000). As an alternative to the use of the PVS2 vitrification solution, physical dehydration is another common approach to the direct immersion in LN of naked or encapsulated callus clumps. The dehydration to which the explants are subjected depends on the method applied, ranging from 4 (*Iris nigricans*; Shibli 2000) to 16 h (*Manihot esculenta*; Danso and Ford-Lloyd 2004) when dehydration is carried out under the sterile air flow of a laminar cabinet, or from 1 (*Fraxinus angustifolia*; Tonon et al. 2001) to 16 hours (*Elaeis guineensis*; Dumet et al. 2000) with silica gel.

9.2.5 Cooling Rates

Reports of cryopreservation protocols based on controlled rate cooling show that cooling at 0.2–0.5°C min^{-1} to about –40°C is effective in the protection of embryogenic cultures of a large variety of plant species from intracellular ice formation (Tables 9.1–9.3). Slow cooling of specimens is generally obtained by using a programmable freezer. However, some authors (Martinez-Montero et al. 1998; Ford et al. 2000) report the use of

Nalgene Freezing Containers, where a cooling rate of 1°C min^{-1} is achieved with the use of chilled isopropyl alcohol in a standard freezer. As an alternative, incubating the embryogenic lines at –20°C (Winkelmann et al. 2004) or at –70/–80°C (Jain et al. 1996; Ford et al. 2000; Hargreaves et al. 2002; Marum et al. 2004) for 1 to 24 h, before plunging the samples into LN, also drops the temperature gradually enough to prevent cryoinjury.

9.2.6 Warming and Rinsing

Cryoinjury can occur not only during the ultra-freezing process, but also during sample thawing, due to recrystallization, (Meryman and Williams 1985). Recrystallization can be avoided by rapid thawing of samples in a water bath at 35 to 45°C. In *Citrus* spp. warming at room temperature is as effective as fast warming for recovery of viable isolated somatic embryos (Marin and Duran-Vila 1988; González-Arnao et al. 2003).

Cryoprotective solutions are rapidly drained off the cryovials to prevent prolonged exposure. Cultures are rinsed with a hormone-free liquid medium containing a high concentration of an osmoregulating agent (generally, 1.2 M sucrose) and finally plated on an appropriate medium. Rapid removal of the cryoprotective solutions may also cause deplasmolysis injuries to the cells (Finkle and Ulrich 1982; Wang et al. 1994). This can be avoided by gently transferring embryogenic samples on to a filter paper placed on a semi-solid culture medium that is replaced after a few days (Cyr et al. 1994; Marum et al. 2004; Valladares et al. 2004).

9.3 Genetic Stability

Some peculiarities of cryogenic technology (e.g., the blocked metabolism of cells and the absence of subcultures) reduce the risks of genetic and epigenetic alterations to a minimum. Presently there is no clear evidence that morphological, cytological or genetic alterations are a consequence of cryopreservation (Harding 2004). Following cryopreservation, embryogenic cultures of *Picea glauca engelmanni* derived from 12 full-sib families, were examined using a single DNA fingerprinting probe, and no genetic variation was evidenced (Cyr et al. 1994). Similarly, no sign of somaclonal variation was detected in embryogenic cultures of *Citrus sinensis* (Marin et al. 1993), *Picea glauca* (Park et al. 1994), *Picea mariana* (Isabel et al. 1993), or *Pinus sylvestris* (Häggman et al. 1998) after the lines were recovered from storage in LN.

Some threats to genetic stability arise from the common practice of using DMSO as cryoprotectant at concentrations of up to 10%. However, in an embryogenic culture of *Abies cephalonica* (Aronen et al. 1999), intraclonal genetic variation detected in the unfrozen embryogenic line (17% of the RAPD profiles) was attributed to a 30-min pretreatment of callus clumps with 10% DMSO. However, as DMSO-treated cryopreserved lines did not show any signs of instability, it was hypothesized that cryopreservation indirectly removed the mutagenic potential of DMSO by eliminating a high proportion of cells bearing genetic alterations.

De Verno et al. (1999) found genetic alterations in embryogenic cultures of *Picea glauca*, 2 and 12 months after they were re-established in culture after cryopreservation. However, as plantlets regenerated soon after callus recovery from cryostorage did not evidence any sign of genetic variation, the authors concluded that the observed genetic instability was presumably a consequence of repeated subculturing rather than of the cryopreservation procedure itself.

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9.4 Protocols

9.4.1 Controlled Rate Cooling of Pinus pinaster *Embryogenic Cultures*

(Marum et al. 2004)

Checklist for slow cooling approach
Items needed for cryopreservation

1. Forceps/tools
2. Sterile filter paper discs (55 mm)
3. Transfer pipette, pipette
4. Ice, LN
5. Cryovials and markers
6. Cryobox or cryocane for immersion of the cryovials in LN
7. Nalgene Freezing Container (Sigma-Aldrich) and isopropanol

Prepare in advance

1. Embryogenic cell masses, harvested 7 days after transfer to fresh proliferation medium and suspended in 20 ml hormone-free medium (corresponding to a suspension density of 150 mg ml^{-1}) inside a 50 ml flask
2. Nalgene Freezing Container filled with isopropyl alcohol and kept at –80°C for 24 h
3. Autoclaved filter paper discs
4. Filter sterilized maltose stock solution (4 M) in liquid MS (Murashige and Skoog 1962) medium
5. PSD cryoprotectant: 10% (w/v) PEG (polyethylene glycol) 4000 + 10% (w/v) sucrose + 10% (v/v) DMSO in liquid proliferation medium, filter sterilized
6. Proliferation medium: MS medium with 200 mg l^{-1} casein hydrolysate, 400 mg l^{-1} glutamine, 10 mg l^{-1} pantothenic acid, 20 g l^{-1} sucrose, 0.7% agar and 5 µM 2,4-D in Petri dishes.

The procedure

1. Carefully disaggregate the embryogenic masses with a transfer pipette and vigorously shake the flasks to break up into a fine suspension.
2. Add maltose stock solution drop wise over a period of 30 min to a final concentration of 0.2 M and incubate on a rotary shaker at 100 rpm in the dark for 24 h.
3. Add maltose stock solution drop wise over 30 min to a final concentration of 0.4 M and incubate on a rotary shaker at 100 rpm in the dark for an additional 24 h.
4. Add PSD cryoprotectant drop wise over a period of 30 min to a final concentration of 5% of the components (i.e., in a 1:1 ratio with the suspension culture).
5. Dispend aliquots of 1.8 ml of the pretreated embryogenic suspension into cryovials.
6. Transfer the cryovials to the Nalgene Freezing Container filled with isopropyl alcohol, previously kept at –80°C for 24 h.
7. Place the container at –80°C and hold for 24 h.
8. After 24 h, directly plunge the cryovials into LN.
9. Transfer the cryovials to a 45°C water bath and hold until the samples thaw completely, and then transfer to an ice bath.
10. Plate the contents of the cryovial on an autoclaved filter paper disc on proliferation medium in a 90 mm Petri dish and incubate in the dark at 22±2°C.
11. After 1 h, transfer the filter paper with the suspension culture to fresh medium and incubate for 18–24 h in the dark at 22±2°C.
12. Transfer the filter paper with the suspension culture to fresh medium again and subculture at 2-week intervals.

9.4.2 Vitrification of Embryogenic Cultures of **Picea mariana**

(Touchell et al. 2002)

Checklist for vitrification

Day 1: Items needed for preculture

1. Forceps/tools
2. Preculture medium

Day 3: Items needed for cryopreservation

1. Forceps/tools
2. Sterile pipettes or syringes for solutions (one for each)
3. 1.2 ml cryovials and markers
4. Nalgene® Labtop Cooler (0°C model) or ice
5. PVS2 (modified) and rinsing solution
6. Embryogenesis induction medium for recovery

Prepare in advance

1. Preculture medium: Semi-solid LM medium (Litvay et al. 1981), containing 0.8 M sorbitol and 3 g l^{-1} Phytagel in Petri dishes
2. PVS2 (modified): 30% (v/v) glycerol, 15% (v/v) ethylene glycol, 15% (v/v) DMSO in ½ strength LM medium
3. Nalgene Labtop Cooler (0°C model) placed at –20°C to chill
4. Rinsing solution: Liquid ½ strength LM with 1.0 M sucrose
5. Embryogenesis induction medium: ½ strength LM with 1 g l^{-1} casein hydrolysate, 500 mg l^{-1} glutamine, 9.1 μM 2,4-D, 4.5 μM 6-benzyladenine, 30 μM sucrose, 3 g l^{-1} Phytagel

The procedure

This is a one-day procedure, following a 48 h preculture.

1. Transfer the embryogenic masses (3 mm diameter) onto preculture medium and incubate for 48 h.
2. Using a sterile pipette or a syringe, put 1 ml of PVS2 (modified) into a cryovial. The ratio of PVS2 to tissue should be about 10:1.
3. Place 10 embryogenic masses in the cryovial. Place the cryovials in Nalgene Cooler (0°C) for 30 min.
4. Directly immerse the cryovials in LN.
5. Warm the samples in a 40°C water bath for 2 min.
6. Drain off the PVS2 solution and add rinsing solution three times for 5 min each.
7. Recover the embryogenic masses on induction medium in the dark at 25°C and subculture at 2–3 week intervals.

9.4.3 Controlled Rate Cooling of Embryogenic Callus of Citrus *ssp.*

(Pérez et al. 1997)

Checklist for cryopreservation
Items needed for cryopreservation

1. Forceps/tools
2. Sterile pipettes for solutions (one for each)
3. 2 ml cryovials and markers
4. Cryobox or cryocane to hold the cryovials
5. Cooling bath and pure methanol as coolant
6. Cryoprotective solution
7. Rinsing solution
8. Solid MS medium for recovery

Prepare in advance

1. Cryoprotective solution: Liquid MS (Murashige and Skoog 1962) with 10% (v/v) DMSO
2. Rinsing solution: Liquid MS
3. Solid basal MS medium for recovery (7 g l^{-1} agar)
4. Cooling bath containing pure methanol

The procedure

1. Add 1.8 ml cryoprotective solution to the cryovials.
2. Transfer the cell cultures (150–200 mg of loose cells from callus) to the cryovials containing liquid medium and DMSO. Resuspend and hold at 4°C for 30 min.
3. Place the cryovials in cooling bath containing pure methanol (cooling rate of about 0.5°C min^{-1}) to –40°C, then immerse in LN.
4. Rewarming: immerse the cryovials in 37°C water bath for 5 min.
5. Remove the cryoprotection solution from the cryovials.
6. Rinse cells 3 times with 1.8 ml rinsing solution.
7. Transfer the cells to fresh-gelled MS medium and keep at 27±1°C and a 16 h photoperiod (43µmol m^{-2} s^{-1}).

9.4.4 Encapsulation Dehydration of Citrus Somatic Embryos

(Dereuddre et al. 1990) Adapted by M.T. Gonzalez-Arnao and N. Duran-Vila (Gonzalez-Arnao et al. 2003).

Checklist for Alginate Bead Cryopreservation
Day 1: Items needed

1. Tools
2. Sterile Petri dishes
3. MS medium in Petri dishes to hold somatic embryos.
4. *In vitro* cultured ovules
5. Alginate solution: 3% alginate, low viscosity, in 0.4 M sucrose calcium-free MS (Murashige and Skoog 1962) medium
6. Calcium chloride solution: 100 mM in MS medium
7. Sterile 250 ml beakers for calcium chloride solution
8. Small sterile beakers for alginate solution
9. Sterile cut pipette tips to make beads (Tip capacity 100-1000μl, approximate cut diameter of the tips: 4 mm depending on the size of tissues)
10. Sterile strainers
11. Preculture medium: MS with 0.75 M sucrose in 125 ml flasks

Day 2: Items needed to dry beads and cryopreserve

1. Sterile strainer
2. Sterile 250 ml beaker
3. Dry filter paper for draining beads
4. Sterile Petri dishes for drying beads (one for each 15 beads)
5. Tools and sterile Petri dishes
6. Cryovials (1.8 or 2 ml) and markers
7. Petri dishes with recovery medium
8. Dewar flask and LN

Prepare in advance

1. Somatic embryos induced *in vitro* from ovules isolated from immature fruits and maintained on MS medium with 0.7% agar under a 16 h light/8 h dark photoperiod at 25°C for 16 h
2. MS medium with 0.7% agar in Petri dishes
3. Alginate solution: It is very difficult to dissolve alginate, so heat medium and add alginate slowly under agitation. Do not boil the solution to dissolve alginate.
4. Calcium chloride solution (100 mM) (in a flask)

5. Sterile pipettes with cut tips for forming beads
6. Sterile 50 ml beaker for alginate solution, sterile 250 ml beakers for forming beads in calcium chloride and for draining beads
7. Preculture medium (75 ml in 125 ml flasks)
8. Sterile 250 ml flask for 0.75 M sucrose solution
9. Sterile sieves or tea strainers for removing beads from solutions
10. Petri dishes with sterile filter paper for draining beads
11. Sterile Petri dishes for holding beads during dehydration
12. Sterile cryovials

The procedure

This is a 2-day procedure once the dissection of embryos is accomplished.

1. Dissect embryos on MS medium plates.
2. Suspend tissues in alginate solution in a small sterile beaker.
3. Mix apices with alginate solution and using a pipette pick up a single embryo and drop into a 250 ml beaker of 100 mM calcium chloride to make beads. Avoid air bubbles in the alginate.
4. Leave the beads in the solution for 20 min to firm up.
5. Place beads in 0.75 M sucrose MS in 125 ml flasks on a shaker for 18–20 h.
6. Drain beads and briefly place on sterile filter paper in Petri dish to absorb excess moisture.
7. Place surface-dried beads in open sterile Petri dishes and expose them to the air current of the laminar flow cabinet for ~4–5 h (20–25% moisture content in the beads, fresh weight basis). They should not touch each other or they will not dry properly. There should be no extra moisture in the dish.
8. Place 10 beads in a cryovial and plunge in LN one at a time, hold under the surface for 30 s, or place on cane and immerse.
9. Warm slowly by placing the encapsulated apices in open Petri dishes and exposing to the laminar flow for 2 min. Place beads on semi-solid medium in Petri dishes.
10. For regrowth, place dishes in the dark for 1 week and then transfer to standard growth conditions.

Possible problems

Some plants are very sensitive to sucrose. Controls should include:

(1) dissection, (2) encapsulated embryos, (3) encapsulated embryos after sucrose treatment (4) Dried beads at 20–25% moisture content.

9.4.5 Controlled Rate Cooling of Banana Embryogenic Cultures

(Panis et al. 1990)

Checklist

Items needed for cryopreservation

1. Graduated centrifuge tube
2. 2 ml cryovials and markers
3. Cryobox or cryocane for storage
4. Teflon tape for sealing cryovials
5. Sterile pipettes for replacing culture medium with cryoprotectant
6. Automatic pipette pump
7. Beaker and sterile water at 40°C for thawing
8. Methanol and methanol bath for the slow cooling of cells
9. Thermometer in the methanol bath
10. Cryoprotectant
11. Recovery medium in 90 mm Petri dish

Prepare in advance

1. Methanol bath
2. Cryoprotectant: 180 g l^{-1} sucrose and 7.5% DMSO in liquid MS
3. Growth medium: ½ MS (Murashige and Skoog 1962), 10 mg l^{-1} ascorbic acid, 5 µM 2,4-D, 1 µM zeatin
4. Recovery medium: MS, 10 mg l^{-1} ascorbic acid, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose, 1 µM 6-benzyladenine (BA), 2 g l^{-1} Gelrite

The procedure

1. Settle 10-day old embryogenic suspension cells in a graduated centrifuge tube and remove culture medium.
2. Add cryoprotectant gradually (over a period of 1 h) at room temperature until a final settled cell volume of 30% is obtained.
3. Transfer 1.5 ml to 2 ml cryotubes and seal with Teflon tape.
4. Cool cryovials in a stirred methanol bath (1°C min) to –7.5°C.
5. Immerse cryovials in LN for 3 s to initiate ice crystallization, then continue to cool cryovials to –40°C and hold 30 min.
6. Plunge the cryovials into LN.
7. Rewarm rapidly in a beaker of 40°C sterile water for 1.5 min.
8. Plate cells recovery medium and place in the dark for 1 week before moving to standard growth room conditions.

9.4.6 Vitrification of **Aesculus hippocastanum** *Embryogenic Callus*

(Lambardi et al. 2005)

Checklist for vitrification
Items needed for cryopreservation

1. Forceps, tools, sterile Petri dishes, cell dissociation sieve
2. Sterile pipettes or syringes for solutions (one for each)
3. 2 ml cryovials and markers, cryobox or cryocane
4. Nalgene Labtop Cooler (0°C model) (or ice)

Prepare in advance

1. Osmotic loading solution: 2 M glycerol and 0.4 M sucrose in MS (Murashige and Skoog 1962) medium
2. PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO (v/v), 0.4 M sucrose in MS medium
3. Rinsing solution (Liquid MS with 1.2 M sucrose)
4. Proliferation medium: MS medium with 50 mg l^{-1} arginine, 500 mg l^{-1} proline, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D)
5. Embryogenic cultures (containing mostly torpedo stage embryos), precultured on proliferation medium and maintained for 5 days at 4°C in the dark for cold acclimation.
6. Nalgene Labtop Cooler (0°C) placed at –20°C (or an ice bath)

The procedure
This is a 1-day procedure following cold acclimation of the cultures.

1. Drain the callus and transfer to the 2 ml cryovials (5 samples, 100–180 mg each).
2. Add osmotic loading solution (1 ml), hold for 30 min at 25°C.
3. Remove the loading solution, add PVS2 and hold for 90 min in the cooler or on ice to maintain the solution at about 0°C.
4. Remove the PVS2 and add 0.6 ml of fresh PVS2.
5. Immerse the cryovials directly in LN.
6. Rapidly warm in a 45°C water bath for 50 s.
7. Drain PVS2, add rinsing solution and hold 20 min at 25°C.
8. Recover the callus samples by filtration with a sterile sieve.
9. Plate the samples on the proliferation medium and maintain in the dark at 23±1°C for 3 weeks.
10. At 3 weeks transfer the embryogenic callus to maturation conditions (hormone free MS medium, 16 h photoperiod) to induce the completion of somatic embryo development.

9.4.7 *Cryopreservation of Embryogenic Callus of* **Prunus avium** *by Dehydration*

(Grenier-de March et al. 2005)

Checklist for dehydration
Items needed for sucrose preconditioning and rehydration

1. Forceps/tools
2. Sterile Petri dishes (40 × 12 mm)
3. MS medium + 0.25 M sucrose (day 1 and 9) in Petri dishes
4. MS medium + 0.50 M sucrose (day 2 and 9) in Petri dishes
5. MS medium + 0.75 M sucrose (day 3 and 8) in Petri dishes
6. MS medium + 1.0 M sucrose (day 5 and 8) in Petri dishes
7. Embryogenic tissues subcultured 3 weeks before cryopreservation

Items needed for dehydration in laminar air flow

1. Forceps/tools
2. Sterile Petri dishes (40 × 12 mm)
3. Sterile Petri dishes with air-vented lids (100 × 20 mm) (Greiner)

Items needed for cryopreservation

1. Forceps/tools
2. Sterile Petri dishes (40 × 12 mm)
3. 1.8 ml cryovials and markers
4. Cryobox or cryocane for storage of the cryovials in LN
5. Ice

Prepare in advance

1. Rinsing solution: liquid MS (Murashige and Skoog 1962) medium with 1.2 M sucrose
2. Preconditioning and rehydration medium: MS (0.5% phytagel) with 0.25 M sucrose, 0.50 M sucrose, 0.75 M sucrose, or 1.0 M sucrose in Petri dishes
3. Multiplication medium: MS salts, Morel vitamins (Morel and Wetmore 1951), 500 mg l^{-1} casein hydrolysate, 88 μM sucrose, 0.2% (w/v) phytagel, 0.54 μM NAA (naphthalene acetic acid), 0.46 μM KIN (kinetin), 0.44 μM BA (6-benzyladenine)

The procedure

This is an 11-day procedure with 7-day step-wise sucrose preconditioning and a 3-day rehydration treatment.

1. Divide the embryogenic callus (with somatic embryos at the globular stage) into clumps of 2–3 mm.
2. Culture the clumps on 0.25 M sucrose MS medium at 23°C for 1 day (20 clumps each per 10 mm Petri dish).
3. Transfer to 0.5 M sucrose MS medium for 1 day.
4. Transfer to 0.75 M sucrose MS for 2 days.
5. Transfer 1.0 M sucrose MS for 3 days.
6. Weigh an empty sterile Petri dish and record the value.
7. Transfer the clumps into the weighed Petri dish (20 clumps per Petri dish) and weigh again to calculate the fresh weight of the clumps.
8. Transfer the clumps to a sterile air-vented Petri dish and desiccate at 25°C under the sterile air of a laminar air flow cabinet.
9. Weigh the samples (with a Petri dish that was previously used for the measurement of the fresh weight) every hour and continue desiccation until their weight reaches 50–60% of the initial fresh weight.
10. After desiccation, place 20 clumps of embryogenic callus in each cryovial filled with LN.
11. Plunge the cryovials into LN.
12. Warm the 1.2 M sucrose solution in a 40°C water bath and pour into a small Petri dish (40 × 12 mm).
13. Take out the cryovials from LN and pour directly into a Petri dish containing warm rinsing solution and hold for 1 min.
14. To avoid overheating transfer the Petri dish to ice for 10 min.
15. For rehydration, transfer the samples to 1.0 M sucrose MS medium in the dark at 25°C for 12 h.
16. Transfer 0.75 M sucrose MS plates in the dark at 25°C for 12 h.
17. Transfer to 0.5 M sucrose MS plates in the dark at 25°C for 12 h.
18. Transfer to 0.25 M sucrose MS plates n the dark at 25°C for 48 h.
19. Transfer to multiplication medium in the dark at 25±1°C. Subculture at 3-week intervals and grow in normal growth room conditions.

9.4.8 Vitrification of Embryogenic Cultures of Chestnut, Common Oak and Cork Oak

By AM Vieitez based on Corredoira et al. 2004; Martinez et al. 2003; Valladares et al. 2004

Items needed

1. Tools
2. Sterile filter paper discs for draining embryo clumps
3. Cryovials and markers
4. Sterile pipettes for replacing solutions from cryovials
5. Petri dishes with sterile filter paper discs for draining
6. Specific recovery medium for each of the three species

Culture information and culture media

1. Chestnut embryogenic lines maintained by repetitive or secondary embryogenesis with subculture at 6-week intervals on MS (Murashige and Skoog, 1962) with half-strength macronutrients, 3 mM glutamine, 0.1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 0.09 M sucrose, 7 g l^{-1} agar.
2. Oak embryogenic lines maintained by secondary embryogenesis with subculture at 5-week intervals on MS, 0.1 mg l^{-1} BA, 0.05 mg l^{-1} NAA, 500 mg l^{-1} casein hydrolysate, 0.09 M sucrose, 6 g l^{-1} agar.
3. Cork oak embryogenic lines maintained by secondary embryogenesis with subculture at monthly intervals on Schenk and Hildebrandt (1972) macronutrients, MS micronutrients, vitamins and Fe-EDTA, 0.09 M sucrose and without plant growth regulators, 6 g l^{-1} agar.

Prepare in advance

1. Preculture medium: (the specific proliferation media defined above without plant growth regulators) with 0.3 M sucrose and 0.7% agar for preculture of embryo clumps (in Petri dishes)
2. PVS2: 30% glycerol, 15% ethylene glycol, 15% dimethylsulfoxide, in liquid basal MS medium with 0.4 M sucrose (50 ml in 100 ml flasks)
3. Embryogenic cultures of for isolation of somatic embryo clumps
4. Rinsing medium: Liquid MS with 1.2 M sucrose (50 ml in 100 ml flasks)
5. Recovery medium in Petri dishes: The corresponding proliferation medium defined for chestnut, oak and cork oak.

The procedure

The procedure for chestnut, oak and cork oak is very similar, and specific differences will be pointed out.

1. Isolate clumps (4–6 mg) of globular and heart-shaped embryos from stock embryogenic cultures, 3–4 weeks after the last subculture.
2. Preculture somatic embryo clumps for 3 days on 0.3 M sucrose MS medium.
3. After preculture, place embryo clumps in 2 ml cryovials (10 clumps to a vial) with 1.8 ml of ice-cold PVS2. Time of exposure at 0°C: 60 min chestnut, 30–60 min oak, and 60 min cork oak.
4. Resuspend the embryogenic clumps in 0.6 ml of fresh PVS2, place the cryovials on cane and plunge in LN.
5. To warm, immerse in a 40°C water bath for 2 min.
6. Drain off the PVS2 and replace it twice with rinsing medium for 10 min each time.
7. Place the embryogenic clumps of each cryovial onto sterilized filter paper discs and transfer to recovery medium in a Petri dish.
8. After 24 h transfer the samples to fresh recovery medium in Petri dishes without paper discs.
9. Determine somatic embryo recovery by assessing the cultures for 6 weeks (chestnut, cork oak) or 8 weeks (oak) for the frequencies of embryo clumps showing secondary embryogenesis.

Post-cryostorage embryogenesis resumption rates were 57–92% for 10 oak embryogenic lines, 88–93% for three cork oak lines and 68–70% for three chestnut lines.

Notes

PVS2 was tolerated well by chestnut, oak and cork oak embryogenic cultures. The developmental stage of somatic embryos to be cryopreserved should be considered. Clumps formed by 2–3 globular or heart-shaped embryos give a better response for withstanding storage in LN than the more differentiated (cotyledonary) embryos.

9.4.9 Controlled Rate Cooling of **Cyclamen persicum** *Suspension Cultures*

(Winkelmann et al. 2004)

Checklist for controlled cooling approach
Day 1: Items needed for preculture

1. 7–10 day old suspension cultures
2. Forceps/tools
3. Sterile plastic pipettes
4. Pipette pump
5. Preculture medium

Day 3: Items needed for pretreatment and cryopreservation

1. Forceps/tools
2. Sterile plastic pipettes for replacing solutions
3. Pipette pump
4. 15 ml centrifuge tubes
5. Cryovials (pre-cooled at –20°) and markers
6. Cryobox or cryocane to plunge the cryovials into LN
7. 0.2 μm filters to sterilize DMSO
8. Syringes to sterilize DMSO

Prepare in advance

1. 7–10 day old embryogenic suspension cells, cultured on suspension medium in Erlenmeyer flasks. It is important to use 7–10 day old cells as the cells are in the linear growth phase.
2. Suspension medium: ½ strength MS salts, full-strength Fe-EDTA, 250 mg l^{-1} peptone, 2 g l^{-1}glucose, 0.09 M sucrose, 2.0 mg l^{-1} 2,4-D, 0.8 mg l^{-1} 2iP
3. Cryoprotectant: Liquid MS (Murashige and Skoog 1962) medium with 0.6 M sucrose and 10% DMSO, filter sterilized
4. Rinsing solution: Liquid MS medium with 0.18 M sucrose, 2.0 mg l^{-1} 2,4-D and 0.8 mg l^{-1} 2iP.
5. Recovery medium: Sterile filter paper on Petri dish of ½ strength MS medium with 0.09 M sucrose, 2.0 mg l^{-1} 2,4-D, 0.8 mg l^{-1} 2-isopentenyl adenine (2iP) and 3.7 g l^{-1} Gelrite.
6. Preculture medium: suspension medium with 0.6 M sucrose
7. Cool empty cryovials to –20°C.
8. Nalgene® Freezing Container (Sigma-Aldrich) filled with isopropyl alcohol and kept at –80°C for 24 h.

The procedure

1. 7–10 days after subculture, allow the embryogenic suspension cells to settle at the bottom of the Erlenmeyer flask.
2. Replace suspension medium with preculture medium with a sterile pipette.
3. Incubate cells for 2 days at 24°C in the dark, shaking at 120 rpm.
4. Replace the preculture medium with cryoprotectant.
5. Incubate for 20 min without shaking, and then shake at 60 rpm for additional 40 min.
6. Divide suspensions into 10 ml portions and pour them into 15 ml centrifuge tubes to settle.
7. Adjust the cell density to 33% sedimented cell volume by removing the surplus supernatant medium.
8. Transfer aliquots of 1 ml to pre-cooled 1.8 ml cryovials.
9. Hold the cryovials for 2 h at –20°C in a freezer and then immerse them in LN.
10. Thaw suspension cells in a 38°C water bath for 3 min and immediately pour cells into a 100 ml flask with 10 ml rinsing medium.
11. Place flasks on a shaker (60 rpm) at 24°C, in the dark for 24 h.
12. Plate 1 ml aliquots on pieces of filter paper on Petri dishes of recovery medium and incubate at 24°C in the dark.
13. After 3 weeks, plate 100 mg samples of callus on differentiation medium (½ strength MS salts, full-strength Fe-EDTA, 250 mg l^{-1} peptone, 2 g l^{-1}glucose, 0.09 M sucrose, 2.0 mg l^{-1} 2,4-D, 0.8 mg l^{-1} 2iP, 3.7 g l^{-1} Gelrite)

9.4.10 *Encapsulation Dehydration and Controlled Cooling of Embryogenic Callus of* **Ipomoea batatas**

(Bhatti et al. 1997)

Items needed

1. Forceps, tools, sterile Petri dishes (90 mm)
2. Small sterile Petri dishes or beakers for alginate
3. Sterile 250 ml beakers for $CaCl_2$
4. Sterile Pasteur pipettes, sieves or tea strainers
5. Sterile Petri dishes for drying beads
6. 2 ml cryovials and markers, Cryobox or cryocane

Prepare in advance

1. Preculture medium: Petri dishes with 25 ml MS with 0.1 M sucrose, 0.4 M sucrose and 0.7 M sucrose, solidified with 0.7% agar (one of each for each culture)
2. Alginate solution: Liquid MS (Murashige and Skoog 1962) medium without calcium and with 3% (w/v) Na-alginate and 0.1 M sucrose
3. Calcium chloride solution: liquid MS medium with 100 mM $CaCl_2$ and 0.1 M sucrose
4. Recovery medium: MS with 0.1 M sucrose and 0.7% agar

The procedure

This is a 9-day protocol, with an initial 8-day sucrose conditioning.

1. Excise embryogenic masses of 1–2 mm diameter.
2. Suspend in the alginate solution. With a sterile Pasteur pipette pick up an embryogenic mass and some alginate solution and drip it into the calcium chloride solution to make beads. Leave the beads in the solution for 15 min to harden.
3. Transfer beads to 0.1 M sucrose MS and culture for 3 days.
4. Transfer to 0.4 M sucrose MS and culture for 3 days.
5. Transfer to 0.7 M sucrose MS and culture for 2 days.
6. Move beads to an open sterile Petri dish and expose them to sterile airflow of a laminar flow hood for 4 h. Then transfer to 2 ml cryovials.
7. Put cryovials in a programmable freezer (10°C min^{-1} to 0°C; 0.5°C min^{-1} to –40°C). Plunge cryovials into LN. Rewarm in a 38°C water bath for 2 min.
8. Transfer beads to recovery medium and incubate at 25°C in the dark for 48 h. Remove embryogenic masses from the beads and incubate at 25°C in the light (50 µmol m^{-2} s^{-1}).

9.4.11 Controlled Cooling of Embryogenic Callus of **Saccharum officinarum**

(Martinez-Montero et al. 1998)

Items needed

15–25 day old callus

1. Tools
2. Preculture medium
3. 2 ml cryovials and markers
4. Thin polypropylene plate with holes to insert cryovials
5. Ethanol bath (a commercial alcohol freezing unit could also be used such as Nalgene or “Mr. Frosty”).
6. 700 ml ethanol precooled at 0°C
7. Cryoprotectant
8. Recovery medium in Petri dishes

Prepare in advance

1. Ethanol precooled at 0°C
2. Preculture medium: Liquid MS (Murashige and Skoog 1962) medium with 0.5 M sucrose
3. Cryoprotectant: DMSO
4. Recovery medium: MS medium with 50 mg l^{-1} arginine, 500 mg l^{-1} proline, 1 mg l^{-1} 2,4-D and 8 g l^{-1} agar

The procedure

1. Place 15–25 day old callus pieces (3-5 mm diameter) in preculture medium for 1 h at 0°C.
2. Place 6 pieces of the pretreated callus in a 2 ml cryovial with 1.6 ml preculture medium. Add 0.2 ml DMSO drop by drop to the vial over a period of 30 min to reach 10% (v/v).
3. Set up an ethanol bath (700 ml precooled at 0°C) with a polypropylene plate on top to hold vials.
4. Insert cryovials in the ethanol bath and place ethanol bath in a –40°C freezer. The cooling rate is 0.4 to 0.6°C min^{-1}.
5. Induce nucleation at about –10°C by touching the base of each tube to LN. Resume cooling for about 2 h and when –40°C is reached hold the cryovials for 2 h more, then plunge cryovials in LN.
6. Rewarm by plunging into a 40°C water bath.
7. Transfer callus directly to recovery medium without washing.

Chapter 10
Cryopreservation of Excised Embryos and Embryonic Axes

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10.1 Introduction

Seeds are categorized into two main groups according to their response to desiccation and their storage physiology: orthodox (desiccation-tolerant) and recalcitrant (desiccation-sensitive) seeds (Roberts 1973). A third category of seeds are those that are relatively desiccation tolerant but do not withstand desiccation down to water contents as low as those tolerated by orthodox seeds. These seeds are freezing sensitive and are referred to as intermediate seeds (Ellis et al. 1990, 1991).

Seed is the most preferred plant propagule for *ex situ* germplasm conservation due to low storage cost, ease of seed handling and regeneration of whole plants from genetically diverse materials (Chin 1994; Pritchard 1995). While orthodox seeds are acquiescent to storage under conventional gene bank conditions for centuries, (i.e. 3–7% seed water content at –20°C) (FAO/IPGRI 1994), cryopreservation is the only available option for long-term storage of non-orthodox seeds. However, in circumstances where storage of the whole seed of a non-orthodox species is constrained by desiccation and freezing sensitivity on one hand, and by its relatively large seeds on the other, excised embryos and embryonic axes are an alternative option. In other cases where viability of lipid-rich orthodox seeds under conventional storage conditions is drastically reduced due to the thin seed

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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coat coupled with lipid peroxidation, as in the case of peanuts (*Arachis hypogaea*), germplasm curators can resort to cryopreservation of the excised embryonic axes (Gagliardi et al. 2002). Several studies on recalcitrant and intermediate species empirically determined that excised embryos and embryonic axes (in most cases) are more tolerant to desiccation and subsequent cryoexposure than whole seeds (e.g. Bajaj 1984; Radhamani and Chandel 1992; Normah et al. 1994; Makeen et al. 2005).

The removal of a large amount of water from the tissues by desiccation coupled with appropriate cooling–rewarming rates, will probably preclude the formation of lethal intracellular ice crystals (e.g. Mazur 1984; Steponkus 1985). Desiccation of excised embryos and embryonic axes is one of the simplest techniques for cryopreservation, whereby explants are dehydrated by airflow in a laminar flow cabinet, over silica gel or by flow of sterile compressed air prior to cryoexposure. Though not commonly used, embryos and embryonic axes can be desiccated via equilibration to a series of controlled relative humidities created by saturated salt solutions at a given temperature. In view of its simplicity and practicability, desiccation is the most commonly used procedure for successful cryopreservation of zygotic embryos and embryonic axes excised from seeds of many non-orthodox plant taxa. These plant taxa are exemplified by rubber (Normah et al. 1986), chayote (Abdelnour-Esquivel and Engelmann 2002), hazelnuts (Normah et al. 1994), coffee (Normah and Vengadasalam 1992; Dussert et al. 2001), olive (Gonzaléz-Rio et al. 1994), almond (Chaudhury and Chandel 1995), tea (Kim et al. 2002) and *Citrus* (Radhamani and Chandel 1992; Cho et al. 2002, 2003; Makeen et al. 2005).

Whole plant recovery from frozen embryos and axes is generally attained at an optimal range of water contents between 7 and 20% on a fresh weight basis (equivalent to 0.08–0.25 g $H_2O.g^{-1}$dw). However, embryos and embryonic axes excised from seeds of several recalcitrant species such as tea, chayote and almond can survive cryogenic exposure after dehydration down to far lower water contents. In contrast, excised embryos of recalcitrant *Zizania palustris* survived cryoexposure at extremely high water content (30–47%; 0.36–0.56 g $H_2O.g^{-1}$dw) obtained under ultra-rapid desiccation conditions (Touchell and Walters 2000).

Though moisture content (MC) of the excised embryos and embryonic axes is the most critical factor for a successful cryopreservation protocol, manipulations of desiccation conditions, particularly the rate of desiccation (Pammenter and Berjak 1999), physiological status, preculture and cryoprotectant treatments, cooling/rewarming rates, recovery medium and viability assessment require optimization for recovery of vigorous plantlets from the cryogen.

10.2 Desiccation Rate

Desiccation rates influence the extent of water loss that recalcitrant seed tissues can tolerate. Excised embryonic axes from non-orthodox seeds survive cryogenic storage at recovery rates commensurate with the acquired desiccation tolerance obtained under rapid (Normah et al. 1986; Berjak et al. 1993; Pammenter and Berjak 1999; Makeen et al. 2005), or ultra rapid drying (Berjak et al. 1990; Vertucci et al. 1991; Berjak et al. 1999; Walters et al. 2001, 2002). Acquisition of desiccation tolerance under fast and ultra-rapid desiccation conditions is likely a result of minimizing the time of intermediate water content in the metabolically active tissues during which deleterious degradative processes take place (Pammenter et al. 1991, 1998; Pritchard and Manger 1998).

Desiccation curves of whole seeds are characterized by their monotonic pattern following a simple exponential function (e.g. Tompsett and Pritchard 1998; Pammenter et al. 1998; Dussert et al. 1999; Makeen et al. 2006). In contrast, for small explants such as excised embryos and embryonic axes, initial desiccation is expected to be faster than exponential, giving rise to biphasic desiccation curves. This is, in fact, seen in excised embryonic axes of *Theobroma cacao* (Liang and Sun 2000), seeds of *Ekebergia capensis* (Pammenter et al. 1998) and excised embryonic axes of *Citrus suhuiensis* cv. Limau langkat (Makeen et al. 2005). This biphasic desiccation curve should be determined for each seed type before cryopreservation.

10.3 Physiological Status

For a successful cryopreservation protocol, the excised embryos and embryonic axes should be in a physiological and developmental state suitable for acquisition of desiccation and freezing tolerance, with the ability to regenerate vigorous plantlets after cryogenic storage. Because recalcitrant seeds do not undergo maturation drying and they are shed wet with variable water contents (Berjak et al. 1990, 1993), there are subtle metabolic differences between seeds that are immature compared with those that are fully mature (Farrant et al. 1993). Developmental stage and metabolic activity may contribute variability among axes of the same seed lot and from one seed lot to another (Berjak et al. 1996). At the intracellular level variability in the degree of vacuolation and nature of vacuole constituents and extent of membrane development may significantly affect response of individual axes to ultra-rapid drying and subsequent cryoexposure (Berjak

et al. 1993, 1996). Therefore it is important to select uniform embryos and embryonic axes at a developmental state that can ensure maximal tolerance to desiccation and cooling. The importance of choosing the proper stage was shown for embryonic axes excised from mature seeds of tea (*Camellia sinensis* L.) and *Zizania texana* (Kim et al. 2002; Walters et al. 2002) that yielded better plantlet recovery compared to early mature and late mature seeds. The choice of maturity stage of the fruit affects recovery after cryopreservation. Higher plantlet recovery was obtained when fruits of *Citrus suhuiensis* cv. Limau langkat (intermediate) and *Prunus amygdalus* (desiccation-sensitive seed with inherently poor germination) were collected at horticultural maturity rather than from less mature fruits (Makeen et al. 2005; Chaudhury and Chandel 1995).

10.4 Cryopreservation

10.4.1 Preculture and Cryoprotectant Treatment

For several recalcitrant species desiccation of embryos and excised embryonic axes under ultra-rapid drying conditions allows their survival to very low water contents (0.28–0.44 g $H_2O.g^{-1}$ dw), close to the point of only non-freezable water remaining in tissues (Berjak et al. 1993; Pammenter et al. 1993). Axes of other species are injured at water contents that are appreciably higher. Freeze drying upon cryoexposure may take place at water contents far above the previously mentioned hydration level (Wesley-Smith et al. 1992, Berjak et al. 1996), a factor that indicates the need for a preculture treatment after embryo and axis excision and before cryopreservation (Berjak et al. 1996).

Preculture involves the culture of the excised embryos and embryonic axes on medium containing sucrose or sugar alcohols followed by exposure to cryoprotectants such as dimethylsulfoxide (DMSO), glycerol and ethylene glycol for few hours to several days. Samples are partially dried to appropriate water contents prior to immersion into the cryogen (Engelmann 1997, 2000; Walters et al. 2002). Osmoprotection is a technique where the embryos and embryonic axes are subjected to a short duration (normally from minutes to few hours) of incubation on medium consisting of various sugars or other osmotically active substances. Preculture and osmoprotection treatments have positive influences on the recovery of the frozen embryos and embryonic axes (Engelmann 1997; Walters et al. 2002). Medium content and exposure duration are two key factors for successful cryopreservation.

Embryos and embryonic axes of several plant species survived cryogenic storage after vitrification treatments using cryoprotectants. *Zizania texana* (Walters et al. 2002) embryos showed a substantial increase in recovery (from 5% to 75%) following preculture in high concentrations of sugars and sugar alcohols (sucrose, glucose, raffinose, sorbitol, mannitol, xylitol and ribitol) and treatment in a cryoprotectant (PVS2), followed by partial drying to the appropriate water content.

10.4.2 Cooling and Warming Rate

Successful cryopreservation protocols entail optimization of cooling rates in conjunction with seed-tissue hydration level, to eliminate or at least minimize, nucleation of lethal intracellular ice crystals. Water loss enhances cytoplasmic viscosity (Buitink et al. 1998; Leprince et al. 1999) and rapid cooling of sufficiently dehydrated embryos and embryonic axes minimizes ice nucleation (Berjak et al. 1996; Wesley-Smith et al. 2004). Vertucci (1989) demonstrated that at higher hydration levels where freezable water is present (> 0.25 g H_2O g^{-1}dw), faster cooling rates are required to maintain plantlet vigor.

Ultra-rapid cooling prevents the growth of intracellular ice crystals (Sakai 1986) and enables flash-dried embryonic axes to successfully survive cryoexposure at moderate and high water contents (Berjak et al. 1996). Substantial recovery of plantlets was obtained from axes of the recalcitrant seed of *Quercus robur* (60%) that were flash-dried and ultra-rapidly cooled (Berjak et al. 1999). Successful cryopreservation of embryonic axes excised from recalcitrant seed can be attained if water content and cooling rates are effectively balanced. In a recent study on embryonic axes excised from a putative intermediate seed of *Citrus suhuiensis* cv. Limau langkat (Makeen et al. 2005), high recovery (83%) was attained under rapid cooling (at about –200°C min^{-1}) at axes with a water content of 0.10 g H_2O g^{-1}dw.

Rewarming is one of the most important post-cryopreservation processes that affect survival. During warming, small ice crystals can coalesce into larger ones inflicting damage to the plasma membrane (Mazur 1984; Bajaj 1985). Rapid cooling followed by slow warming is almost invariably detrimental to standard plant tissues, but slow warming is considered less damaging to slowly-cooled embryonic axes (e.g. Wesley-Smith et al. 2004). In the majority of cases, embryos and embryonic axes are warmed rapidly by immersing the cryotubes in a water-bath at 38±2°C. Most protocols use the fast warming method, immersing cryovials or aluminum envelopes in a water bath at 37–40°C for 1–5 min.

10.4.3 Recovery

It is widely accepted that suboptimal recovery conditions of the cryopreserved embryos and embryonic axes adversely affect their recovery. Factors that arise in the recovery medium adversely influence recovery rates of frozen axes that are already suffering desiccation and freezing stresses. A variety of culture media are used for recovery of frozen embryos and axes. MS medium (Murashige and Skoog 1962) with varying growth regulators is the most commonly used. Modification of growth regulator balance in the recovery medium was beneficial for coffee zygotic embryos (Normah and Vengadasalam 1992). Damage incurred by photo oxidative stress and free radicals are suggested to adversely influence recovery (Touchell and Walters 2000). Touchell and Walters (2000) demonstrated that recovery of *Zizania palustris* axes improved from 35% to 56% when cultures were maintained in the dark as compared to light. Recovery media that suppress production of free radicals, or provide free radical scavenging elements will sustain high recovery rates (Touchell and Walters 2000). Culture medium with suitable antioxidants and frequent subcultures are also important (Chandel et al. 1996).

10.4.4 Viability Assessment

The only definitive assessment of viability is regrowth of the embryos and embryonic axes into normal seedlings. However, it is very important to know as soon as possible if the material is living after cryopreservation, because in many cases regrowth is very slow. Vital stains such as fluorescein diacetate (FDA) and triphenyl tetrazolium chloride (TTC) are frequently used to determine viability. TTC is often used for embryos and axes. TTC is reduced into red colored formazan by respiration in mitochondria of the living cells. This test is qualitative for large tissues and organs.

10.5 Summary

Air desiccation, either in a laminar airflow cabinet or over silica gel, is normally used for cryopreservation of embryos and embryonic axes. This is the simplest method and should be tried first before going on to other techniques. The optimum moisture for cryopreservation ranges from 8 to 20% (period of desiccation, usually 1–2 h) depending on the species. Vitrification is used for embryos and axes that are sensitive to desiccation (i.e. *Citrus macroptera*). Most protocols use cryovials as storage containners.

The protocol for rubber however, requires the use of aluminum foil envelopes. This is one of the other alternatives that can be considered when low survival is obtained after cryoexposure. Rubber embryos did not survive cryoexposure when stored in cryovials (Normah et al. 1986).

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10.6 Protocols

10.6.1 Almond (Prunus amygdalus) *Air Desiccation Cryopreservation*

By R Chaudhury based on Chaudhury and Chandel 1995

Items required a day before initiating cryopreservation

1. Sterile glass Petri dishes, 100 ml flasks, filter paper discs
2. Scalpel blade holder, scalpel blades, forceps, needles
3. Sterile cryovials and cryomarkers
4. MS (Murashige and Skoog 1962) culture medium in glass test tubes
5. Dewar flasks containing liquid nitrogen (LN)

The procedure

1. Crack open the hard endocarp to extract the seeds. Surface sterilize in sodium hypochlorite (2–2.7%) for 10–15 min and rinse four times with sterile distilled water.
2. Remove brown seed coat and gently separate out the cotyledons, snapping one of the attached connections with embryonic axes.
3. Make an incision at the other connection to separate it out.
4. Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
5. Spread rest of the axes on sterile filter paper discs in the laminar flow cabinet for desiccation to between 6.8 and 7.5% moisture (fresh weight basis).
6. Place 10–15 axes in sterile 2 ml cryovial and plunge into LN.
7. Rewarm cryovials in a water bath at +38°C for 5 min.
8. Culture on MS medium with 1 $mg \cdot ml^{-1}$ each of 6-benzylamino purine and napthelene acetic acid, 2 $g \cdot l^{-1}$ charcoal and 0.7% agar. Maintain at 25±2°C with a 16 h photoperiod under light intensity of 35 $\mu E\ m^{-2}s^{-1}$.

Notes

1. If the seeds are hard and it is not easy to separate out the cotyledons; they may be first soaked in water for 20 min.
2. The excision of attachment points of axis to cotyledons should be done carefully to avoid damage to shoot or root apex.

The highest survival (normal seedling percentage) of axes was 66.6% at a water content of 7% (= 0.075 g $H_2O\ g^{-1}$dw) after 3 h desiccation.

10.6.2 Cryopreservation of **Arachis (A. hypogaea** *and Wild Relatives*)

By RF Gagliardi and E Mansur based on Gagliardi et al. 2002

Air desiccation

Checklist for desiccation
Items needed to dissect embryonic axes

1. Tools
2. Sodium hypochlorite (5%) for surface sterilization of seeds
3. Sterile distilled water
4. Sterile Petri dishes

Prepare in advance

MS (Murashige and Skoog 1962) medium with 8.8 μM BAP (6-benzylaminopurine) and 0.7% agar

The procedure

This is a 1-day procedure.

1. Surface sterilize seeds by immersion in sodium hypochlorite (5%) with agitation for 20 min. This may need to be adjusted if there is a high level of contamination.
2. Wash the seeds four times with sterile distilled water.
3. Carefully separate cotyledons with a scalpel and dissect embryonic axes from the seeds.
4. Place axes in Petri dishes for exposure to the laminar flow cabinet for 1–2 h to reach 60–65% moisture reduction.
5. For MC determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
6. Place the embryonic axes in cryovials and submerge in LN.
7. Warm cryovials in 38–40°C water for 2 min.
8. Culture the embryonic axes individually in culture tubes (25 × 150mm) containing 10 ml of MS medium with 8.8 μM BAP and 0.7% agar under light.

Vitrification

Checklist for vitrification

Items needed to dissect embryonic axes

1. Tools
2. Sodium hypochlorite (2–2.7%) for surface sterilization of seeds
3. Sterile distilled water
4. Sterile Petri dishes

Prepare in advance

1. Osmotic loading solution (MS medium with 2 M glycerol and 0.4 M sucrose) (Sakai et al. 1991)
2. PVS2: [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulphoxide] (Sakai et al. 1991)
3. Rinsing solution: Liquid MS medium with 1.2 M sucrose
4. Liquid MS medium with no growth regulators (MS0)
5. MS medium with 8.8 μM BAP and 0.7% agar

The procedure

This is a 1-day procedure.

1. Surface sterilize seeds and dissect axes as described above.
2. Place axes in cryovials with 1 ml osmotic loading solution and hold for 15 min at 25°C.
3. Remove the solution and add 1 ml PVS2, hold for 1–2 h to reach 60–65% moisture reduction. Test the time for new accessions.
4. Submerge cryovials in LN.
5. Warm in 38–40°C water for 2 min.
6. Replace the PVS2 immediately with rinsing solution for 10 min.
7. Gradually reduce the sucrose concentration by removing 0.5 ml of this solution and adding 0.5 ml of liquid MS0. Repeat this procedure successively six times.
8. Culture the embryonic axes individually in culture tubes (25×150mm) containing 10 ml of MS medium with 8.8 μM BAP and 0.7% agar.

Possible problems

Controls should be regrown after the steps involving air desiccation and incubation with PVS2. Excessive dehydration may result in undesired cal-logenesis during the recovery step.

10.6.3 Air-Desiccation Cryopreservation of Embryos of Chayote (Sechium edule)

By A Abdelnour-Esquivel based on Abdelnour-Esquivel and Engelmann 2002

Items needed to excise and cryopreserve the embryos

1. Tools (scalpels, blades and forceps), detergent
2. Liquid nitrogen
3. 1.5 ml sterile cryotubes
4. A water bath preset at 40°C
5. An oven preset at 103°C

Prepare in advance

1. Freshly harvested fruits
2. MS medium (Murashige and Skoog 1962) with 0.5 $mg \cdot l^{-1}$ benzyladenine (BA), 30 $g \cdot l^{-1}$ sucrose and 2 $g \cdot l^{-1}$ Phytagel
3. Sterile distilled water, Petri dishes and filter papers
4. Calcium hypochlorite solution (~4% active chlorine)

The procedure

Day 1: Seed extraction and disinfection

1. Thoroughly wash the fruits with running tap water and detergent.
2. Extract the seeds and immerse in calcium hypochlorite for 10 min.
3. Rinse three times with sterile distilled water.
4. Excise and place the embryos on semi-solid MS in the dark at 24°C for 24 h before cryopreservation.

Day 2: Cryopreservation of the excised zygotic embryos

1. Desiccate embryos in laminar airflow cabinet for 4 h (19% moisture content MC). The highest regrowth of embryos was 30% with a water content of 19% (= 0.23g H_2O g^{-1}dw) after 4 h desiccation.
2. For MC determination: Determine the fresh weight of 10 axes replicated 3 times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
3. Place the desiccated embryos inside cryotubes (10 embryos in each cryotube) and directly immerse into LN.
4. Rewarm by rapid immersion in a water bath at 40°C for 60–90 sec. Culture warmed embryos at 24±1°C in the dark for 2 days, then under a 16 h light/8 h dark photoperiod (34 μE m^{-2} s^{-1}). Assess the growth after 3 weeks.

10.6.4 Vitrification of Embryonic Axes of **Citrus macroptera**

By R Chaudhury based on Malik and Chaudhury 2006

Items required a day before initiating cryopreservation

1. Sterile glass Petri dishes, 100 ml flasks, filter paper discs
2. Scalpel blade holder, scalpel blades, forceps, needles
3. Sterile cryovials and cryomarkers, Dewar with LN

Prepare in advance

1. Preculture medium: MS medium (Murashige and Skoog 1962) with 0.3 M sucrose, 2 M glycerol and 0.7% agar
2. Osmotic loading solution: 0.4 M sucrose, 2 M glycerol in MS
3. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in MS medium
4. Rinsing solution: 1.2 M sucrose in MS medium
5. Culture medium: MS with 1 $g \cdot l^{-1}$ activated charcoal, 0.17 $g \cdot l^{-1}$ NaH_2PO_4 and 1 $mg \cdot l^{-1}$ each of 6-benzylamino purine (BAP) and naphtlene acetic acid (NAA) after Chin et al. (1988)

The procedure

1. Collect seeds from ripe fruits within 4–5 days of harvest.
2. Remove the seed coat from the seeds just before experimentation.
3. Surface sterilize the seeds with sodium hypochlorite (2–2.7%) for 10 min.
4. Rinse four times with sterile distilled water.
5. Gently separate out the cotyledons, snapping one of the attached connections with the zygotic embryonic axes. Scoop it out by making another incision at the other joining point.
6. For moisture content determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
7. Preculture axes on preculture medium for 16 to 24h.
8. Transfer 15–25 axes to 1.2 ml sterile cryovials and treat with 0.5 ml loading solution for 20 min at 25°C.
9. Replace loading solution with 0.5 ml PVS2 for 30 min at 25°C.
10. Plunge cryovials into LN (LN).
11. Rewarm in a 38±1°C water bath for 1 min with vigorous shaking.
12. Replace the PVS2 with 0.5 ml rinsing solution at 25°C for 20 min.
13. Drain the solution and blot the axes dry on sterile filter papers.
14. Culture the axes in culture medium (above) and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35 $\mu E\ m^{-2}s^{-1}$.

10.6.5 Cryopreservation of Embryonic Axes of **Citrus suhuiensis** *cv. Limau langkat through Desiccation*

By AM Makeen and MN Normah based on Makeen et al. 2005

Desiccation under laminar flow

Items needed

1. Sterile plastic Petri dishes, glass Petri dishes and beakers/ bottles
2. Sterile propylene cryovials, cryocanes and cryovials rack
3. Sterile filter papers
4. Tools (scalpels, sterile blades and forceps)
5. 95% (v/v) ethanol for flaming the tools
6. Stereomicroscope

Prepare in advance

1. Freshly harvested fruits
2. Sterile distilled water
3. 200 ml 80% (v/v) ethanol (for seed sterilization)
4. 200 ml of 20% (v/v) bleach (~5% sodium hypochlorite) and a few drops of Tween 20
5. Aluminum foil boats for water content determination
6. Growth medium: MS medium with 0.1 $g \cdot l^{-1}$ 6 benzylaminopurine (BAP) and 0.7% agar in Petri dishes
7. Liquid nitrogen
8. Water bath preset at 40°C
9. An oven preset at 103°C
10. A desiccator to cool down the dried embryonic axes (for moisture content determination)

The procedure

This is a 1-day procedure.

1. Remove the seeds and wash in running tap water for 1 h.
2. Place the whole seeds inside a bottle or a beaker and pour the 200 ml 80% ethanol, leave for 2 min, shake gently. Pour off the ethanol and submerge the seeds in 20% bleach solution for 20 min with shaking every 5 min.

3. Remove the disinfectant and rinse the seeds with sterile distilled water three times until the foam made by Tween 20 disappears.
4. Keep the sterilized seeds in Petri dishes sealed with Parafilm.
5. Place one or two layers of filter paper on a glass Petri dish and place a few seeds on the filter paper. Under a stereomicroscope with the aid of a pair of forceps and a scalpel excise the biggest embryonic axes (seeds are highly polyembryonic) leaving a small block of the cotyledon attached to the axes. Place the excised embryonic axes (in a row of 10 axes) inside another Petri dish with a filter paper moistened with few drops of sterile distilled water. Close the Petri dish with its cover. This is done until all axes are excised for desiccation.
6. Transfer the axes to glass Petri dishes with dry filter papers and desiccate for 2 h in laminar airflow cabinet (using high airflow).
7. For moisture content (MC) determination: Determine the fresh weight of 10 axes (replicated three times) in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh (Fig. 10.1).
8. Place 10–30 axes inside cryovials (5 axes in each vial), mount the cryovials in the cryocanes and plunge into the LN.
9. Rapidly warm the cryovials in a 40°C water bath for 2 min, then culture the axes on growth medium.
10. Seal all Petri dishes with Parafilm and keep the cultures in the culture room (Culture conditions: 25±1°C under 16 hours light/ 8 h dark photoperiod with light intensity of 25 µmol $m^{-2}s^{-1}$).
11. Score viability when axes develop into normal seedlings (shoot and root).

Desiccation over silica gel

Items needed to dissect embryonic axes
Use the same list as for air desiccation in laminar flow hood.

Prepare in advance

1. Silica gel: wrap batches of 15 g silica gel in aluminum-foil bags; place them in an autoclavable container and autoclave for 20 min. After that place silica gel packages inside the oven set at 103°C for 24 h or until use.
2. Follow the same procedures for embryonic axis excision as for the desiccation under laminar airflow.

The procedure

1. Place 15 g of sterile, dry silica gel in a glass Petri dish (7 cm diameter) and cover with a sterile filter paper.
2. Place the axes on the filter paper covering the silica gel.
3. Seal each Petri dish with Parafilm and leave for the prescribed desiccation period.
4. For moisture content (MC) determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh (Fig. 10.2).
5. Follow the previously described procedure for cryopreservation, rewarming and culture.

Optimal hydration for cryopreservation
The most normal seedlings regrown from cryopreserved axes was 83.3% obtained at MC of 7.8 (= 0.095 g H_2O g^{-1}dw) after 2 h of desiccation in the laminar airflow cabinet and 62% at 11.5% MC (0.139 g H_2O g^{-1}dw) after one hour of desiccation over silica gel.

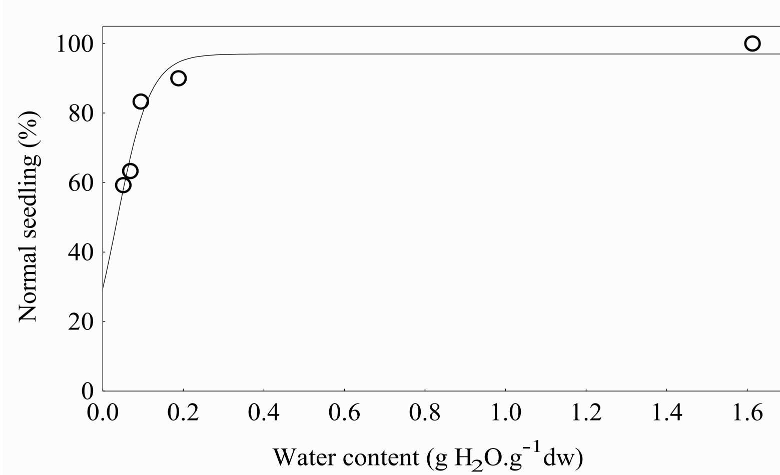

Fig. 10.1 Observed normal seedling percentage (o) of the excised embryonic axes of *C. suhuiensis* cv Limau langkat desiccated in laminar airflow cabinet to various water contents. The fitted pattern of the desiccation sensitivity model was computed by Quasi-Newton method. (From Makeen et al. 2005)

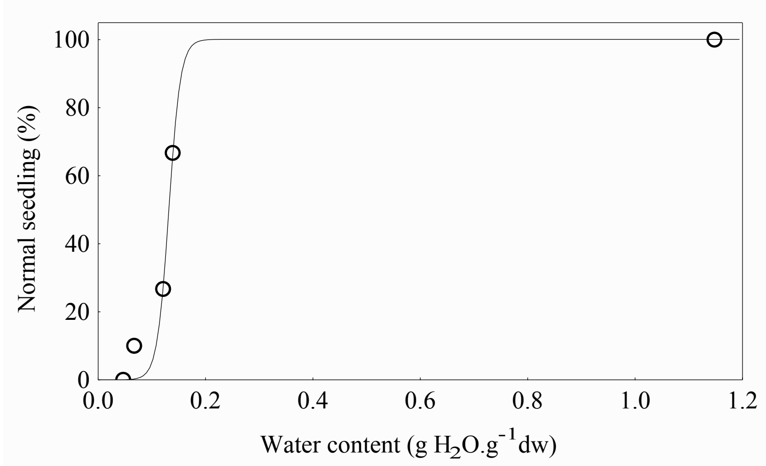

Fig. 10.2 Observed normal seedling percentage (o) of the excised embryonic axes of *C. suhuiensis* cv Limau langkat desiccated over silica gel to various water contents. The fitted pattern of the desiccation sensitivity model was computed by Quasi-Newton method. (From Makeen et al. 2005)

10.6.6 *Cryopreservation of Coffee* (Coffea liberica) *Embryos through Air Desiccation*

By MN Normah based on Normah and Vengadasalam 1992

Items needed to excise and to cryopreserve the embryos

1. A laminar airflow cabinet
2. Tools (forceps, blades, scalpels)
3. Polypropylene cryovials
4. LN tank
5. Sterile glass Petri dishes and beakers/bottles
6. An oven preset at 103°C, desiccator for moisture determination

Prepare in advance

1. Sterile distilled water
2. A 40°C water bath
3. 5% solution of commercial bleach (final concentration 0.27% sodium hypochlorite)
4. Recovery medium: MS medium (Murashige and Skoog 1962) with 0.1 mg·l^{-1} kinetin, 0.1 mg·l^{-1} 2,4-D, 30 g·l^{-1} sucrose, 2 g·l^{-1} activated charcoal and 8 g·l^{-1} Difco Bacto Agar
5. Subculture medium: MS with 0.1 mg·l^{-1} 6 benzyl adenine (BA), 0.5 mg·l^{-1} indole 3 butyric acid (IBA), 7 g·l^{-1} agar

The procedure

This is a 1-day procedure.

1. Rinse coffee fruits in water and remove the exocarp and mesocarp.
2. Isolate the embryos under laminar airflow.
3. Desiccate embryos in open Petri dishes in the laminar airflow for 30–50 min.
4. Determine the moisture content (MC) of the excised embryos after desiccation treatment (optimal is 20% = 0.25 g H_2O g^{-1}dw). Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
5. Place embryos in cryovials and immerse directly into LN.
6. To warm: rapidly immerse vials in a 40°C water bath for 2–3 min.
7. Sterilize embryos with bleach for 6 min followed by 5 rinses with sterile distilled water and directly culture on the recovery medium.
8. Maintain cultures at 27±1°C with 8 h photoperiod (25 µmol $m^{-2}s^{-1}$).
9. Score viability when embryos develop into normal seedlings (shoot and root). The highest regrowth was 83–86% at 25–20% MC.

10.6.7 Cryopreservation of Embryonic Axes of Hazelnut (Corylus avellana) *by Air and Silica Gel Desiccation*

By MN Normah and BM Reed based on Reed et al. 1994 and Normah et al. 1994

Air desiccation

Items needed to dissect and cryopreserve the embryonic axes

1. Sterile propylene cryovials, cryocanes and cryovials rack
2. Tools (scalpels, sterile blades and forceps)
3. Sterile filter papers
4. Welled culture plates or Petri dishes of recovery medium
5. LN in a storage dewar
6. Commercial bleach (5.3% sodium hypochlorite)

Prepare in advance

1. Freshly harvested hazelnuts (nuts can be stored for several months in 70 l burlap bags at 20°C and relative humidity of 20–40%)
2. Aluminum foil boats for weighing the embryonic axes
3. An oven preset at 103°C, a desiccator
4. Basal NCGR-COR medium (Yu and Reed 1993)
5. Perlite, moistened with tap water, autoclaved for 30 min and cooled

The procedure

Week 1: Stratification of whole seeds

1. Surface sterilize fresh or stored whole nuts in 20% bleach for 10 minutes and rinse in tap water.
2. Plant intact seeds in the perlite, close tray with plastic bag and chill at 4°C for 2 weeks.

Week 3: Cryoexposure of hazelnut excised embryonic axes

1. Crack nuts to remove the pericarps and excise the embryonic axes.
2. Surface sterilize the embryonic axes in 10% bleach (final concentration 0.53% sodium hypochlorite) for 10 min and rinse in sterile water.
3. Blot dry on sterile filter paper. Dry in an open Petri dish in a laminar flow hood for 1.5–2 h (8–10% moisture = 60% regrowth).
4. Moisture content (MC): Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
5. Place 10 axes in each cryovial, immerse directly into LN for 1 h.
6. Warm cryovials in a water bath at 45°C for 1 min.

7. Culture axes on NCGR-COR medium (Yu and Reed 1993) at 25°C with a 16 hr photoperiod (25 µmol m^{-2} s^{-1}).
8. Score viability when axes develop into normal seedlings.
9. This technique was used to store axes from four species of *Corylus*.

Silica gel dehydration

Embryonic axes can also be excised from directly cryopreserved seeds of *Corylus.* The protocol is as follows:

Items needed for cryopreservation of seeds and excision of embryonic axes

1. A seed germinator with alternating temperatures of 10°C for 14 h and 25°C for 10 h under 12 h photoperiod
2. An oven preset at 103°C, a desiccator
3. Cryostorage rack
4. Tools (scalpels, sterile blades and forceps), sterile filter papers
5. Recovery medium: Basal NCGR-COR medium (Yu and Reed 1993)
6. LN storage unit

Prepare in advance

1. Freshly harvested or stored hazelnuts
2. Silica gel in batches of 125 g
3. Gibberellic acid (GA_3) solution (10 mg/100 ml) for soaking the embryonic axes
4. Basal NCGR-COR medium (Yu and Reed 1993)
5. TTC solution: 2,3,5-triphenyltetrazolium chloride (1.0%)

The procedure

Day 1: Desiccation of hazelnut seeds

1. Desiccate whole nuts (with pericarp) over the silica gel for 32 to 48 h.
2. Use 10 nuts with four replicates for moisture content (MC) determination and 25 nuts in triplicates for desiccated-frozen nuts and germination tests (in a seed germinator).
3. For MC determination: Determine the fresh weight of nuts (cut into small pieces) in an aluminum foil boat, then dry in an oven set at 103°C for 16 h, cool in a desiccator and reweigh.

Day 2: Cryoexposure of hazelnut seeds and survival of excised embryonic axes

1. Place nuts into a cryo storage rack and immerse directly into the LN tank.
2. Warm the nuts at room temperature.
3. Crack nuts to remove the pericarps.
4. Perform the tetrazolium test on a sample of the axes to determine their viability.
5. Excise the embryonic axes of the remaining nuts.
6. Surface sterilize the embryonic axes in 10% bleach (final concentration 0.53% sodium hypochlorite) for 10 min and rinse in sterile water.
7. Soak embryonic axes in the GA_3 solution for 5 min and culture on NCGR-COR medium.

Optimal hydration for cryopreservation

The highest regrowth of axes was 97% attained by cryopreserving whole hazelnuts dried to a water content of 15% (= 0.176 g H_2O g^{-1}dw) after desiccation over silica gel for 32 h. Optimal water content for excised axes was 3% (= 0.03 g H_2O g^{-1}dw).

10.6.8 *Air-Desiccation Cryopreservation of Embryonic Axes of Neem* (Azadirachta indica)

By R Chaudhury based on Chandel et al. 1996

Items required a day before initiating cryopreservation

1. Sterile glass Petri dishes and 100 ml flasks
2. Sterile filter paper discs
3. Scalpel blade holder, scalpel blades, forceps, needles
4. Sterile cryovials and cryomarkers
5. MS medium (Murashige and Skoog 1962) with 0.1 mg ml^{-1} each of 6-benzylamino purine (BAP) and naphthlene acetic acid (NAA), 0.7% agar in test tubes
6. Dewar flasks containing LN
7. Seeds: The seeds should be harvested only from ripe yellow fruits. Complete the extraction process within 4–5 days of harvest to obtain optimal results in cryopreservation.

The procedure

1. Collect seeds from ripe yellow neem fruits. Break open the endocarp to extract the seeds just before experimentation. Surface sterilize the seeds using sodium hypochlorite (2–2.7%) for 10 min.
2. Rinse four times with sterile distilled water.
3. Remove brown seed coat and gently separate out the cotyledons snapping one of the attached connections with the embryonic axis.
4. Make an incision at the other attached connection of the axis and scoop it out.
5. For moisture content determination: Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
6. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for desiccation to moisture levels between 11 and 16% (on fresh weight basis).
7. Moisture content (MC): Determine the fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
8. If the initial moisture content of neem axes is about 45%, it may require 3.5–4 h of desiccation to achieve target moisture level.
9. Place about 10–15 desiccated axes in sterile 1.2 ml cryovial and plunge rapidly in LN.
10. Warm the cryovials in a water bath at 37–38°C for 5 min, and culture on MS medium under growth room conditions.

10.6.9 Desiccation Cryopreservation of Isolated Embryos of Olive (Olea europaea)

By MA Revilla based on Gonzaléz-Rio et al. 1994

Items needed

1. Sterile tools (scalpels, blades, forceps)
2. A laminar airflow cabinet
3. An oven set at 70°C for moisture content determination
4. Sterile 2 ml polypropylene cryovials
5. LN tank
6. A 45°C water bath

Prepare in advance

1. Fruits with intact mesocarps (to reduce chances for contamination)
2. A solution of 70% ethanol
3. A solution of 1.2% active sodium hypochlorite
4. Sterile distilled water, filter papers
5. MS (Murashige and Skoog 1962) medium with 30 $g{\cdot}l^{-1}$ sucrose and 7.5 $g{\cdot}l^{-1}$ agar

The procedure

Day 1: Remove the fleshy mesocarp of the fruit and break the endocarp using a vice. Soak seeds in water overnight.

Day 2

1. Make a longitudinal excision in the endosperm and remove embryo.
2. Immerse embryos in 70% ethanol for 2 min, followed by 1.2% sodium hypochlorite for 10 min, then rinse twice with sterile water.
3. Place embryos on filter paper and desiccate for 13 and 18 h in the laminar flow cabinet. Determine the moisture content.
4. The highest normal seedling percentages (70%) were obtained when embryos were desiccated for 18 h to a moisture content of 3% (0.03g $H_2O.g^{-1}$dw) and for 13 h with recovery of 53% and 10% MC (0.11g $H_2O.g^{-1}$dw).
5. For moisture content determination: take fresh weight of 10 axes replicated three times each in an aluminum foil boat, then dry in the oven for 24 h, cool in a desiccator and reweigh.
6. Place five embryos in each cryovial and plunge into LN.
7. Rewarm by immersing the cryovials in a 45°C water bath for 2 min.
8. Culture embryos on MS medium at 26°C with 16 h light (30–45 µE $m^{-2}s^{-1}$)/8 h dark. Embryos turn green after 15 days of culture.

10.6.10 Desiccation Cryopreservation of Embryonic Axes of Rubber (Hevea brasiliensis)

By MN Normah based on Normah et al. 1986

Items needed

1. An oven set at 103–105°C, desiccator
2. Sterile tools for excision
3. Sterile aluminum foil envelopes
4. LN
5. A water bath set at 37±2°C

Prepare in advance

1. Fresh seeds: Seeds from fruits collected from trees give better regrowth compared to those dispersed on the ground.
2. 20% solution of commercial bleach (final concentration 1% sodium hypochlorite) with a few drops of Tween 20 per 500 ml.
3. Sterile distilled water
4. MS medium (Murashige and Skoog 1962) with 0.6–0.7 µM kinetin, 1.0 µM naphthelene acetic acid (NAA), 1.4 µM gibberellic acid (GA_3) and 4 $g{\cdot}l^{-1}$ activated charcoal. Softer agar (6–7 $g{\cdot}l^{-1}$) for the recovery medium produces better growth of the seedlings.

The procedure

This is a 1-day procedure.

1. Immerse the seeds in bleach solution for 30 min. Rinse with sterile distilled water three to four times.
2. Crack the seeds and isolate the embryonic axes by completely removing the endosperm and the cotyledons (in laminar airflow cabinet).
3. Desiccate the excised embryonic axes for 3–4 h at 26–28°C. (Optimal MC 16.2% = 0.19 g H_2O g^{-1} dw)
4. After each desiccation period, determine moisture content of the axes; use 40 axes (10 in 4 replicates).
5. For moisture content determination: Determine the fresh weight axes in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
6. Place desiccated axes inside sterile aluminum foil envelopes and directly immerse into the LN tank for 16 h.
7. Warm the frozen axes by immersing the foil envelopes in the water bath (at 37± 2°C) for 1–2 min.
8. Culture the axes under a 12 h photoperiod with light intensity of 25 µmol $m^{-2}s^{-1}$.

10.6.11 Flash Drying Tea Zygotic Embryos (Camellia sinensis)

By HH Kim based on Kim et al. 2002, 2005

Checklist for flash drying of tea zygotic embryos

Items needed to prepare plant material

1. Sterile filter papers in sterile Petri dishes
2. Tea seeds
3. Sterile (autoclaved) distilled water
4. Scalpel, forceps for inoculation
5. 80% ethyl alcohol for surface sterilization
6. 1% sodium hypochlorite solution with 3 drops of detergent

Items needed to dehydrate and cryopreserve

1. Sterile standard sieves (850 µm) to dry embryos
2. Petri dishes with sterile filter paper to remove moisture following surface sterilization
3. Cryovials (2 ml) and markers
4. Recovery medium: ½ strength MS medium (Murashige and Skoog 1962) with 0.5 $mg{\cdot}l^{-1}$ benzylaminopurine (BAP), 0.01 $mg{\cdot}l^{-1}$ indole butyric acid (IBA), 1.5 $g{\cdot}l^{-1}$ activated charcoal and 2.5 $g{\cdot}l^{-1}$ Phytagel
5. Canes, canisters and long-term storage Dewar
6. A 40°C water bath
7. Vertical laminar airflow to dry embryos

The procedure

Step 1. Plant material

1. Harvest tea seeds at mid-maturity (mid-October in Korea).
2. Store the seeds with pericarp at 5–8°C under humid conditions.
3. Use seeds within 1 month after harvest. Germination of dried cotyledonary embryonic axes (CEAs) decreases rapidly after seed storage.
4. Remove the pericarp and seed coats with a scalpel.
5. Dissect axes with fragments of cotyledons attached to the embryonic axis (CEAs) forming explants of pyramidal shape 5–6 mm long.
6. Sterilize in 1% sodium hypochlorite with 2–3 drops of detergent for 15 min with shaking, and then rinse three times with sterile water.
7. Place CEAs on sterile filter paper in a Petri dish to absorb moisture.

Step 2. Dehydration and cryopreservation of CEAs

1. Place sterilized standard sieve (pore size: 850 μm) in the air current of the vertical laminar airflow.
2. Place the CEAs on sieves and dry for ~4 h to embryo moisture content (MC) of 16–17% (fresh weight basis).
4. Place CEAs in 2 ml cryovials (10 CEAs per vial) and submerge in vapor phase LN.
5. Rapidly warm samples in a 37°C water-bath for 2 min.
6. Culture at 22–25°C with 16 h light (35 μmol m^{-2} s^{-1})/8 h dark.
7. Expansion of embryonic axes is noticeable after 1 week.
8. CEAs expand and produce a small green shoot of at least 3 mm by 10 days after inoculation. CEAs develop normal shoots and roots within 1 month.

Notes

The following are critical for successful cryopreservation:

1. Seed harvesting stage: Early- to mid-maturity seeds showed higher survival than late-maturity seeds.
2. Dissection of CEAs: Dissection area and dissection angle (depth) should be precisely controlled. The location (in depth) is different among individual seeds.
3. Drying to moisture of 16–17%: The MC of CEAs is the most critical factor. The hydration window for tea zygotic embryos is narrow. CEAs with higher or lower moisture content are damaged due to freezing or desiccation injury.

10.6.12 Desiccation of Zygotic Embryo Axes of Chestnut (Castenea)

By AM Vieitez based on Corredoira et al. 2004

Items needed

1. Tools
2. Sterile filter paper
3. Decoated chestnut seeds for excision of embryonic axes
4. 70% ethanol
5. Chlorine solution at 5% (Millipore® chlorine tablets) plus a few drops of Tween 80
6. Sterile distilled water
7. Sterile 2-l beakers for seed sterilization
8. Sterile Petri dishes
9. Cryovials and markers
10. Recovery medium

Prepare in advance

1. Sterile 2-l beakers for surface sterilization of chestnut seeds
2. Sterile distilled water in flasks or bottles
3. Sterile Petri dishes (one for each 25 axes)
4. Recovery medium: Murashige and Skoog (1962) medium (with half-strength nitrates), 0.5 mg l^{-1} 6-benzyl adenine (BA), 1 ml l^{-1} Plant Preservative Mixture (PPM) and 0.09 M sucrose. Dispense in Petri dishes (25 ml of 5 $g \cdot l^{-1}$ agar and 20 × 150 mm tubes (16 ml of 6 $g \cdot l^{-1}$ agar).

The procedure

1. Collect seeds and store in paper bags at 4°C for up to 1 month.
2. Remove seed coats and surface sterilize in 70% ethanol (2 min) and 5% chlorine solution (30 min). Shake from time to time.
3. Rinse three times with sterile distilled water.
4. Aseptically dissect embryonic axes from the cotyledons.
5. Desiccate axes on open Petri dishes in a laminar flow hood for 4–5 h (20–24% moisture content on a fresh weight basis).
6. For moisture content determination: Determine the fresh weight of 10 axes replicated 3 times each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
7. Place axes into 2 ml cryovials (five axes to a vial) plunge into LN.
8. Warm vials in a 40°C water bath for 2 min.

9. For rehydration place embryonic axes in Petri dishes of recovery medium, and incubate in darkness at 25°C for 24 h.
10. Transfer axes to culture tubes (one axis per tube) with fresh recovery medium, but with agar concentration increased to 6 $g{\cdot}l^{-1}$.
11. After 2 weeks place under a 16 h photoperiod (30 µmol $m^{-2}s^{-1}$).
12. Assess plant recovery (whole plants developing directly from embryonic axes) 8 weeks after warming (Table 10.1).

Comments

Replicate samples should be enough to determine the fresh and dry weights before and after Step 5. Controls (desiccated and non-cryopreserved axes) should be included. Appropriate procedures should be developed for the *in vitro* culture of embryonic axes in recovery medium prior to the cryopreservation experiments.

In this protocol, most non-cryostored embryonic axes develop as whole plantlets (100% when post-desiccation moisture content was 29–35%). Following cryopreservation plant recovery increases from zero for non-desiccated axes to 63% for those with moisture contents ≅ 20% (5 h desiccation). In addition, 36% of the axes produce only roots. Desiccation to moisture contents less than 18% results only root development.

Table 10.1 Shoot recovery (% ± standard error) of cryopreserved shoot apices of six chestnut genotypes from juvenile- and mature-tree origin. Assessment made 8 weeks after cryopreservation and plating on recovery medium

Genotype	Origin	Shoot recovery (%)
812	Juvenile	53.2 ± 1.6
818	Juvenile	53.3 ± 3.3
12	Juvenile	37.5 ± 9.8
LA1	Mature	35.4 ± 4.4
LA3	Mature	54.4 ± 8.1
Pr5	Mature	42.5 ± 3.8

Chapter 11
Cryopreservation of Monocots

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11.1 Introduction

Monocotyledonous plants comprise the majority of agricultural plants in terms of biomass produced. Estimates of the number of species within this group range from 50,000 to 60,000. By far the largest monocot family is the orchid family, with some 20,000 species. Economically the most important family in this group (and in the flowering plants) is the grasses, family *Gramineae* or *Poacea*. Since many monocots are propagated through seed and monocot seed is often orthodox (can be dried to low moisture content see Chap. 19), seed conservation is the method of choice to store the diversity of many members of this group. Storage of desiccated seeds at low temperature is not applicable to crops that do not produce seed (e.g., bananas) or that produce recalcitrant seed (i.e. seed that can not be dried, see Chaps. 10, 18). Other plant species are propagated vegetatively to preserve the unique genomic constitution of cultivars (for crops such as yam, taro and garlic and ornamental plants such as lily and orchids). Vegetative tissues can be preserved in field collections and *in vitro*. Cryopreservation, however, is the ultimate preservation method since under these conditions material can be preserved for unlimited periods without alteration. In this chapter cryopreservation protocols for vegetative tissues of monocotyledonous plants will be discussed.

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

11.2.1 Starting Material

Since the main goal of cryopreservation is to store material for the long term and subsequently produce true-to-type plants from the stored material, shoot-tips or meristems are the preferred experimental material. Meristematic cells contain relatively small vacuoles with small amounts of water. Less water needs to be removed to prevent lethal ice crystallization compared to non meristematic cells (Panis et al. 2001). In the literature only one example can be found that deals with cryopreservation of monocot root tips and regeneration into new plants was problematic (Bouman et al. 2003).

In the large majority of the reports *in vitro* tissues are used for cryopreservation. For most of the herbaceous monocotyledonous species for which cryopreservation protocols are described (Table 11.1), shoot tips excised from *in vitro* plants are used. Meristematic tissues that survive cryopreservation include shoot primordia of orchid (Na and Kondo 1996) and multiple bud clusters of banana (Agrawal et al. 2004; Panis et al. 2002; Panis et al. 1996) and *Asparagus* (Kohmura et al. 1992). Cryopreservation of *ex-vitro* material is reported in only two cases. In banana it is possible to cryopreserve shoot tips excised from greenhouse plants (Panis, unpublished), and shoot tips can be extracted from garlic cloves (Baek et al. 2003; Kim et al. 2004a, b; Kim et al. 2005; Volk et al. 2004).

Table 11.1 Cryopreservation protocols applied in monocot plants

Plant	Technique	Reference
Asparagus	Droplet	Mix-Wagner et al. 2000 Protocol 11.6.2
	Desiccation	Uragami et al. 1990 Protocol 11.6.1
	Controlled cooling	Suzuki et al. 1997, 1998 Jitsuyama et al. 2002, Protocol 11.6.3
	Preculture	Uragami et al. 1990
	Vitrification	Kohmura et al. 1992
Australian	Droplet vitrification	Turner et al. 2001
Banana	Preculture	Panis et al. 1996, 2002 Protocol 11.6.6
	Encapsulation dehydration	Panis 1995
	Vitrification	Thinh et al. 1999; Panis, Thinh 2001
	Droplet vitrification	Agrawal et al. 2004; Panis et al. 2005 Protocol 11.6.7
Garlic	Vitrification	Keller 2005, Protocol 11.6.9
	Droplet vitrification	Kim et al. 2004a, b; Kim et al. 2005;

		Baek et al. 2003; Volk et al. 2004; Ellis et al. 2006, Protocol 11.2.8
Grass	Controlled cooling	Chang et al. 2000
	Encapsulation dehydration	Reed et al. 2006; Chang et al. 2000, Protocol 11.6.13
	Vitrification	Chang et al. 2000
Lily	Encapsulation dehydration	Matsumoto and Sakai 1995
	Vitrification	Bouman et al. 2003; Matsumoto, Sakai 1995; Matsumoto et al. 1995
	Encapsulation vitrification	Matsumoto and Sakai 1995
		Protocol 11.6.12
Orchid	Desiccation	Na and Kondo 1996
		Protocol 11.6.4
	Vitrification	Thinh and Takagi 2000
		Protocol 11.6.5
Pineapple	Vitrification	González-Arnao et al. 1998
		Protocol 11.6.10
	Encapsulation vitrification	Gamez-Pastrana et al. 2004
Sugarcane	Encapsulation dehydration	González-Arnao et al. 1996; Paulet et al. 1993
		Protocol 11.6.8
Taro	Vitrification	Takagi et al. 1997
	Droplet vitrification	Protocol 11.6.11
Yam	Droplet	Leunufna and Keller 2003
	Vitrification	Mandal 2000
		Kyesmu and Takagi 2000
	Droplet vitrification	Leunufna and Keller 2003, 2005
	Encapsulation vitrification	Mandal 2000

11.2.2 Preconditioning and Preculture

Most hydrated tissues do not withstand dehydration to the moisture content that is needed to prevent crystallization during cooling (i.e. 20–30%). Naturally low moisture tissues that cryopreserve well are pollen and orthodox seeds. The key to successful cryopreservation lies not in the induction of freezing tolerance, but rather on dehydration tolerance (Panis and Lambardi 2005). Acclimation is a physiological process that increases a plant's ability to survive unfavorable environmental stress. Acclimation is triggered by environmental parameters like reduction in temperature and shortening of day length. Osmotic changes and abscisic acid (ABA) treatments can have similar effects.

Three main strategies are followed to prepare monocot plant tissues for the dehydration stress to which they will be exposed during the cryopreservation process: ABA, cold, and sugar treatments. *Asparagus* shoot tips

(Kohmura et al. 1992; Mix-Wagner et al. 2000) and banana apical meristems (Panis et al. 2005) do not require preconditioning or preculture treatments before cryopreservation. A 3-day preculture in liquid medium with 1 mg l^{-1} ABA proved to be essential to obtain recovery of shoot primordia of the orchid *Vanda pumila* that were then desiccated to 25% water content and rapidly frozen (Na and Kondo 1996). Cold acclimation is often applied to temperate species. Monocot examples include species of temperate and subtropical grasses (Chang et al. 2000; Reed et al. 2006), garlic (Volk et al. 2004), and lily (Bouman et al. 2003). One report mentions a positive effect of cold acclimation in a tropical species, yam (Leunufna and Keller 2005). The conditions for cold acclimation can vary considerably, from a cold regime of –1°C 16 h dark/22°C 8 h light for 2–4 weeks (Chang et al. 2000; Reed et al. 2006) to a preculture treatment of excised shoot-tips for 48 h at 5°C (Volk et al. 2004). Sugar treatments are commonly applied to prepare plant tissues for cryopreservation. Sugars act as osmolytes, are good glass formers, and stabilize membranes and proteins. They can induce the production of certain compounds like proteins, other sugars, glycerol, proline, glycine betaine and polyamines, which have colligative as well as non-colligative effects. Sugar treatments are applied to whole *in vitro* plants and excised meristems. Sucrose concentrations range from 0.3 to 1 M and treatment is for 16–4 weeks. In the encapsulation-dehydration protocol sugar concentrations up to 1 M are applied (Malaurie et al. 1998a). For lily (Bouman et al. 2003) and garlic (Kim et al. 2004a), cold acclimation and sugar hardening are applied simultaneously.

11.3 Cryoprotection and Cryopreservation

Since the nature of the cryoprotective treatment is dependent on the cryopreservation method that is applied, cryoprotection and cryopreservation are discussed together. Eleven plant species (or groups) were identified belonging to the monocot herbaceous species for which a cryopreservation protocol for vegetative tissues was developed (Table 11.1). For these, eight different protocols are applied. Some protocols are only reported in a few instances (droplet vitrification, desiccation, slow cooling and preculture) while others are more widely applied; the vitrification protocol was used for 10 species.

11.3.1 Controlled Rate Cooling

Controlled rate cooling is often used for unorganized tissues like callus and cell suspensions (Panis and Lambardi 2005), but is also successful for meristems of temperate crops like rye grass (*Lolium*) (Chang et al. 2000) and *Asparagus* (Suzuki et al. 1997; Suzuki et al. 1998). For *Asparagus*, the cryoprotective mixture contained 0.2 M sorbitol and 8% sucrose while for grasses PGD (Finkle and Ulrich 1979) containing 10% each of polyethylene glycol, glucose and Dimethyl sulfoxide (DMSO) was used. The cooling rates were 0.5°C min^{-1} for *Asparagus* and 0.1°C min^{-1} for grasses (Chap. 5).

11.3.2 Sucrose Preculture

The preculture method relies on a sucrose preculture, after which the material is plunged directly into liquid nitrogen (LN). Higher recovery could be obtained in *Asparagus* if the sucrose preculture was followed by a drying period, but culture for 2 days on 0.7 M sucrose without drying resulted in regrowth (Uragami et al. 1990). Banana meristem clumps belonging to the ABB genomic group required 2 weeks preculture on 0.4 M sucrose before LN plunge (Panis et al. 2002; Panis et al. 1996).

11.3.3 Encapsulation Dehydration

The encapsulation-dehydration method was successfully applied to *Cynodon, Zoysia,* and *Lolium* grasses (Chang et al. 2000; Reed et al. 2006), sugarcane (González-Arnao et al. 1993, 1996; Paulet et al. 1993), yam (Malaurie et al. 1998a; Malaurie et al. 1998b; Mandal et al. 1996), lily (Matsumoto et al. 1995) and banana (Panis 1995) After encapsulation in alginate beads, meristems are exposed to liquid medium with 0.75 M sucrose. In yam, sugar concentrations are increased step-by-step to 0.9 or 1 M, then beads are dehydrated in a container with dry silica gel or in the laminar airflow bench to achieve moisture contents between 19% and 26% and then plunged into LN (Chap. 4).

11.3.4 Desiccation

Two examples illustrate the desiccation protocol. For the orchid, *Vanda pumila*, treatment with a medium that contains 1 mg/l ABA before desiccation is required for acceptable regrowth (Na and Kondo 1996). For axillary buds of *Asparagus* preculture on 0.7 M sucrose for 2 days was crucial

(Uragami et al. 1990). After preculture, buds are dried over silica gel to about 19% moisture content, then plunged into LN.

11.3.5 Encapsulation Vitrification

Encapsulation vitrification combines the encapsulation of meristems in alginate beads with the application of vitrification solutions (Chap. 3). It is described for 3 genera: pineapple (Gamez-Pastrana et al. 2004), yam (Mandal 2000) and lily (Matsumoto et al. 1995).

11.3.6 Vitrification

There are several variations of the vitrification technique (Chap. 3). The standard vitrification protocol takes place in cryotubes while the droplet method is done on aluminum foil strips (Panis et al. 2005). Encapsulation vitrification adds an encapsulation step to the standard protocol. PVS2 (plant vitrification solution 2)(Sakai et al. 1990) is widely used while PVS3 is used for garlic (Keller 2005) and pineapple (Martinez Montero et al. 2002). In *Aginozanthos* and *Conostylis* spp., endangered Australian species, a modified PVS2 was used (Turner et al. 2001a). Duration of vitrification treatment ranges from 15 min at room temperature for orchids (Thinh and Takagi 2000) to 7 h at 0°C for pineapple apices (González-Arnao et al. 1998). Droplet vitrification of banana increased regrowth by 40–50% over standard vitrification (Panis et al. 2005). The droplet method was also useful for taro (Sant, current chapter), garlic (Kim et al. 2004a) and yam (Leunufna and Keller 2005).

11.3.7 DMSO Droplet Freezing

This technique was originally designed for *in-vitro* shoot tips of potato (Schäfer-Menuhr et al. 1996). Shoot tips are treated with a 10% DMSO (dimethyl sulfoxide) solution and frozen ultra rapidly in a droplet of this solution that is placed on a small piece of aluminum foil. Meristems of yam (Leunufna and Keller 2003) and A*sparagus* (Mix-Wagner et al. 2000) respond well with this cryopreservation protocol.

11.4 Rewarming

Rapid warming is essential to prevent devitrification, i.e. the formation of damaging ice crystals from a previously vitrified solution upon rewarming. This is usually achieved by exposing the frozen cryotubes to a 37°C to 45°C water bath for 1–2 min. In encapsulation dehydration the material is transferred from LN to room temperature (Dereuddre et al. 1990; Malaurie et al. 1998b) or transferred to 45°C water for faster warming (Reed et al. 2006). For dried axillary buds of *Asparagus officinalis*, room temperature warming is applied (Uragami et al. 1990). Ultra-rapid warming rates are used with droplet freezing and droplet vitrification (Mix-Wagner et al. 2000), and in droplet vitrification the recovery solution contains 1.2 M sucrose (Panis et al. 2005; Volk et al. 2004).

11.5 Recovery and Viability Assessment

After warming, meristems cryopreserved using the vitrification method are rinsed in a 1.2 M sucrose solution to remove the cryoprotectant and prevent osmotic shock. Sometimes meristems are placed on medium containing 0.3 M sucrose for 1–2 days after which they are placed on normal regrowth medium. In most cases, transfer of shoots to normal solid medium is appropriate. Exceptions are the use of liquid medium for banana meristem clumps (Panis et al. 2002), orchid shoot primordia (Na and Kondo 1996) and softer than normal medium for encapsulated-dehydrated grass meristems (Chang et al. 2000; Reed et al. 2006). In some cases medium additives such as choline chloride (Turner et al. 2001a), or gibberellic acid (Turner et al. 2001b) and cytokinins (Turner et al. 2001b; Volk et al. 2004; Kim et al. 2004a) are added to enhance recovery.

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11.6 Protocols

11.6.1 Dehydration of Asparagus *Axillary Buds*

By A Uragami based on Uragami et al. 1990

Checklist for *Asparagus* stem segment cryopreservation

1. Tools (forceps and scalpels)
2. Sterile Petri dishes, empty and with sterile filter paper
3. Preculture medium: MS (Murashige and Skoog 1962) with 0.7 M sucrose and 0.25% Gelrite, no hormones
4. Petri dishes (6 cm) with 15 g dry silica gel
5. Small (3 cm) sterile Petri dishes with sterile coarse nylon mesh (500 μm)
6. Cryotubes and markers
7. Plant ID numbers and names of accessions
8. Cell well culture plates with growth medium for each accession
9. Growth medium: MS with 3% sucrose, 0.25% Gelrite

Procedure

1. Dissect 5 mm stem segments with one lateral bud in the center and place on preculture medium for 2 days.
2. Wipe segments briefly on sterile filter paper in Petri dish to remove attached medium.
3. Place stem segments separately on nylon mesh in small Petri dish. Place small sterile Petri dish in the center of large Petri dish with silica gel around the edge.
4. Cover the large Petri dish with a lid and dry segments for about 16 h (to 19% moisture content). The moisture content of the segments after drying depends on a number of factors, i.e. volume of segments and silica gel, temperature, dish size and drying period.
5. Determine the fresh weight in an aluminum foil boat; dry in an oven set at 103°C for 24 h; cool in a desiccator and reweigh.
6. Place 15 segments in each vial, place vials on aluminum canes and submerge in LN.
7. Rewarm at room temperature.
8. Place segments on regular growth medium to recover and grow.

11.6.2 Droplet Freezing of Asparagus *Shoot Tips*

Mix-Wagner et al. 2000 with minor improvements by AJ Conner

Checklist of items

1. Tools (forceps and scalpels)
2. Sterile Petri dishes (9 × 1.5 cm)
3. Sterile filter paper (e.g. 7 cm Whatman No. 1)
4. Pipette (e.g. Gilson P20) plus sterile tips
5. Cryogenic vials (e.g. Corning, 1.2 ml)
6. Rack for the cryogenic vials placed inside a polystyrene box
7. LN
8. Strips of sterile aluminum foil cut to fit into the cryogenic vials (e.g. 15 mm × 5 mm)

Prepare in advance

1. In vitro cultures of *Asparagus* (minicrown cultures with proliferating shoots or complete plants) as a source of shoot tips
2. Shoot-inducing medium: liquid MS salts and vitamins (Murashige and Skoog 1962) with 200 mg l^{-1} L-glutamine, 0.1 mg l^{-1} kinetin, and 30 g/L sucrose at pH 5.8
3. Cryoprotectant solution: 10% dimethylsulfoxide (DMSO) in shoot-inducing medium

Procedure

Day 1: Preparation of *Asparagus* shoot tips

1. Remove shoots from cultures and place in the base of a Petri dish.
2. Dissect the 1.5–2 mm tip from each shoot and place in a Petri dish on sterile filter paper soaked in shoot-inducing medium.
3. Close the Petri dish and hold overnight at room temperature.

Day 2: Cryopreservation

1. Transfer shoot tips to a Petri dish with filter paper soaked in cryoprotectant solution. Close the Petri dish and hold at room temperature for 2 h.
2. Label the cryogenic vials, fill with LN and place in the rack in a polystyrene box containing LN.

3. Evenly place five to eight 5 µL droplets of cryoprotectant solution onto each sterile strip of aluminum foil.
4. Place one shoot tip into each droplet on the aluminum foil.
5. Plunge the aluminum foil with adhering droplets containing the shoot tips vertically into the LN-filled cryogenic vials.
6. Transfer the vials to a cryogenic storage unit.
7. To warm: open tube remove foil strip with a fine forceps and plunge into a Petri dish of liquid shoot-inducing medium at room temperature.
8. Close Petri dish and maintain at room temperature for 1 h to allow diffusion of DMSO from the tissue.
9. Transfer the warmed shoot tips to another Petri dish with filter paper soaked in liquid shoot-inducing medium.
10. Close and seal the Petri dish (e.g. with Parafilm) and incubate at 25–26°C under light from cool white fluorescent lamps (50–80 µmol m^2 s^{-1}; 16 h light: 8 h dark).

Recovery of shoot cultures from cryopreserved shoot tips

Once regrowth is evident (shoot tips should elongate to about 5 mm within 7–10 days), transfer the recovered shoots to shoot-inducing medium with 0.7% agar and micropropagate plants as described by Abernethy and Conner (1992).

Results

1. The 'droplet' method was successfully applied to eight cultivars.
2. Successful regrowth ranges from 36–93% and varies with genotype and infection with *Asparagus* virus II.
3. Genotypes with regrowth less than 50% are known to perform poorly in tissue culture. The low frequency of recovery following cryopreservation in these genotypes is likely due to tissue culture rather than cryopreservation.
4. In direct comparison of genotypes with and without *Asparagu*s virus II, viral infection reduces the frequency of regrowth following cryopreservation by 14–27%.

11.6.3 *Controlled Rate Cooling of* Asparagus *Meristems*

By Y Jitsuyama (Jitsuyama et al. 2002; Suzuki et al. 1997; Suzuki et al. 1998)

Checklist of items needed

1. Dissecting tools
2. Cryo straws, LN
3. Preculture medium: Modified MS medium (Murashige and Skoog 1962) (1/2 nitrogen) including 0.4 M glucose and 0.7 % agar (pH 5.7)
4. Cryoprotectant solution: 0.2 M sorbitol and 8% DMSO in liquid MS
5. Recovery medium: modified MS (1/2 nitrogen) with 3% Sucrose, 0.1 mg l^{-1} napthelene acetic acid (NAA), 0.1 mg l^{-1} kinetin and 0.7% agar (pH 5.7)

Procedure

Day 1: Dissect lateral meristems (about 5 mm) and incubate on modified MS medium for 2 days at 26°C (16 h photoperiod).

Day 3

1. Suspend meristems in cryoprotectant solution in cryo straws and hold at room temperature for 2 h.
2. Cool straws at 0.5°C min^{-1} to –40°C (initiate exotherm at –8°C). Plunge in LN.
3. Warm straws in 38°C water for 2 min.
4. Transfer to recovery medium and culture at 26°C (16 h photoperiod).
5. Regrowth is noted 4 weeks after warming (Fig. 11.1). Generally recovery is 70% or more.

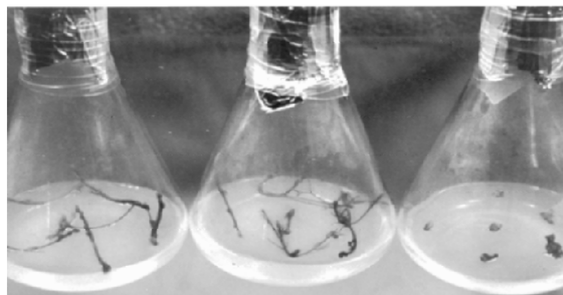

Fig. 11.1 Shoot meristems 2 months after cryopreservation following preculture with: Left: no sugar. Center: 0.5 M glucose. Right: 1.0 M glucose

11.6.4 Cryopreservation of Orchid Shoot Primordia

By K Kondo based on (Na and Kondo 1996).

Procedure

Preparation of tissue-cultured shoot primordia of orchids

1. Tissue-cultured shoot primordia (Fig. 11.2) of orchid accessions are subcultured in B5 (Gamborg et al. 1968) or in MS (Murashige and Skoog 1962) liquid medium with 0.02 $mg{\cdot}l^{-1}$ 6-benzylamino purine (BAP) and 2% sucrose at 21 day intervals.
2. Shoot apices 1–2 mm diameter are individually harvested from 60-day-old protocorms and transferred to Hyponex-peptone, MS or B5 liquid medium (Table 11.2).
3. Culture in 30 × 200 mm test tubes maintained on a rotary culture apparatus at 2 cycles per minute.
4. Environmental conditions are 22°C and a continuous halogen lamp illumination of about 50 $\mu E{\cdot}m^{-2}{\cdot}s^{-1}$.

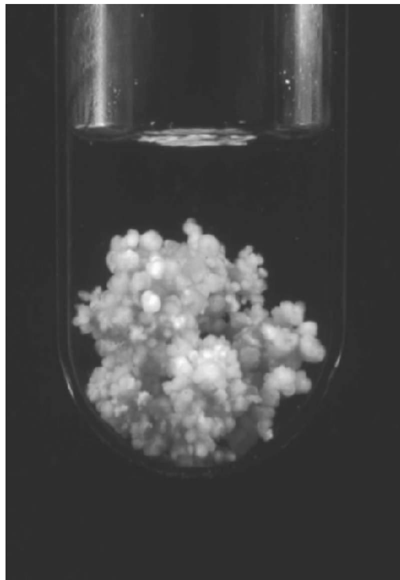

Fig. 11.2 Tissue-cultured shoot primordia of *Dendrobium* cv. 'Yukidaruma'

Cryopreservation

1. ABA preculture: shoot primordia are grown in liquid medium with 1.0 mg l^{-1} ABA for 3 days to induce tolerance to dehydration and cryopreservation.
2. Desiccation: ABA-treated clumps are placed inside a sterilized, polycarbonate culture box that has two ventilation holes on the lid designed and prepared especially for the desiccation experiment. The two holes are covered with Milliseal membrane filters (pore size of 0.5µm; Millipore), allowing ventilation under aseptic conditions. This box is placed in a desiccator with dry silica gel. The relative humidity in the desiccator is monitored with a hygrometer and kept at 45% by occasionally adding new dry silica gel.
3. Determination of water content: Forty blocks of shoot primordia are taken daily from the desiccator, weighed, and oven-dried at 70°C for 48 h to determine dry weights. The maximum recovery was obtained at the relative water content of 24%.
4. Ten dried clumps of shoot primordia are placed per 1.8 ml cryotube and cryotubes are cooled rapidly by immersion into LN.

Recovery

1. Rapidly warm cryotubes in a 40°C water bath for 1.5 min.
2. Shoot primordia are placed on B5 medium with 0.4% Gelrite for rehydration and regaining their initial size.
3. Rehydrated cultures are transferred to ½ strength liquid growth medium.
4. Rooted plants develop within two months.

Table 11.2 Orchid species or cultivars for which shoot primordia were induced and subsequently cryopreserved

Species or Cultivar	Medium to produce shoot primordia
Cattleya loddigesii var. harrisoniana	B5 liquid + 2% sucrose
C. walkeriana	B5 liquid + 0.02 mgl^{-1} NAA or 0.20 mg^{-1} BAP + 2% sucrose
Dendrobium cv. 'Yukidaruma'	3 gl^{-1}Hyponex + 2 gl^{-1} peptone liquid + 3% sucrose
Vanda pumila	B5 liquid + 0.02 mgl^{-1} BAP + 2% sucrose

11.6.5 Vitrification of Cymbidium *Apical Meristems*

By NT Thinh based on (Thinh and Takagi 2000)

Checklist for cryopreservation of apical meristems by vitrification

Day 1: Items needed to dissect apical meristems and their preculture

1. Protocorm-derived mericloned plants, ideally at three leaf stage
2. Growth medium: Morel (Morel 1960) medium with 0.2% gellan gum and 0.1 M sucrose
3. Dissection tools (forceps and scalpels)
4. Stereo binocular microscope
5. Pretreatment medium: Semi-solid Wimber medium (Wimber 1963) with 0.3 M sucrose and 0.2% gellan gum in small (3.5cm) Petri dishes
6. Parafilm strips

Day 2: Vitrification

1. Tools and small sterile Petri dishes
2. Small sterile pieces (1.5 × 1.5 cm) of tissue paper
3. Sterile filter paper for draining solutions
4. Sterile cryotubes and markers
5. Parafilm strips
6. Timers
7. Sterile plastic pipettes for drawing off solutions
8. Rinsing solution: Liquid Wimber medium with 1.2 M sucrose
9. Recovery medium: Wimber medium with 0.3 M sucrose and 0.2% gellan gum in small Petri dishes and with a filter paper on the surface
10. Osmoprotection (loading) solution of 2 M glycerol plus 0.4 M sucrose prepared in MS medium (Murashige and Skoog 1962)
11. PVS2: 30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide (DMSO), 15% (w/v) ethylene glycol and 0.4 M sucrose prepared in MS medium (Sakai et al. 1990)
12. LN

The procedure

This is a 2 day procedure once the plants are ready.

1. Dissect meristems and transfer onto preculture medium. Seal the dishes with Parafilm strips and keep them in darkness or dim light overnight. The ideal apical meristems to be cryopreserved are those with two leaf primordia and where the apical domes can still be partly observed from one side of the 2nd leaf.
2. Wet a sterile tissue paper with some drops of osmoprotection solution and transfer the precultured meristems onto it.
3. Wrap the meristems in the wet tissue paper to form a small package (±3×6 mm) and submerge in loading solution for 20 min.
4. Blot the package on sterile filter paper to remove excessive loading solution then transfer it into PVS2 and hold for another 15–20 min.
5. Place the PVS2-treated package into a cryotube, cover it with fresh PVS2 and close the cryotube.
6. Plunge the cryotube under LN.
7. To warm: Shake the cryovial in 40°C water for 1.5 min, then quickly remove the package and blot for a few sec on a sterile filter paper.
8. Transfer the blotted package into rinsing medium for 20 min.
9. Unwrap the package in a sterile Petri dish to release the LN-treated meristems and transfer them onto a filter paper on recovery medium.
10. Incubate treated meristems overnight in darkness.
11. Transfer the meristems onto fresh growth medium for further formation of plantlets/protocorm clumps.
12. The protocol was successfully applied to 4 commercial clones (Table 11.3).

Table 11.3 Commercial mericlones and wild species of *Cymbidium* successfully cryopreserved by vitrification. Part of this work was generously funded by Professor A. Sakai via the cooperation with the 'Osaka Fund'

Clone/species	PVS2 dehydration time (min.)	Recovery (%)
C. insign var. "Xuan 88"	15	76
C. insign var "Trang Luat"	20	86
C. Suva Royal Velvet	20	93
C. Nandy Green Mist	15	81

11.6.6 Cryopreservation of Banana Meristem Clumps

(Panis et al. 2002; Panis et al. 1996) with minor improvements by B Panis

Checklist of items

1. Tools (forceps and scalpels).
2. LN source
3. Cryotanks, safety equipment: gloves and goggles
4. Dewar vessels
5. Sterile 2 ml cryotubes
6. Warm water bath
7. Thermometer

Plant tissue culture media

1. *p5 medium*: MS (Murashige and Skoog 1962) salts and vitamins, 10 µM benzyladenine (BA), 1 µM indole acetic acid (IAA), 10 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 30 g l^{-1} sucrose (pH 5.8).
2. *p4 medium*: MS with 100 µM BA, 1 µM IAA, 10 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 30 g l^{-1} sucrose (pH 5.8).
3. *Preculture medium*: MS with 10 µM BA, 1 µM IAA, 10 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 136.8 g l^{-1} (0.4 M) sucrose (pH 5.8).
4. *Regrowth medium*: MS 100 µM BA, 1 µM IAA, 1 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 30 g l^{-1} sucrose (pH 5.8).

Cryoprotective solutions

1. *Loading solution*: contains 2 M (14.73 ml/100 ml) glycerol and 0.4 M (13.68 g/100 ml) sucrose and is filter sterilized. This solution can be stored at –20°C and must always be stirred before use.
2. *PVS2 (Plant Vitrification Solution 2):* contains 30% (24 ml/100 ml) glycerol, 15% (13.5 ml/100 ml) ethylene glycol and 15% (13.6 ml/100 ml) DMSO (dimethylsulphoxide) dissolved in 0.4 M (13.68 mg/100 ml) sucrose solution (so not dissolved in water). This solution is filter sterilized.
3. *Reloading solution:* MS medium with 1.2 M (41.08 g/100 ml) sucrose. This solution is filter sterilized and can be stored at –20°C.

Procedure

Production of 'cauliflower-like' meristem clumps of banana (Strosse et al. 2006; Strosse et al. 2003)

1. Cutback roots and leaves (about 0.5 cm above the level of the apical meristem) and place explants on p5 medium at 27°C in darkness for 1 month.
2. Cutback roots (if present), and place explants on p4 to reduce outgrowth of shoots, and incubate at 27°C in darkness.
3. Subculture monthly, selecting only groups of closely packed meristems (surface ~0.5 cm^2).
4. Repeat step 3 until 'cauliflower-like' meristem cultures are obtained (Fig. 11.3). This can take 4–12 months.

Fig. 11.3 Proliferating meristem clumps of the cultivar 'Bluggoe' (ABB group) (From Helliot et al. 2002)

Preculture

1. Excise white meristematic clumps (4 mm diameter) containing at least 4 apical domes from 'cauliflower-like' cultures, 4-6 weeks after the last subculture. Place on preculture medium.
2. Preculture for 2 weeks at 27°C in darkness.

Cryopreservation

1. Excise 2–3 mm diameter clumps from the precultured clumps containing at least five meristematic domes, remove brown tissues and retain only white-yellowish tissues.
2. Transfer 7 to 10 clumps to each 2 ml cryovial.
3. Seal the cryovials with a layer of Teflon tape and plunge them directly into LN.

Warming and recovery

1. Warm cryovials in a beaker of 40°C sterile water for 1.5 min.
2. Transfer clumps to 30 ml liquid regrowth medium in 100 ml Erlenmeyer flasks and place on a rotary shaker at 70 rpm under standard culture conditions.
3. After 1 week of culture in the dark transfer flasks to continuous light (50 μE m^{-2} s^{-1}) and keep at 27°C, RH >70%.
4. Four weeks after warming determine regrowth using an inverse binocular microscope.
5. Transfer recovering clumps to 25 × 150 ml test tubes containing 25 ml regrowth medium for further development of whole plants.
6. As soon rooted plants reach the top of the test tube they can be planted in the soil.

Results

The preculture protocol was applied to 36 banana cultivars belonging to eight genomic groups (Panis et al. 2002). The results were very genotype dependent. Most ABB cultivars responded favorably with regrowth up to 66%, while East African highland bananas showed almost no regrowth.

Possible problems

Quality of 'cauliflower-like' meristem clumps may be too poor for use in cryopreservation (meristematic tissue versus corm tissue is too low and/or the explant shows too much blackening). This can be due to the cultivar belonging to a 'difficult' genomic group (for example East African highland bananas and many plantains). *Solution:* Use alternative (and stronger) cytokinins like thidiazuron (TDZ) to increase proliferation or use a cryopreservation protocol (droplet vitrification) that does not rely on proliferating meristem clumps.

11.6.7 Droplet Vitrification of Banana Apical Meristems

(Panis et al. 2005; Agrawal et al. 2004; Panis and Thinh 2001; Thinh et al. 1999) with minor improvements by B Panis.

Checklist of items required

1. LN source, cryotanks, Dewar vessels
2. Timers, tools (forceps and scalpels)
3. Styrofoam boxes with lids
4. Crushed ice and ice packs
5. Sterile 2 ml cryovials, cryovial holder (12 × 12)
6. Sterile Pasteur pipettes
7. Binocular stereoscopic microscope (with good light source)
8. Safety equipment: gloves and goggles

Plant tissue culture media

1. p5 medium: MS (Murashige and Skoog) macro elements and iron, MS microelements, MS vitamins, 10 µM benzyladenine (BA), 1 µM indole acetic acid (IAA), 10 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 30 g l^{-1} sucrose (pH 5.8).
2. Rooting medium*:* MS with 0.5 g l^{-1} activated charcoal, 10 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 30 g l^{-1} sucrose (pH 5.8).
3. Recovery medium: MS with 10 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 102.7 g l^{-1} (0.3M) sucrose (pH 5.8).
4. Regrowth medium: MS with 100 µM BA, 1 µM IAA, 1 mg l^{-1} ascorbic acid, 3 g l^{-1} Gelrite, and 30 g l^{-1} sucrose (pH 5.8).

Cryoprotective solutions

1. Osmoprotective loading solution: 2 M (14.73 ml/100 ml) glycerol and 0.4 M (13.69 g/100 ml) sucrose in MS and filter sterilized.
2. PVS2: 30% (24 ml/100 ml) glycerol, 15% (13.5 ml/100 ml) ethylene glycol and 15% (13.6 ml/100 ml) DMSO and 0.4 M (13.68 mg/100 ml) sucrose in MS medium. Filter sterilize.
3. Rinsing solution: 1.2 M (41.08 g/100 ml) sucrose in MS. Filter sterilize, can be stored at –20°C, stir before use.

Procedure

Production of 'strong' *in vitro* plants and excision of apical meristems

1. Place *in vitro* shoots on p5 medium for multiplication under standard culture conditions of 27°C with continuous light.
2. Place shoots on rooting medium (1 explant / 25 × 150 ml test tubes containing 25 ml medium).
3. After 1 month, carefully remove leaves, one by one, until the glassy apical dome of the meristem can be clearly observed. Reduce the corm tissue to a base diameter of 0.5–1 mm. The apical domes may be 2/3 covered by the youngest leave primordia (Fig. 11.4). Tips that are slightly damaged or are not in the correct stage (the meristem is too much or too little covered by leaf primordia) are excluded from cryopreservation.

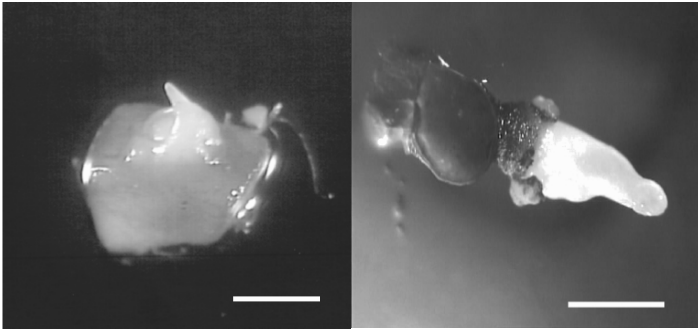

Fig. 11.4 Left: Freshly isolated apical meristem from the cv. Williams (bar = 500 µm). Right: Regrowth of apical meristem from the cv. Williams, 1 month after cryopreservation (bar = 1 mm)

Cryopreservation

1. After excision, transfer meristems directly to 5 ml of osmoprotective loading solution in a 20 ml sterile vessel at room temperature (at least 20 min exposure is required).
2. Replace the loading solution with 5 ml of ice-cooled PVS2 and hold on ice for 30–50 min.
3. Five minutes before the end of the treatment, transfer 8 meristems to a droplet of PVS2 (of about 15 µl) on a strip of aluminum foil (5 × 20 mm). Place the foil in a plastic Petri dish placed on ice.
4. After PVS2 treatment the aluminum strip is plunged into LN with a fine forceps. For permanent cryostorage, the foil is quickly transferred to a 2 ml cryotube filled with LN.

Warming, unloading and regrowth

1. Open tubes, remove foil strip with a fine forceps and place in 10 ml room temperature rinsing solution in a small Petri dish for 15 min.
2. Place meristems on two filter papers on the recovery medium.
3. After 2 days transfer the meristems onto regrowth medium without filter papers. The first week of culture is in the dark.
4. Regrowth is determined at 4–6 wks (Fig. 11.5).

Possible problems

Low regrowth may be due to low quality meristems (tips that are slightly damaged or are not in the correct stage or too much or too little covered by leaf primordia). Solution: Improve the quality of the donor plants (more light, fewer plantlets in containers), improve excision skills.

Results

This protocol was applied to 256 Banana accessions from genetically distinct groups (Fig. 11.5). This includes wild accessions such as *Musa schizocarpa, Musa laterita, Musa balbisiana, Musa acuminata* as well as edible bananas belonging to the AA, AAA, AAAA, AAAB, AAA East African highland banana, AAB, AAB plantain, AB and ABB groups. The related genus, *Ensete*, responds favorably to the same protocol. Regrowth is relatively independent of the group to which the cultivar belongs.

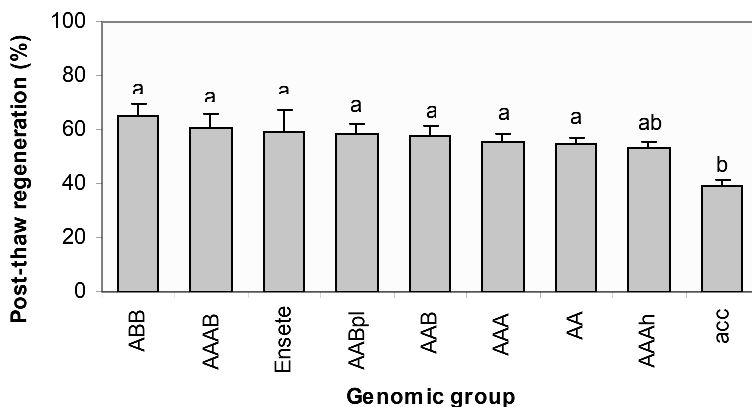

Fig. 11.5 Mean regrowth of 56 banana accessions grouped by genomic constitution (from Panis et al. 2005). Error bars represent the standard errors of regenerable regrowth. Groups marked by the same letter are not significantly different according to Duncan's test after arcsine transformation ($P < 0.05$)

11.6.8 Encapsulation Dehydration of Sugarcane Apices

Dereuddre et al. (1990) adapted by MT González-Arnao and F Engelmann (González-Arnao et al. 1996; González-Arnao et al. 1999)

Checklist for Alginate Bead Cryopreservation
Day 1: Items needed to dissect apices

1. Tools (forceps and scalpels)
2. MS growth medium in Petri dishes to hold apices overnight

Day 2: Items needed to make beads and for sucrose preculture

1. Alginate solution
2. Calcium chloride (saturated) solution (100 mM)
3. Sterile 50 ml beaker for alginate solution
4. Sterile 250 ml beakers for calcium chloride solution
5. Sterile cut pipette tips to make beads (100-1000µl tips cut to a 4 mm diameter so the tissues will pass through).
6. Sterile strainers
7. Pretreatment medium: 0.75 M sucrose in MS medium

Day 3: Items needed to dry beads and to cryopreserve

1. Sterile strainer
2. Sterile 250 ml beaker
3. Sterile Petri dishes for drying beads (1 for each 15 beads)
4. Tools (forceps and scalpels) and sterile Petri dishes
5. Cryovials (1.8 or 2 ml) and markers
6. Petri dishes with recovery medium
7. Petri dishes with sterile filter paper for draining beads
8. Dewar flask and LN

Items to be prepared in advance

1. *In vitro* plants subcultured 15 days before dissection of apices with a 16 h light/8 h dark photoperiod at 25°C
2. Growth medium: MS (Murashige and Skoog 1962) with 2 $mg \cdot l^{-1}$ 6 benzylamino purine, 0.3 $mg \cdot l^{-1}$ naptheleacetic acid, 30 $g \cdot l^{-1}$ sucrose and 10 $g \cdot l^{-1}$ agar
3. Growth medium in Petri dishes for dissection and regrowth
4. Alginate solution: 3% low viscosity sodium alginate in MS without calcium and with 34 $g \cdot l^{-1}$ sucrose (in a flask). It is very difficult to dissolve alginate, so add alginate slowly under agitation and gentle warming.

5. Calcium chloride solution: MS with 100 mM calcium chloride
6. Pretreatment medium: liquid MS with 0.75 M sucrose (100 ml in 250 ml flasks, one for each plant type)

The procedure

This is a 3-day procedure after dissection of meristems.

1. Dissect apices on growth medium plates until enough are collected, and leave them overnight for recovering from the dissection stress.
2. Suspend apices in alginate solution in a small sterile beaker.
3. Pick up a single apex and some alginate with a pipette, drop apices into a 250 ml beaker of saturated calcium chloride solution to produce the beads. Avoid air bubbles in the alginate.
4. Leave the beads in the solution for 20 min to firm up. Pour through strainer to remove solution.
5. Transfer to 125 ml flasks of MS with 0.75 M sucrose on a shaker for 18–20 h.
6. Drain through strainer beads and place on filter paper in Petri dish to absorb moisture.
7. Place surface-dried beads in open sterile Petri dishes and expose them to the air current of the laminar flow cabinet for ~ 4–5 h (to 20–25% moisture content, fresh weight basis). Spread beads evenly over the surface of the dish, so they do not touch. You may perform desiccation in air-tight containers with silica gel instead. Desiccation with silica gel will result in more reproducible conditions.
8. Place ten beads per cryovial. Place vial on aluminum cane or drawer of Dewar and immerse in LN.
9. To warm, place in open Petri dishes in the laminar flow for 2 min.
10. Place 10 beads on semi-solid medium per Petri dish. Place in the dark for 1 week, then transfer to the usual controlled illumination conditions.
11. Recovery varied from 24% to 91% (average of 62%) depending on the cultivar.

Possible problems

Some plants are sensitive to specific steps in the protocol. Controls are needed for (1) dissection, (2) encapsulated apices, (3) encapsulated apices after sucrose treatment, (4) dried beads at 20–25% moisture content.

11.6.9 PVS3 Vitrification of In Vitro *Garlic Meristems*

By ERJ Keller, based on (Niwata 1995; Makowska et al. 1999; Keller 2002; Keller 2005)

Checklist of items required

Day 1: Items needed

1. Tools (forceps and scalpels)
2. Sterile glass Petri dishes, filter paper
3. Dissection microscope
4. Cold acclimated *in-vitro* plantlets (alternatively: bulbils or cloves)
5. Preculture medium in Petri dishes, Parafilm

Day 2: Items needed

1. Sterile marked cryotubes (accession number, date)
2. Sterile tweezers, pipette, sterile pipette tips
3. LN in Dewar vessel
4. Sterile filter paper
5. Gyratory shaker for glass vessels, shaker (Vortex)
6. Tweezers or forceps for cryo tubes (non-sterile)
7. Parafilm
8. Osmoprotectant solution, PVS3

Day 3: Items needed

1. Sterile tweezers
2. Recovery medium
3. Water bath at 40°C
4. Rinsing solution
5. Parafilm

Prepare in advance

1. Transfer well-developed plantlets at the end of a subculture (4 weeks at 25°C and 16-h light) to cold acclimation.
2. Cold acclimate plantlets for 8 weeks at 25°C with 16-h light and –1°C 8 h in the dark.
3. Growth medium: MS (Murashige and Skoog 1962) with 0.1 mg/l NAA, 0.5 mg/l 2-isopenenyl adenine (2-iP), 1% agar, 3% sucrose, pH 5.8
4. Preculture medium: growth medium with 10% sucrose
5. Osmoprotectant solution: 0.4 M sucrose and 2 M glycerol in MS at pH 5.8

6. PVS3: 50% (w/v) sucrose, 50% (w/v) glycerol in MS at pH 5.8, filter sterilize
7. Rinsing solution: MS with 1.2 M sucrose
8. Recovery medium: MS with 3% sucrose and 1% agar in Petri dishes

Procedure
Day 1: Preparation of plant material
A: In-vitro plants as source

1. Isolate the 1 cm base (remove the leaves and the roots).
2. Remove the leaf bases until a final diameter is 1 mm (1-2 long leaf bases), cut the explants to about 5 mm and remove the root initials
3. Place 30 explants upright (bases downwards) in Petri dishes of preculture medium and seal with Parafilm.
4. Cultivate at 25°C and 16 h illumination until next day.

B: Bulbils or cloves as sources

1. Remove outer dry scales from bulbils or cloves.
2. Sterilize in 70% ethanol by dipping and shaking (30 s.)
3. Disinfection in sodium hypochlorite solution (3% active chlorine) with two drops of Tween 20, on a shaker for 20 min.
4. Rinse 3–4 times with sterile water.
5. Cut transversally and longitudinally to obtain a 5 mm cubic block containing the meristem.
6. Remove storage scales and leaf sheaths until the final diameter is 1 mm (1–2 long leaf bases remaining), cut to about 5 mm final length; remove the root initials without damaging the meristem.
7. Place 30 explants upright in preculture medium and seal with Parafilm. Cultivate at 25°C, 16 h illumination until next day.

Day 2: Cryopreservation

1. Transfer ten explants to each cryotube.
2. Add 1 ml osmoprotectant solution, close the tube; vortex; hold for 20 min.
3. Remove solution, add 1 ml PVS 3; vortex; hold for 2 h.
4. Remove PVS 3, add 0.5 ml PVS 3 solution; vortex; plunge into Dewar filled with LN and hold for at least 1h.
5. Transfer tubes to the cryo tank for storage.
6. To warm, plunge into 40°C water bath for 2–2.5 min.
7. Open tubes and remove PVS 3 solution.
8. Add 1 ml rinsing solution, vortex and hold for 10 min.

9. Remove solution, remove from tube and blot dry on filter paper.
10. Place explants upright on preculture medium and hold at 25°C in the dark until the next day.

Further recovery

1. Transfer explants to Petri dishes of recovery medium.
2. Cultivate at 25°C in the dark for 7 days.
3. Move Petri dishes into light at 25°C, 16 h illumination for 7 days.
4. Identify contamination and discard those explants.
5. Transfer all non-infected explants into tubes of growth medium.
6. Cultivate at 25°C and 16 h illumination for 6–10 weeks.
7. Final record: count the plantlets (regrowth). Discard dead and abnormal explants.

Notes

1. PVS 3 is highly viscous and needs long stirring and warming to dissolve.
2. The meristem can be cut off if too much of the root-forming region is removed; study the morphology of the explants by cutting one explant longitudinally.
3. If doing many explants at one time the removal of the outer dry scale and sterilization should be done the day before and the bulbils/cloves obtained kept overnight in closed vessels in the refrigerator.
4. Transfer all non-infected explants to give any slow growing plants a chance to develop. Discard hyperhydric (watersoaked) explants.

Results

IPK storage standards: an accession is considered safely stored when it is represented by two times 100 explants AND two times 30 controls have a mean regrowth higher than 30%. If the regrowth is between 10% and 30% in the first two repetitions, another set of 100 explants plus 30 explants control is included. If the mean of regrowth of these three sets is lower than 10%, experiments will be initiated to increase regrowth. Garlic has high labor requirements so the standard was modified so that sets consisting of 30 explants plus ten explants of control are done until the final storage amount is reached. IPK has stored 11 accessions from bulbils (mean regrowth 34%) and 5 from virus-free *in vitro* plants (mean regrowth 44%). The best regrowth of a genotype was 68%.

11.6.10 Vitrification of Pineapple (Ananas comosus) *Meristems*

Based on Sakai et al. 1990 with modification by MT Gonzalez-Arnao (González-Arnao et al. 1998) and ME Martinez-Montero (Martinez Montero et al. 2002)

Checklist

Day 1: Items needed to dissect and preculture apices

1. Tools (forceps and scalpels)
2. Sterile Petri dishes
3. Preculture medium

Day 3: Items needed for the osmoprotection (loading) treatment and vitrification

1. Sterile Petri dishes, empty, and with filter paper for drying apices
2. Osmoprotection (loading) solutions in 125 ml flasks
3. Sterile pipette tips
4. PVS2 or PVS3, filter sterilized
5. Cryovials (1.8 or 2 ml) and markers
6. Dewar flask and LN
7. Rinsing solution

Items to prepare in advance

1. *In vitro* mother plants subcultured 15 days before dissection of apices; maintained on MS (Murashige and Skoog 1962) medium with 10 $g \cdot l^{-1}$ agar; a 16 h light/8 h dark photoperiod at 25°C
2. Preculture medium: MS with 0.3 M sucrose, 10 $g \cdot l^{-1}$ agar
3. Osmoprotection solution (for PVS2 protocol): MS with 0.75 M sucrose and 1 M glycerol (75 ml in 125 ml flasks), filter sterilize
4. PVS2: MS medium with 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% dimethyl sulfoxide (DMSO), 0.4 M sucrose; filter sterilize
5. Osmoprotection solution (for PVS3 protocol): MS with 0.4 M sucrose, 2 M glycerol (75 ml in 125 ml flasks), filter sterilize
6. PVS3: MS medium with 50% (w/v) glycerol + 50% (w/v) sucrose, filter sterilize
7. Rinsing solution: MS with 1.2 M sucrose
8. Regrowth medium: MS with 2 $mg \cdot l^{-1}$ 6 benzylaminopurine, 0.3 $mg \cdot l^{-1}$ NAA, 30 $g \cdot l^{-1}$ sucrose and 10 $g \cdot l^{-1}$ agar

The procedure

This is a 2-day procedure once the dissection of apices is accomplished.

1. Dissect meristems and place on preculture medium for 2 days.
2. Move precultured apices to two filter papers in a Petri dish containing the osmoprotective solution (as designated for PVS2 or PVS3) for 25 min at room temperature. Filter papers should be very wet but the apices should not float.
3. Remove solution, place ten apices in each cryovial with ice-cold cryoprotectant (PVS2 or PVS3) and hold for 7 h at 0°C
4. Replace the cryoprotectant solution once during the hold time.
5. Place vials on aluminum cane and immerse in LN.
6. Warm in 40°C water for 2 min, then remove the solution and rinse twice with rinsing solution at room temperature (for about 30 min total rinse time).
7. Place on two filter papers in a Petri dish to absorb excess moisture, and then transfer to recovery medium.
8. Place dishes in the dark for 1 week then transfer to standard conditions.
9. Results for several genotypes are shown in Table 11.4.

Table 11.4 Effect of the PVS3 vitrification protocol on apices of 7 pineapple accessions and one *Bromelia* sp. from the Cuban Pineapple Germplasm Bank before (–LN) and after cryopreservation (+LN). M. Martínez-Montero 2002

Accessions	Recovery	(% ± S.E.)[a]
	–LN	+ LN
Cayenne of Puerto Rico	80.2 ± 9.5	65.5 ± 11.4
Perolera	49.9 ± 5.4	33.8 ± 7.9
Smooth Cayenne of Serrana	50.3 ± 11.1	25.3 ± 6.3
Cabezona	61.5 ± 9.8	27.9 ± 8.1
Piña Blanca	57.9 ± 3.2	24.7 ± 9.0
P3R5	53.1 ± 5.8	20.0 ± 5.3
Red Spanish	45.5 ± 4.5	12.1 ± 5.9
Bromelia sp.	33.1 ± 4.0	6.3 ± 3.5
Average	53.9 ± 6.7	27.0 ± 7.2

[a]Six shoot tips per replicate, four replicates per treatment. Each experiment was repeated three times

11.6.11 Droplet Vitrification of Taro (Colocasia esculenta)

By R Sant based on (Panis et al. 2005)

Checklist

1. Aluminum foil to wrap plates
2. Sterile aluminum foil strips, $2 \times 0.8\ cm^2$
3. Food wrapping polyethylene sheet (Glad wrap) to seal plates
4. Sterile McCartney bottles with lids (or other small container)
5. Timers
6. Petri dishes (9 cm and 15 cm)
7. Sterile filter paper (cut to fit 9 cm Petri dish)
8. Cryo vials (1.8 or 2.0 ml)
9. Cryo vial holder (12 x 12)
10. Two styrofoam boxes with lids
11. Crushed ice, frozen cooling element (cool pack)
12. LN
13. Sterile Pasteur pipettes
14. Tissue culture tools (small soft forceps; sharp-pointed blades)
15. Binocular dissection microscope and light

Prepare in advance

1. Medium for taro: MS (Murashige and Skoog 1962) with 3% sucrose and 7.5 $g \cdot l^{-1}$ agar Type A (Sigma) at pH 5.8
2. Preculture medium: MS with 0.3 M sucrose (in 9 cm Petri dish)
3. Recovery medium: MS with 0.1 M sucrose (in 9 cm Petri dish)
4. Cryoprotective solutions
   a. Osmoprotective loading solution: 2 M glycerol + 0.4 M sucrose in liquid MS medium at pH 5.8
   b. PVS2: 30% (3.26 M) (w/v) glycerol, 15% (2.42 M) (w/v) ethylene glycol, 15% (1.9 M) (w/v) DMSO in 0.4 M sucrose in liquid MS at pH 5.8
   c. Rinsing solution: liquid MS with 1.2 M sucrose at pH 5.8

Procedure

Production of 'strong' *in vitro* plants and excision of apical meristems

1. *In vitro* stock plants of taro are cultured on MS medium with 30 $g\ l^{-1}$ sucrose but no growth regulators in 100 ml glass jars for three months (produces plants with large and robust shoot tips).

2. Excise 2 cm explants, including some basal corm tissue and an adjoining length of leaf cluster bases.
3. Culture on MS medium with 90 $g \cdot l^{-1}$ sucrose for 4–7 weeks.
4. To dissect, cut the corm end so that the excised explant stump can stand on its base. Slice the corm into a square cube (this ensures the explant can be held steadily for dissection).
5. Start removing the leaves layer by layer. Cut longitudinally on the thick side of the outer most leaf, followed by a very careful horizontal cut at the base so that the inner leaves are not damaged (Fig. 11.6). Remove the cut leaf.
6. Repeat with the inner leaf layers until the meristematic dome with two leaf primordia remains (0.8 mm–1.0 mm).
7. Make four straight cuts around the shoot tip followed by a horizontal cut at the bottom so that the base of the shoot tip comes off as a $1 \times 1 \times 1$ mm^3 (Fig. 11.7)
8. Place carefully on the preculture plate (MS + 0.3 M sucrose) until all are excised.

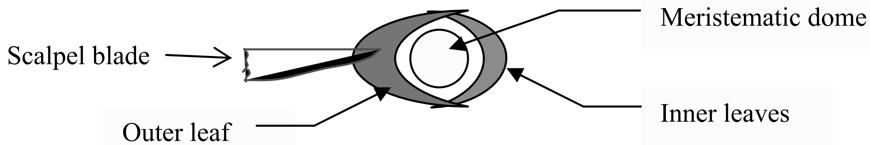

Fig. 11.6 Schematic of a taro shoot tip dissection (not drawn to scale)

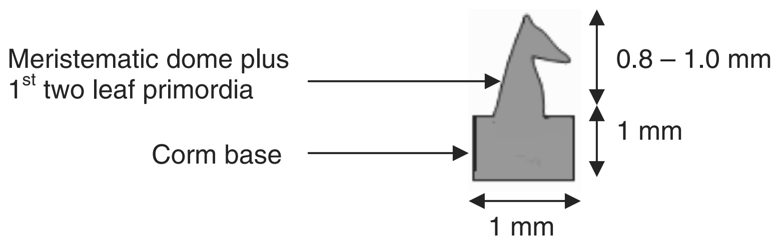

Fig. 11.7 Schematic of a taro shoot tip

Cryopreservation

1. Immerse shoot tips (5–10) in 5 ml of osmoprotective loading solution for 20 min at 25°C.
2. Remove solution with a pipette and replace with 5 ml chilled PVS2 on ice for 20–40 min.
3. Place aluminum strips on a Petri dish on ice 5 min before use.
4. Place drops of PVS2 on a strip of aluminum foil (5×20 mm^2). Two minutes before the end of the PVS2 exposure time, move shoot tips to the drops, one tip per drop.
5. Lift the strip and immerse in LN. After bubbling stops, transfer the strip with droplets into a cryo tube in a holder placed in a Styrofoam box of LN.

Warming, unloading and regrowth

1. To warm, remove cap and remove foils strip with fine forceps; immerse the foil strip in 15 ml of 25°C rinsing solution for 15 min.
2. Looking under the binocular microscope, lift shoot tip with scalpel blade tip and place carefully on two layers of sterile filter paper on solid 0.3 M sucrose MS plate. Leave overnight in the dark at 25°C.
3. The following day move ST to plates of solid 0.1 M sucrose MS without the filter paper. Maintain cultures in the dark for 3 days and then transfer to dim light [3.5 μMol $m^{-2}s^{-1}$] for 2 wks before exposure to normal culture conditions.
4. Regrowth is recorded after 4–6 wks when shoot tips have developed into plantlets with leaves and roots.

Results

This protocol was successful for 18 accessions that are stored under *in vitro* conditions. Regrowth varied from 73% to 100 %.

11.6.12 Encapsulation Vitrification of Lily Shoot Tips

By T Matsumoto based on (Matsumoto and Sakai 1995)

Checklist

Day 1: Items needed to dissect shoot tips and preculture

1. Tools (forceps and scalpels).
2. Cold acclimated bulb-scale segments with adventitious buds

Day 2: Production of beads, vitrification and recovery

1. Sterile pipettes for removing solutions
2. Sterile 250 ml beakers for the calcium chloride solution
3. Small sterile Petri dishes or beakers for the alginate solution
4. Sterile syringes without needle (1 ml) for producing beads
5. Cryotubes (about 2 ml)
6. Dry filter papers, tools and sterile Petri dishes

Prepare in advance

1. To obtain adventitious buds, bulb-scale segments (1 × 1 cm) are placed on MS (Murashige and Skoog 1962) medium with 0.8% agar under a 12 h photoperiod at 25°C for 16 h and cultured for about 40 days.
2. Cold acclimate adventitious bud segments at 0°C for 21 days.
3. Preculture medium: MS medium with 0.3 M sucrose, no hormones, in a Petri dish
4. Calcium chloride solution (modified): (100 mM) in MS with 2 M glycerol and 0.4 M sucrose
5. Alginate solution (modified): 2% sodium alginate in MS medium without calcium with 2 M glycerol and 0.4 M sucrose
6. PVS2: 30 % glycerol, 15 % ethylene glycol and 15 % DMSO and 0.4 M sucrose in liquid MS, cooled to 0°C
7. Rinsing medium: 1.2 M sucrose MS liquid medium
8. Recovery medium: hormone-free MS with 3% sucrose and 0.8% agar

Procedure

1. Select adventitious buds with 2–3 leaves for shoot tip excision.
2. Dissect meristems; preculture on 0.3 M sucrose medium for 16 h.
3. Transfer meristems to the alginate solution, using a sterile syringe.

4. With the syringe, pick up one meristem at a time with some alginate and drip into the calcium chloride solution to produce a bead of about 3 mm in diameter. Avoid air bubbles in the alginate.
5. Leave the beads in the solution for 30 min to firm up.
6. Place beads in the PVS2 in 125 ml flasks on a shaker for 100 min at 0°C (beaker is placed in a larger vessel filled with crushed ice). Drain PVS2 and add fresh PVS2 after 30 min.
7. Transfer 10 beads to a cryotube and add 0.5 ml of fresh PVS2 1–2 min before of the end of PVS2 treatment.
8. Immerse cryotubes into LN and hold for 1 hr.
9. Warm cryotubes in 40°C water for 1 min while stirring.
10. Immediately drain PVS2 and add rinsing solution. Hold at 25°C for 20 min.
11. Transfer meristems onto recovery medium.

Possible problems

1. Beads containing meristems are easily injured during draining and adding new solutions using a pasture pipette so pipette carefully.
2. The time of exposure to PVS2 is very important as it can be very harmful for the plant material, especially at 25°C.

Cold acclimation of plants resulted in an increase in shoot formation of 57–118 % (Table 11.5).

Table 11.5 Shoot formation of cold-acclimated or non-acclimated meristems of lily cooled to –196°C through encapsulation vitrification. (From Matsumoto, and Sakai 1995)

Species or cultivars	Shoot formation (%)	
	Cold acclimated	Non acclimated
Japanese pink lily (*L. japonicum* Thunb.)	93.0	56.0
Golden-banded lily (*L. auratum* var. platyphyllum Baker)	82.5	43.3
Star gazer (*L.* hybrid cv. Star Gazer)	70.0	33.3
Summer dress (*L. parkmanii* cv. Summer dress)	87.1	40.0
Snowy lily (*L. speciosum* Thunb.)	68.1	40.0
Kraetzeri (*L. speciosum* cv. Kraetzeri)	57.5	36.7

11.6.13 *Encapsulation Dehydration of Grasses* (Cynodon, Zoysia, Lolium)

By BM Reed and Y Chang adapted from Chang et al. 2000 and Reed et al. 2006

Preconditioning

1. *In vitro* shoot cultures grown on MS (Murashige and Skoog 1962) medium with 3% sucrose and 7 g l^{-1} agar with: for *Cynodon* 0.5 mg/L kinetin; for *Zoysia* 1 mg/L kinetin and 0.1 mg l^{-1} indole 3 acetic acid; *Lolium* 1 mg l^{-1} kinetin
2. Plants grown for 4 week in cold-acclimation conditions with 8 h low light at 22° C and 16 h dark at –1°C

The procedure takes parts for 2 days
Day 1: Items needed to dissect shoot tips and make beads

1. Tools
2. Sterile Petri dishes, sterile filter papers
3. MS medium to hold shoot tips until beads are made
4. Alginate solution: MS liquid medium (without calcium) with 256.73 g l^{-1} sucrose and 30 g l^{-1} alginic acid (low viscosity). Stir slowly to dissolve, autoclave in advance.
5. Sterile pipettes for bead formation
6. 50 ml sterile beaker for mixing meristems with alginate
7. Calcium chloride (saturated) solution: liquid MS with 14.6 g l^{-1} $CaCl_2 \cdot 2\,H_20$
8. Sterile 250 ml beakers for calcium chloride
9. Sterile strainers for removing beads from solutions
10. Preculture medium: 0.75 M sucrose MS in 125 ml flasks
11. Cold-acclimated plants

You will need a separate flask of 0.75 M sucrose, 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile Petri dish and pipette per treatment (or genotype).

Day 2: Dry beads and cryopreserve

1. Sterile strainers (metal tea strainers)
2. Sterile 250 ml beakers
3. Dry filter paper for draining beads

4. Sterile Petri dishes for drying beads (one for each 25 beads)
5. Tools and sterile Petri dishes
6. Rehydration medium: Liquid MS (10–25 ml)
7. Cryotubes and markers
8. Information for labels
9. Recovery medium in 24 cell plates for each accession: MS medium appropriate for each genus with 6 g l^{-1} agar.

The procedure

1. Dissect 1 mm meristems onto MS agar plates.
2. Suspend meristems in alginate solution in a small sterile beaker.
3. Using a sterile pipette pick up one meristem with some alginate and drip into saturated calcium chloride solution to make beads. Avoid air bubbles in the alginate.
4. Leave the beads in the solution for 20 min to firm up. Pour solution through sterile strainer to remove liquid medium.
5. Place beads in preculture medium in 125 ml flasks on a shaker for 18–22 h.
6. Drain beads using sterile strainer and briefly place on sterile filter paper in Petri dish to absorb excess moisture.
7. Place beads in open sterile Petri dishes and dry in the air flow for ~5 hours (20% moisture content). They should not touch each other or they will not dry properly. There should be no extra moisture in the dish (absorb with sterile filter paper if necessary).
8. Moisture determination: Determine the fresh weight of 10 beads replicated three times, each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
9. Place beads in cryovials; place on cane and submerge in LN.
10. Warm for 1 minute in 45°C water and 2 min in 25°C water, then add liquid MS medium to the tube for 10 minutes to rehydrate. Place beads on recovery medium to grow. Use a softer than normal recovery medium (1 g l^{-1} less agar) for initial growth (4 weeks).
11. Place under low light for 1 week and move to higher intensity light and monitor for regrowth for another 3 weeks. Determine viability by the formation of normal shoots and multiple sets of leaves by week 4 of recovery. It is not necessary to remove the shoots from the beads if they are rehydrated.

Test results

Encapsulation dehydration recovery was good (60–100%) for all accessions using cold acclimation as a pretreatment. Over 30 *Cynodon* species and cultivars (Reed et al. 2006) and five *Zoysia* cultivars were stored with this technique.

Comparisons of encapsulation dehydration, controlled rate cooling and vitrification indicated that cooling rate cooling procedures work as well or better than encapsulation dehydration for *Lolium* (Table 11.6).

Notes

Recovery of shoots should be 80% or greater for the first three controls. Cold acclimation improves the desiccation tolerance of most genotypes. Controls should include the following steps.

1. Dissection
2. Encapsulated shoot tips
3. Encapsulated shoot tips after sucrose treatment
4. Beads dried to 18–20% moisture content

Table 11.6 The regrowth of *Lolium* and *Zoysia* meristems following cryopreservation by controlled cooling, encapsulation dehydration, or vitrification. All materials were cold acclimated for 4 weeks before cryopreservation. (From Chang et al. 2000)

Genus/cultivar		**Regrowth (%)**		
		Technique[z]		
		CC	**ED**	**VIT**
Lolium	Elka	90 ± 7.4^{a}	85 ± 5.6^{a}	3.7 ± 1.7^{e}
	Linn	65 ± 12.7^{b}	45 ± 3.5^{c}	15.5 ± 2.6^{d}
Zoysia	DH 96–12	13.3 ± 3^{d}	60 ± 6.5^{b}	14.5 ± 2.8^{d}
	Palisades	15 ± 2.3^{d}	80 ± 7.8^{ab}	5 ± 2.5^{e}

[z]CC: controlled cooling. ED: encapsulation-dehydration with 4-h air-dehydration. VIT: vitrification in PVS2 for 20 min. ($n = 40$). Means in a column with different letters are significantly different at $P < 0.05$ by Duncan's multiple range test

Chapter 12
Cryopreservation of Herbaceous Dicots

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12.1 Introduction

In tissue culture herbaceous dicots play an important role as model species for various fundamental questions such as somatic embryogenesis (carrot, alfalfa), hormone interrelationships (tobacco), and anther culture (tobacco and *Datura*). This may be facilitated by high plasticity in some groups like the Solanaceae. Crops that must be maintained vegetatively are candidates for cryopreservation. Many important species belong to this group of herbaceous dicots: potato, cassava, and sweet potato, staple foods in many parts of the world, medicinal, and aromatic plants like mint, foxglove, chamomile and St. John's Wort and ornamentals such as *Chrysanthemum*, *Pelargonium*, *Dianthus*, and *Gentiana*. In contrast to monocots which hide their meristems in the basal inner part of the shoot or bulb, most dicots present exposed shoot buds making their isolation easier and enabling the lab workers to more easily collect explants for cryopreservation. With respect to the other factors that influence cryopreservation, herbaceous dicots are as diverse as other plant groups, and generalization is difficult.

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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12.2 Cryopreservation Techniques for Herbaceous Dicots

A wide range of herbaceous dicot plants have been cryopreserved using an equally wide range of techniques (Table 12.1). Plant culture techniques and the propagules used for cryopreservation vary with the crop type.

12.2.1 Root and Tuber Crops

Many important tuber crops are propagated vegetatively using their storage organs as propagules. Potato (*Solanum tuberosum* L.) is the most advanced herbaceous dicot with respect to its large-scale cryopreservation. In IPK Gatersleben around 1000 accessions are cryopreserved (Keller et al. 2005, 2006). The first cryopreservation studies came from Bajaj (1977a), who used ultra-rapid freezing. Donor plants are derived from *in vitro* slow-growth cycles including storage of microtubers (Thieme 1992). In gene-banks, virus-free plants are essential for storage due to strict quarantine regulations for potato. Apical buds of *in vitro* plants are used for isolation of shoot tips (Schäfer-Menuhr et al. 1994; Keller and Dreiling 2003). Several protocols use an additional subculture of nodal cuttings to obtain lateral buds (Bouafia et al. 1996; Grospietsch et al. 1999; Kryszczuk et al. 2006). In contrast to potato, sweet potato (*Ipomoea batatas* [L.] Lam.), is much more difficult. It is a tropical crop sensitive to dehydration by PVS2 and possessing no cold adaptation (Takagi et al. 1998). Towill and Jarret (1992) first published results on this species. Cassava (*Manihot esculenta* Crantz), botanically a semi-shrubby species, is included here because of the cropping practice of using field plants to produce tubers with a relatively short rotation. The first publication of cassava cryopreservation was by Bajaj (1977b, 1990). Stem cuttings are used for primary explants (Kartha et al. 1982; Escobar et al. 1997). A minor tuber crop is the Madagascar potato [*Solenostemon rotundifolius* (Poir.) J. K. Morton], the most profitable tuber crop in Sri Lanka known as innala (Niino et al. 2000). Nodal segments consisting of a pair of leaves and 2–4 mm stem segments produce a large number of uniform shoots of 1–1.5 cm length, the buds of which are used for cryopreservation.

12.2.2 Seed-Propagated crops

In several crops with orthodox seeds, storage of breeding lines is needed, thus justifying cryopreservation. In sugar beet (*Beta vulgaris* L.) male sterile lines, inbred types, and tetraploid material must be kept vegetatively for

several years. Braun (1995) used inflorescence pieces to induce plantlets from which explants were taken for cryopreservation. Several *Brassica* species (*B. napus* L., *B. rapa* L. *[syn. B. campestris* L.*], B. oleracea* L.—Benson 1995) and chicory (*Cichorium intybus* L.—Vandenbussche et al. 2002) are used in this way. Brussels sprouts are started with cold-acclimated winter buds (Harada et al. 1985), and chicory with root explants. Tomato (*Lycopersicon esculentum* Mill.) with similar breeding needs employed shoot tips from *in-vitro* germinated seedlings (Grout and Crisp 1995). In peanut (*Arachis hypogaea* L.) and chickpea (*Cicer arietinum* L.—Bajaj 1979; Gagliardi et al. 2003), cryopreservation is the only conservation method for material coming from *in-vitro* induced variability (Bajaj 1995a). A wild relative to peanut (*A. burchelli* Baker) was also cryopreserved (Gagliardi et al. 2003). Both *Arachis* species were introduced from embryo axes and cotyledons.

In forage crops, cryopreservation is useful to preserve superior white clover (*Trifolium repens* L.) genotypes in breeding. Shoot tips were taken from 2–3 weeks old seedlings after *in-vitro* sowing (Yamada et al. 1991a). *Arachis glabrata* Benth., a relative of the peanut, a high-yielding and high-quality forage crop from South America is propagated from rhizomes due to low seed set. Cryopreservation of this species has been reported from the National Center for Genetic Resources Preservation (NCGRP), Fort Collins, Colorado (Towill 1993).

Chayote (*Sechium edule* [Jacq.] Sw.) is a unique case in the Cucurbitaceae family in which the seeds are recalcitrant. Fruits contain only one large seed which germinates in it, and it is damaged when it is extracted from the fruit. This species is an important crop that originated in South America and is now cultivated in all warm regions. Plantlets were obtained from extracted embryos. (Abdelnour-Esquivel and Engelmann 2002).

12.2.3 Medicinal and Aromatic Crops

Mint is one of the most common medicinal and aromatic crops. Several 'species' are actually sterile hybrids such as peppermint (*Mentha* × *piperita* L.), *M.* × *carinthiaca* Host, *M.* × *dalmatica* Tausch, whorled mint (*M.* × *verticillata* L.), ginger mint (*M.* × *gracilis* Sole), and *M.* × *villosa* Huds. These hybrid forms are seed sterile and require vegetative maintenance in plant collections. The explants for cryopreservation are usually taken from nodal cultures (Towill 1988; Volk 2000; Staats et al. 2006; Senula et al. 2007). In chamomile (*Matricaria recutita* L.), vegetative maintenance is needed to support breeding of varieties with high bisabolol contents (Diettrich et al. 1990). Foxglove species (*Digitalis lanata* Ehrh.,

D. purpurea L., and *D. obscura* L.) are used for their cardiotonic glycosides (Seitz 1995). Most attempts of cryopreservation were done on cell cultures. However, shoot tips of *D. lanata* were used by Diettrich et al. (1987) and of *D. obscura* by Sales et al. (2001). For similar reasons (use of cultivars with high contents of hypericin and pseudohypericin) Saint John's Wort (*Hypericum perforatum* L.) is cultivated *in vitro* starting with *in vitro* seedlings (Urbanova et al. 2002). Wasabi [*Eutrema wasabi* (Siebold) Maxim., syn. *Wasabia japonica* (Miq.) Matsum.] is an important spice plant cultivated in Japan for its pungency. It belongs to the Cruciferae family (Matsumoto et al. 1995). Hops, *Humulus,* is another important food, medicinal and industrial crop, which is highly heterozygous. Therefore, superior clones have to be maintained *in vitro* or in cryopreservation (Martinez et al. 1999; Martinez and Revilla 1998; Reed et al. 2003). Lists of endangered plants contain also many species of medicinal value that are vanishing from nature due to exhaustive collection. Amongst them are the endangered species *Holostemma annulare* (Roxb.) K. Schum. (Asclepiadaceae - Decruse et al. 1999, 2002), and *Picrorhiza lindleyana* Royle ex Benth, (syn. *P. kurroa* - Plantaginaceae—Sharma and Sharma 2003).

12.2.4 Ornamental Plants

Ornamentals have a high impact in cryopreservation research and application because the plants represent a higher value. Maintenance of special genotypes is economically well justified. *Chrysanthemum* × *sinense* Sabine ex Sweet (syn. *C. morifolium* Ramat.; *Dendranthema* × *grandiflorum* [Ramat.] Kitamura) is used as an ornamental and also as a vegetable. Fukai (1990) compared the source material of excised shoot tips; better results were obtained from material excised from greenhouse-grown plants in contrast to *in vitro* plantlets (Fukai et al. 1994). A relative of chrysanthemum is *Pyrethrum cinerariifolium)* (*Tanacetum cinerariifolium* [Trevir.] Sch. Bip. syn. *Chrysanthemum cinerariaefolium),* an ornamental and medicinal plant (Hitmi et al. 1999, 2000). Several wild chrysanthemum species and hybrids were cryopreserved by Fukai et al. (1991). Carnations (*Dianthus caryophyllus* L.), well known for their good survival, were some of the first plants cryopreserved (Fukai 1989). Three species of gentian are ornamentals but also used for medicinal purposes (Tanaka et al. 2004). Pelargoniums are another group of highly demanded ornamentals which are entirely vegetatively propagated. Greenhouse-grown plants are the source of explants for cryopreservation (Grapin et al. 2001). Some wild relatives were cryopreserved by Grapin et al. (2003). The florist's auricula (*Primula* x *pubescens*) Jacq.) had periods of very high value in its history. In

this hybrid species, the cultivars lose vigor when propagated vegetatively for 10–15 years, perhaps by virus accumulation (Hornung et al. 2001). Hybrid statice (*Limonium altaicum* hort. ex G. Don. × *L. bellidifolium* [Gouan] Dumort. *(syn Statice caspia* Willd.) was also successfully cryopreserved (Matsumoto et al. 1997, 1998).

12.2.5 Endangered Species

Cryopreservation is important for several endangered wild species with only a few remaining individuals. Some endangered Spanish wild species were cryopreserved: *Centaurium rigualii* Esteve (Gentianaceae) and *Antirrhinum microphyllum* Rothm. (Plantaginaceae) (González-Benito and Pérez 1997). *Cosmos atrosanguineus* (Hook.) Voss, a very rare species from Mexico was investigated as a model case in Kew Gardens (Wilkinson et al. 1998, 2003). Another endangered wild species is *Arachis burchelli* Krapov. and W. C. Greg. (Gagliardi et al. 2003).

12.3 Cryopreservation

12.3.1 Plant Factors

For several species it was found that the position of the shoot tips used is important; apical tips from the main axes are much better than nodal ones. In carnation, not only the apex itself was superior but also the four uppermost nodal explants gave similar and better values than the nodes below (Dereuddre et al. 1988). Shoot tips were best for sweet potato (Pennycooke and Towill 2000), potato (Schäfer-Menuhr 1996), cassava (Escobar et al. 1997). This may be correlated with apical dominance effects and the resulting state and size of the buds (Pennycooke and Towill 2000). Explants are taken at a set number of weeks after start of the subculture; however, sometimes new cultures are established from which young shoots are taken for explant isolation one week after transfer (Grospietsch et al. 1999, Zhao et al. 2005, Kryszczuk et al. 2006). Sometimes even seedlings are used for explants (Grout and Crisp 1995, Yamada et al. 1991 a,b). Plant quality may be of deciding importance. Undiscovered latent infections may reduce regeneration dramatically (Senula et al. 2007). When taking final explants for cryopreservation the explant size is important and varies with the plant type. Generally shoot tips are 0.5–2 mm.

Table 12.1 Cryopreservation methods used for herbaceous dicots

Plant species	Technique	Reference
Antirrhinum microphyllum	Encapsulation dehydration	González-Benito 1998
Arachis hypogaea	Vitrification 5% sucrose + 5% glycerol + 5% DMSO	Bajaj 1995a
Arachis sp.	Vitrification PVS2	Gagliardi et al. 2003
Beta vulgaris	Controlled rate cooling 0.8°C/min to –40°C	Braun 1995
	Encapsulation dehydration, Vitrification PVS2	Vandenbussche et al. 2000
Brassica napus	Controlled rate cooling, ultrarapid freezing	Withers et al. 1988, Benson 1995
Brassica oleracea, Brussels sprouts	Controlled rate cooling, 0.5°C/min to –30°C	Harada et al. 1985
Centaurium rigualii	Vitrification, Encapsulation dehydration	González-Benito 1998
Chrysanthemum × *sinense*	Controlled rate cooling 0.2–0.3C/min to –40°C; PVS2 vitrification	Ahn 1995: Fukai and Oe 1990; Fukai et al. 1994; Fukai 1995
	Controlled rate cooling 0.25°C/min; ED; Droplet-freezing 7.5% DMSO; Droplet vitrification PVS2	Halmagyi et al. 2004
Cicer arietinum	Vitrification 5% sucrose + 5% glycerol + 5% DMSO	Bajaj 1995a
Cichorium intybus	Controlled rate cooling 0.5°C/min to –40°C; Encapsulation dehydration	Demeulemeester et al. 1992
Cosmos atrosanguineus	Encapsulation dehydration with alginate strips	Wilkinson et al. 1998
Dianthus caryophyllus	Rapid freezing 10% DMSO 400°C/min	Seibert and Wetherbee 1977
	Controlled rate cooling 0.5°C/min to –40°C (1); Encapsulation dehydration (2); Vitrification (3); rapid freezing in bull semen straws 10% DMSO + 3% glucose (4)	(1) Dereuddre et al. 1988; (2) Tannoury et al. 1991 (3) Langis et al. 1990; (4) Fukai 1989
Digitalis lanata	Controlled rate cooling 0.5°C/min; Ultrarapid cooling	Diettrich et al. 1987
Digitalis obscura	Encapsulation dehydration	Sales et al. 2001

Table 12.1 continued

Plant species	Technique	Reference
Eutrema wasabi	Vitrification PVS2; Encapsulation-vitrification	Matsumoto et al. 1994, 1995
Gentiana spp.	Vitrification and encapsulation-vitrification PVS2	Tanaka et al. 2004
Holostemma annulare	Encapsulation dehydration	Decruse et al. 1999
Humulus lupulus	Controlled rate freezing 1°C/min to –40°C; Encapsulation dehydration	Reed et al. 2003; Martinez and Revilla 1998
Hypericum perforatum	Controlled rate cooling 0.5°C/min to –10°C, 1°C/min to –40°C	Urbanova et al. 2002
Ipomoea batatas	Ultra-rapid vitrification PVS2 and filter paper strips	Towill and Jarrett 1992
	Aluminum foil strips with PVS 2 plunge in partially solidified LN (–208°C)	Pennycooke and Towill 2000, 2001
	Vitrification cryoprotectant after Steponkus	Schnabel-Preikstas et al. 1992; Plessis and Steponkus 1996
	Encapsulation vitrification	Hirai and Sakai 2003; Pennycooke and Towill 2001
Limonium hybrid	Vitrification PVS2, Encapsulation-vitrification PVS2, Encapsulation dehydration	Matsumoto et al. 1997, 1998
Lycopersicon esculentum	Droplet freezing on aluminum foil; 15% DMSO	Grout and Crisp 1995
Manihot esculenta	Controlled rate cooling, various speed steps	Kartha et al. 1982; Escobar et al. 1997
	Vitrification PVS2	Charoensub et al. 1999; 2003; Escobar et al. 2000; Ng and Ng 2000
	Freezing with filter paper wrapped in aluminium foil, 10% DMSO	Bajaj 1995b
	Encapsulation dehydration	Benson et al. 1992; Escobar et al. 2000
Matricaria recutita	Controlled rate cooling 0.5°C/min to –40°C; Rapid freezing on filter paper strips	Diettrich et al. 1990

Table 12.1 continued

Plant species	Technique	Reference
Mentha spp.	Ultra-rapid freezing in filter paper	Towill 1990
	Vitrification PVS2	Volk 2000, Towill and Bonnart 2003
	Droplet vitrification PVS2	Volk 2000, Senula et al. 2007
	Encapsulation vitrification with PVS2	Hirai and Sakai 1999a, Sakai et al. 2000
	Controlled rate cooling, PVS2 vitrification, Encapsulation dehydration	Uchendu and Reed 2007
Picrorhiza lindleyana	Vitrification PVS2	Sharma and Sharma 2003
Primula × *pubescens*	Encapsulation dehydration	Hornung et al. 2001
Sechium edule	Vitrification PVS2	Abdelnour-Esquivel and Engelmann 2002
Solanum tuberosum	(1) Controlled rate cooling, 0.3–0.4°C/min to –30°C (2) 1°C/min to 0°C; –0.5°C to –10°C	Bajaj 1977a, (1) Towill 1981, 1984, (2) Benson et al. 1989, Harding and Benson 1994
	Droplet method aluminum foil 10% DMSO	Schäfer-Menuhr et al. 1994, 1997a, b
	Vitrification, Steponkus' solution	Golmirzaie and Panta (2000)
	Vitrification PVS2 or PVS2 modified	Sarkar and Naik 1998, Zhao et al. (2005); Kryszczuk et al. (2006)
	Droplet/vitrification on aluminum foil PVS2	Halmagyi et al. 2005
	Encapsulation dehydration	Fabre and Dereuddre 1990, Bouafia et al. 1996, Grospietsch et al. 1999
	Encapsulation vitrification with PVS2	Hirai and Sakai 1999b
Solenostemon rotundifolius	Vitrification PVS2	Niino et al. 2000
Trifolium spp.	Controlled rate cooling; vitrification PVS2	Yamada et al. 1991a,b, 1992

12.3.2 Preconditioning

Preconditioning is important in many cryopreservation protocols (Table 12.2). These treatments of the mother plants can have profound effects on cryopreservation regrowth. Cold acclimation can double or triple regrowth after cryopreservation (Reed 1988). However, in some tropical species like sweet potato, cold acclimation is harmful (Hirai and Sakai 2003). Abscisic acid (ABA) and an increase of sucrose contents in the culture media are further factors which can increase cryopreservation success. Preconditioning on sugar solutions is commonly used for vitrification or encapsulation dehydration. Preconditioning of St. John's Wort explants was performed in liquid shaking cultures for up to 14 days, and the medium included either mannitol or ABA (Urbanova et al. 2002). Dimethyl sulfoxide (DMSO) is occasionally used for 3–5 day preconditioning (Bajaj 1995a).

12.3.3 Preculture

DMSO (5–10%) in the preculture medium for one or several days is commonly used in controlled rate cooling (Bajaj 1995b). It is sometimes combined with ABA or sorbitol (Escobar et al. 1997). Generally vitrification methods start with overnight incubation of the explants either on growth medium or on increased sucrose concentrations like in mint (Towill 1988) and sometimes the incubation on high sucrose is prolonged up to 2 days. Encapsulation-dehydration techniques normally employ an 18–22 h sucrose preculture before the air dehydration step.

12.3.4 Osmoprotection

Most vitrification protocols include a step to condition shoot tips to the osmotic stress of the vitrification solutions. This is osmoprotection or sucrose loading. In the PVS2 protocols osmoprotection is provided by LS, a solution of 2 M glycerol + 0.4 M sucrose in liquid culture medium (Sakai et al. 1990). Treatment is often for 20 min (Matsumoto et al. 1994). Osmoprotection for encapsulation dehydration starts with increasing sucrose concentrations. Controlled rate cooling protocols provide osmoprotection by slow addition of the cryoprotectant solution.

Table 12.2 Preconditioning treatments for herbaceous dicots (CA - cold acclimation).

Plant species	Technique	Reference
Arachis hypogaea	3–5% DMSO 1 week before vitrification	Bajaj 1995a
Arachis spp.	2.8 µM ABA, 2 months	Gagliardi et al. 2003
Beta vulgaris	CA 1 week 5°C, 8 h light, 0.3 M sucrose	Vandenbussche et al. 2000
Brassica oleracea	CA field plants in winter (−3°C to–9°C)	Harada et al. 1985
Cicer arietinum	3–5% DMSO 1 week before vitrification	Bajaj 1995a
Dianthus caryophyllus	CA 4°C, 3 days	Seibert and Wetherbee 1977
Digitalis lanata	CA 4°C, 8 weeks	Diettrich et al. 1987
Digitalis obscura	CA 4°C, 15 days, 12 h light; 1 µM ABA	Sales et al. 2001
Gentiana spp.	CA 5°C, 10–50 days, 8 h light	Tanaka et al. 2004
Ipomoea batatas	8 mm stem segments, 25°C 2 weeks	Hirai and Sakai 2003
Holostemma annulare	CA 4°C 5–7 days, 0.5 M sucrose	Decruse and Seeni 2002
Humulus lupulus	(1) CA 4°C, dark 1–2 weeks; (2) alternating temperatures 22°C/−1°C, 8 h light 2–5 weeks	(1) Martínez and Revilla 1998; (2) Reed et al. 2003
Hypericum perforatum	0.076 µM ABA 10–14 days	Urbanova et al. 2002
Limonium hybrid	Shoot primordia culture: CA 4°C, 10 days in liquid medium	Matsumoto et al. 1997, 1998
Manihot esculenta	1 M sorbitol, 0.1 M DMSO, 0.1 M sucrose medium, 3 days, 26–28°C	Escobar et al. 1997
Matricaria recutita	CA4°C, 8 weeks	Diettrich et al. 1990
Mentha spp.	CA 22/−1°C (8 h light), 3 weeks	Volk 2000
	CA 25/−1°C (16 h light), 2–6 weeks	Senula et al. 2007
	CA 4°C, 1–3 weeks, 12 h light	Hirai and Sakai 1999; Sakai et al. 2000
	CA 2 weeks with 8 h light 22°C /−1°C	Uchendu and Reed 2007
Picrorhiza lindleyana	CA 4°C, dark, 4 weeks, 35% relative humidity	Sharma and Sharma 2003
Primula × pubescens	CA 10°C, 16 h light, 2 weeks	Hornung et al. 2001
Sechium edule	0.3 M sucrose 22 days	Abdelnour-Esquivel and Engelmann 2002
Solanum tuberosum	(1) CA 10°C, 8 h light, 4 weeks (2) CA alternating temperature 21/8°C, 8 h light, 7 days; (3) CA 4°C 12 h light, 3 weeks	(1) Zhao et al. 2005; (2) Kryszczuk et al. 2006; (3) Hirai and Sakai (1999b)

12.4 Cryoprotection and Cryopreservation

Cryopreservation can be classified into several main methods: controlled rate cooling (Chap. 5), vitrification, droplet method, droplet vitrification, and encapsulation vitrification (Chap. 3), and encapsulation dehydration (Chap. 4). In some crops all methods were successfully applied. For ex ample in potato many techniques were employed: controlled rate cooling, vitrification, the droplet method, vitrification, droplet vitrification, encapsulation dehydration, and encapsulation vitrification. Several methods were compared for wasabi, chrysanthemum, and statice. A list of herbaceous plants and the method of cryopreservation is detailed in Table 12.1.

12.4.1 Controlled Rate Cooling

Most controlled rate cooling protocols use DMSO alone or in combination with other cryoprotectant chemicals (See Chap. 5). PGD, a solution of 10% each polyethylene glycol, glucose and DMSO in MS medium, was used for controlled cooling of hops (Reed et al. 2003) and mint (Uchendu and Reed 2007). The best cryoprotectant for peanut and chickpea was a combination of 5% each of sucrose, glycerol and DMSO, which was even improved by addition of proline (Bajaj 1995a). In St. John's Wort, a cryoprotectant solution of 20% (w/v) sucrose, 10% (v/v) glycerol and 10 (v/v) DMSO was effective (Urbanova et al. 1999). DMSO at 15% is applied for *Brassica napus* (Withers et al. 1988), chicory (Demeulemeester et al. 1992), mint (Towill 1988), and chrysanthemum (Ahn 1995). DMSO is increased stepwise in tomato (Grout and Crisp 1995) and used with glucose in Brussels sprouts (Harada et al. 1985) and carnation (Fukai 1989). A gradual addition of DMSO is reported in carnation (Dereuddre et al. 1988) and clover (Yamada et al. 1992).

12.4.2 Vitrification

PVS2 (Sakai et al. 1990) is the most commonly used vitrification solution and may be used at room temperature or at 0°C. PVS2 is used in plastic cryotubes or in drops on aluminum foil for faster vitrification (See Chap. 3). PVS2 is also used in encapsulation vitrification protocols. Sometimes Steponkus' solution (50% [w/v] ethylene glycol, 15% [w/v] sorbitol, 6% [w/v] bovine serum albumin - Steponkus et al. 1992) is used as an alternative. Encapsulation vitrification and droplet vitrification were developed

by combining established methods with some modifications. Both employ PVS2 as the vitrification solution.

12.4.3 DMSO Droplet Method

The droplet method with DMSO is also used for cryopreservation of several plant types. Drops of 10% (Schäfer-Menuhr et al. 1994) or 15% DMSO (Grout and Crisp 1995) are placed on aluminum foil strips and plunged into liquid nitrogen (LN). Although the cooling rate is extremely fast, no vitrification is obtained within the cell. Similar techniques involving drops of PVS2 or DMSO cryoprotectant on filter paper were used by Towill (1990), Towill and Jarrett (1992), and Bajaj (1995b).

12.4.4 Encapsulation Dehydration

No cryoprotectant solutions are needed for encapsulation dehydration but preconditioning, preculture, and a desiccation step are required (See Chap 4). Air drying in the laminar flow hood or desiccation over silica gel decrease the moisture content of the beads and shoot tips to about 20%.

12.5 Rewarming

In most published protocols rewarming is done rapidly to avoid ice recrystallization. The warming solution temperature varies from 25°C to 45°C, but is usually about 40°C. In controlled rate cooling and vitrification the whole vials are plunged into a water bath. Beads from encapsulation are sometimes warmed by placing them at room temperature. In other cases the beads and aluminum foils from the droplet method are removed from the vial or poured directly into a Petri dish of warm liquid medium. Detailed studies on rewarming were performed with pelargoniums from encapsulation dehydration (Dumet et al. 2002). No significant differences were observed when beads had low water contents (0.25 g/g dry weight) and the vials were directly plunged into a water bath at 40°C (120 s) or 95°C (30 s). Beads also survived well if dropped into a 0.75 M sucrose solution held at 20°C.

12.6 Recovery

As dehydration is a concomitant factor of cryopreservation, and many cryoprotectants are toxic, some rinsing or transfer steps are needed to recover the explants. With controlled cooling the shoot tips are rinsed with standard growth medium for a few minutes before plating. Vitrification techniques normally employ a 1.2 M sucrose medium for rinsing. The first cultivation step usually takes place on the standard medium of the crop. It may contain increased sucrose (1–1.2 M) to allow the explants to slowly acclimate. Sometimes the first medium is liquid or, in potato, agarose drops (Schäfer-Menuhr et al. 1994). Beads from encapsulation are placed directly on recovery medium allowing the explants to grow out of them. Sometimes explants are extracted from the beads (Tanaka et al. 2004) or rehydrated in liquid medium for a few minutes after rewarming (Gupta and Reed 2006). After these steps, the plants are usually grown on standard medium.

The effect of growth regulators on the recovery phase was documented by Withers et al. (1988). Growth regulators had different influences depending on whether *Brassica* shoot tips were cryopreserved by controlled rate cooling or vitrification. Benzyladenine was favorable for organized regrowth after vitrification, but not after controlled rate cooling. Ethylene may play a role in these complicated interactions. Callus formation was mentioned for potato (Benson et al. 1989) and sweet potato (Pennycooke and Towill 2000), and was reduced on recovery medium without growth regulators or with low growth regulator concentrations (Pennycooke and Towill 2001). Auxins may be excluded from the recovery medium to decrease callus formation (Chang and Reed 1999). Culture in the dark for a few days to 1 week is often used to avoid photo oxidation of explants.

12.7 Viability Assessment

Actual regrowth of plantlets is the true test of the success of a cryopreservation protocol. This is usually assessed 4–8 weeks after plating. One of the main features in assessment of cryopreservation success is the determination of the morphogenic pathways. This is important because callus may develop either primarily or only at cut surfaces. Callus formation endangers the genetic stability of the regenerants. Therefore, material forming callus at the early stages must be regarded cautiously, whereas secondary callus should not make problems if it is thoroughly cut off from the explants

and secondary regeneration is not allowed. Callus production or detailed regenerant analyses were mentioned by Fukai (1990, 1995) and Fukai and Oe (1990) for chrysanthemum, and by Demeulemeester et al. (1992) for chicory. Wilkinson et al. (1998, 2003) studied topological ultrastructure and genetic stability of *Cosmos*. Chimeric chrysanthemum mostly lost their chimeric character because regrowth came from adventitious regeneration from the epidermis, not from meristems (Fukai et al. 1994).

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12.8 Protocols

12.8.1 Vitrification of Potato Shoot Tips

By A Panta based on Steponkus et al. 1992, Golmirzaie and Panta, 2000

Day 1: Materials for shoot tip isolation and incubation

1. Tools
2. Sterile Petri dishes
3. Preculture medium # 1: liquid MS (Murashige and Skoog 1962) with 0.04 mg/L kinetin, 0.5 mg/L indole acetic acid, 0.2 mg/L gibberellic acid and 0.09 M sucrose.
4. Sterile filter paper
5. Microscope for shoot tip dissection
6. Sterile hypodermic needles
7. Forceps
8. Sterile pipettes
9. *In vitro* plants for shoot tips

Day 2: Materials for cooling in LN and rewarming

1. Sterile Petri dishes
2. Sterile filter paper
3. Sterile pipettes
4. Propylene straws 0.25 mL
5. Preculture medium # 2: liquid MS medium with 0.04 mg/L kinetin, 0.5 mg/L indole acetic acid, and 0.2 mg/L gibberellic acid and 0.06 M sucrose
6. Vitrification solution: ethylene glycol: sorbitol: bovine serum albumin (50:15:6 wt %) (Steponkus et al. 1992)
7. Hypertonic sorbitol solution (1.5 M)
8. LN and Dewar
9. Semi-solid potato meristem medium: MS with 0.04 mg/L kinetin, 0.1 mg/L gibberellic acid, 25 g/L sucrose, and 2.8 g/L phytagel

Prepare in advance

1. *In vitro* plants 30–45 days old
2. Preculture medium #1

3. Preculture medium #2
4. Vitrification solution
5. Potato meristem medium
6. Hypertonic sorbitol solution
7. Sterile tools (Petri dishes, filter paper, pipettes, forceps)

The procedure
This is a 2 day process.

Day 1
Remove axillary shoot tips (4–5 leaf primordia and apical dome, 1.5 mm long) from *in vitro* plants and put into preculture medium #1. Incubate for 24 h in the growth room.

Day 2
1. Incubate shoot tips for 5 h in preculture medium #2.
2. Place shoot tips in vitrification solution for 50 min at room temperature.
3. Transfer ten shoot tips to 0.25 mL propylene straws with 150 μl of vitrification solution.
4. Cool straws rapidly in LN.
5. Rewarm straws and expel shoot tips into a hypertonic (1.5 M) sorbitol solution at room temperature and incubate for 30 min.
6. Plate shoot tips on meristem medium and maintain them under proper conditions for micropropagation.

Analysis for regrowth should be done 4–6 weeks after rewarming. This protocol was applied to 80 genotypes of potatoes. The explants were successfully recovered for 69% of accessions tested. Of those that recovered the average survival was about 40%.

Further vitrification protocols with only slight modifications

Kryszczuk et al. 2006
Steponkus et al. 1992

12.8.2 Encapsulation Dehydration of Potato Shoot Tips

By A Kaczmarczyk based on Fabré and Dereuddre et al. 1990 ; Bouafia et al. 1996

Checklist for encapsulation dehydration
Materials

1. Sterile Petri dishes
2. Sterile filer papers
3. Microscope for shoot tip dissection
4. Hypodermic needles or scalpels for shoot dissection
5. Sterile beaker
6. Sterile pipettes
7. In vitro plantlets for shoot tips
8. Growth media: 1, 2A, 2B, and 2C
9. Calcium-free medium 2A (with 3% [w/v] sodium alginate)
10. Liquid medium 2A containing 100 mM calcium chloride
11. Sucrose solution (0.75 M) in medium 2A
12. Silica gel
13. Cryotubes
14. Liquid nitrogen and Dewar

Prepare in advance

1. Two week old nodal cuttings grown on medium 1.
2. Medium 1 (standard medium with macroelements: (Tendille and Lecerf 1974) 808.8 mg/L KNO_3, 160.1 mg/L NH_4NO_3, 141.7 mg/L $Ca(NO_3)_2 \cdot 4\,H_2O$, 123.3 mg/L $MgSO_4 \cdot 7\,H_2O$, 104.4 mg/L KCl, 81.7 mg/L KH_2PO_4, 7.0 mg/L K_2HPO_4; Murashige & Skoog (1962) micronutrients; Morel and Wetmore (1951) vitamins: 100 mg/L inositol, 1 mg/L thiamine·HCl, 1 mg/L nicotinic acid, 1 mg/L pyridoxine·HCl, 1 mg/L Ca pantothenate, 0.01 mg/L biotin, Na-Fe-EDTA, 30 g/L sucrose, 8 g/L agar, pH 5.8.
3. Medium 2A Morel and Müller (1964) macronutrients: 500 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 125 mg/L $MgSO_4 \cdot 7\,H_2O$, 1000 mg/L KCl,

125 mg/L KH_2PO_4, 1000 mg/L $(NH_4)_2SO_4$; Heller (1953) micronutrients: 0.1 mg/L $MnSO_4 \cdot 4H_2O$, 1 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L H_3BO_3, 0.01 mg/L KI, 0.03 mg/L $CuSO_4 \cdot 5H_2O$, 0.03 mg/L $AlCl_3$, 0.03 mg/L $NiCl_2 \cdot 6H_2O$, 1 mg/L $FeCl_3 \cdot 6H_2O$, Morel and Wetmore (1951) vitamins (see medium 1).

4. Initial recovery medium 2B = 2A + 0.01 mg/L BA + 0.001 mg/L NAA + 5 mg/L GA_3
5. Final recovery medium 2C = 2A + 0.0001 mg/L GA_3
6. Alginate solution: Calcium-free medium 2A with 3% [w/v] low viscosity sodium alginate.
7. Calcium chloride solution: 100 mM calcium solution in medium 2A.
8. Flasks of 0.75 M sucrose solution.
9. Sterile tools (Petri dishes, filter paper, pipettes, and forceps).

The procedure

1. Remove axillary and terminal shoot tips (0.5 mm long) from 14 day-old nodal cuttings.
2. Incubate the shoot tips for several hours in medium 2A.
3. Suspend them afterwards in alginate solution
4. With a sterile pipette, pick up a shoot tip and some alginate and drip into the calcium chloride solution to form beads (3–4 mm in diameter).
5. Preculture the beads for 2 days in 0.75 M sucrose solution on a shaker (100 rpm).
6. Drain beads and place on sterile filter paper in Petri dish to absorb excess of moisture.
7. Dry the beads over silica gel in airtight boxes for 4.5 h at constant temperature of 20°C ± 1°C to obtain 0.20–0.22 g water per g DW [determination of DW by drying 20 beads devoid of shoot tips in an oven (85°C) to constant weight].
8. Put beads into cryotubes and submerge directly in LN.
9. Rewarm beads by removing to room temperature.
10. Transfer beads with shoot tips to regrowth medium # 2B.
11. Regrowth is on medium # 2C.

12.8.3 DMSO Droplet Method for Potato Shoot Tips

By A Schäfer-Menuhr based on Schäfer-Menuhr et al. 1994

Checklist for droplet method cryopreservation

Day 1: Material for shoot tip isolation and incubation

1. Sterile Petri dishes, filter paper, forceps, Parafilm
2. Liquid MS (Murashige and Skoog 1962) with 30 g/L sucrose, 0.5 g/L zeatin riboside, 0.5 mg/L IAA, 0.2 m/L GA_3
3. Sterile hypodermic needles or scalpels for shoot dissection
4. Sterile pipettes
5. In vitro plantlets for shoot tips

Day 2: Material for cooling in LN and rewarming

1. Filter-sterile DMSO
2. Cryoprotectant: 10% DMSO in MS with 30 g/L sucrose
3. Sterile Petri dishes, filter paper, pipettes
4. Cryotubes
5. Sterile aluminum foils (5 ×25 × 0.03 mm)
6. LN and Dewar
7. Polystyrene box with holes for cryotubes
8. Pipette with sterile tips for 2 µl droplets
9. Sterile 100 mL beakers for rewarming
10. Rewarming medium: MS with 30 g/L sucrose
11. Agarose 1% in MS with 30 g/L sucrose
12. Water bath
13. Sterile MS medium (above)

Prepare in advance

1. Plants 3–4 weeks-old and grown on solidified MS with 20 g/L sucrose and maintained under a 16 h photoperiod at 22°C
2. Sterile MS medium
3. Sterile agarose solution
4. Sterile liquid MS with 30 g/L sucrose
5. Filter-sterile DMSO
6. Sterile Petri dishes, filter paper, pipettes, forceps, aluminum foils

The procedure
This is a 2-day experiment.

Day 1

1. In a Petri dish with one filter paper add 2 mL of medium 1.
2. Dissect shoot tips (2–3 mm long and 0.5 – 1.0 mm thick) and put into the prepared Petri dish.
3. The Petri dish is sealed with Parafilm and held over night (ca. 24 h) at 22°C.

Day 2

1. Make fresh cryoprotectant and filter sterilize.
2. Into a Petri dish with one filter paper add 2 mL cryoprotectant.
3. Additional cryoprotectant is held in a cryotube to make the droplets later.
4. Transfer shoot tips into Petri dish with the cryoprotectant and incubate for 1–3 h at room temperature.
5. Prepare LN, put aluminum foils on a sterile Petri dish and prepare the polystyrene box, label the cryo tubes.
6. Fill the polystyrene box with LN, open cryo tubes and put them into the box and fill them with LN.
7. Put five–six drops (each 2 μL) cryoprotectant on each aluminum foil.
8. Pick up shoot tips with forceps put into each drop.
9. Refill the cryo tubes with LN.
10. Put one aluminum foil into the cryotube, holding it down for some seconds until the foil has the temperature of LN.
11. Refill the cryotubes with LN.
12. Repeat for additional aluminum foils.
13. Close the cryotube and move it from the polystyrene box into the Dewar with LN.
14. Warm the agarose in a water bath (70°C). After melting set the temperature to 50°C.
15. Fill the polystyrene box with LN.
16. In sterile beaker add 50 mL room-temperature MS (30 g/L sucrose); label one for each foil.
17. Put the cryo tubes from the Dewar into the box.

18. Open the tubes, take out one aluminum foil with forceps and transfer it quickly into the warming solution and shake it so that the shoot tips will rewarm very quickly.
19. Repeat with the second foil.
20. In a Petri dish place ten drops of agarose solution.
21. Place one rewarmed shoot tip in each agarose drop.
24. Let the agarose solidify for about 30–60 min.
22. Add 2.5 mL medium 1 into the Petri dish, label, and seal with Parafilm.
23. For regrowth Petri dishes are grown at 22°C with a 16 h photo-period.

Analyze for survival and regrowth 3, 6, and 8 weeks after rewarming.

The droplet method is used for long term storage at the IPK (Leibniz Institute of Plant Genetics and Crop Plant Research) and about 1000 accessions are conserved with this method. It is a very simple method with low costs (Fig. 12.1). The average survival of potato shoot tips after cryopreservation by the droplet method is about 80% and average regrowth is about 40%. The genetic stability was confirmed by comparing morphological parameters, flow cytometric measurements and restriction fragment length polymorphism (RFLP) analysis.

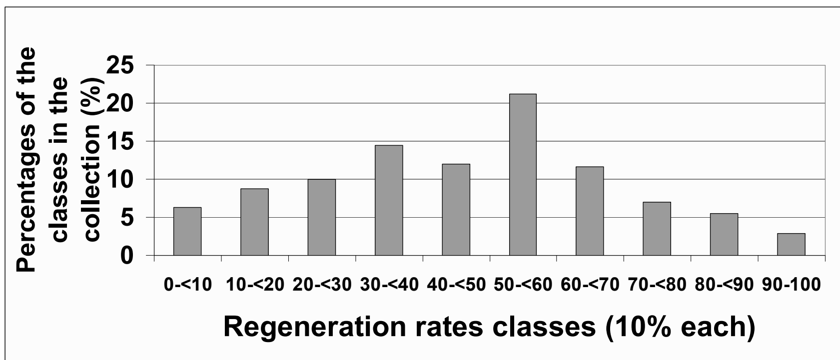

Fig. 12.1 Distribution of the regrowth of IPK's potato cryo-collection summarized as means of the introductory and a later control test and arranged in an overall-histogram showing the percentages of each regeneration-rate class in the collection. The total is 968 accessions.

12.8.4 Droplet Vitrification of Potato Shoot Tips

By A Halmagyi based on Halmagyi et al. 2005

Day 1: Materials for shoot tip isolation, incubation, and preculture

1. Sterile Petri dishes, filter paper, forceps
2. Liquid MS (Murashige and Skoog 1962) medium with 0.4 mg/L GA_3, 0.5 mg/L zeatin and 0.2 mg/L IAA
3. Microscope for shoot tip dissection
4. Sterile hypodermic needles, pipettes
5. In vitro plantlets for shoot tips
6. MS medium with 0.5 M sucrose

Day 2: Materials for cooling in LN and rewarming

1. Sterile Petri dishes, filter paper, pipettes
2. PVS2: 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) DMSO in liquid MS basal medium (pH 5.8)
3. Sterile aluminium foil strips (0.6 × 1.5 cm)
4. Cryotubes
5. Liquid nitrogen (LN) and Dewar
6. Liquid MS medium
7. MS medium with 3.5 g/L agar (semi-solid)

Prepare in advance

1. Provide *in vitro* plants cultured for 1–2 months after last transfer at 24°C, 16 h light/8 h dark photoperiod, 40 $\mu E\, m^{-2} s^{-1}$.
2. Liquid and semi-solid MS medium
3. MS medium with 0.5 M sucrose
4. PVS2
5. Sterile aluminium foil strips (0.6 × 1.5 cm)
6. Sterile tools (Petri dishes, filter paper, pipettes, forceps)

The procedure

This is a 2-day procedure.

Day 1

Note: In the original protocol, shoot tips are put into P1 medium after isolation and kept there for 24 h at 24°C.

1. Prepare a Petri dish with liquid 0.5 M sucrose MS medium and one filter paper.

2. Isolate the shoot tips (3–4 mm long) and place in the Petri dish.
3. Incubate for 24 h at 24°C.

Day 2

1. Place 4 µl droplets of PVS2 on sterile aluminium foil strips. With forceps add a shoot tip to each drop.
2. Hold for 10–30 min at room temperature.
3. Transfer aluminium foils with shoot tips to precooled cryotubes.
4. Freeze cryotubes directly in LN.
5. Rewarming: Open the tubes, take out one aluminum foil with forceps and transfer it quickly into liquid MS medium at room temperature and shake it so that the shoot tips will rewarm very quickly.
6. Place shoot tips on semisolid MS medium for 20 days at 24°C.
7. Elongated shoot tips can be planted on solid MS medium (7 g/L agar).

The best regrowth was 55% for ‘Desiree’, 51% for ‘Ostara’ and 46% for ‘Sante’.

Best results were found when

1. Apical shoot tips were used.
2. Shoot tips were 3–4 mm.
3. PVS2 incubation was 10–15 min.
4. Preculture 0.5 M sucrose for 24 h.
5. No cold acclimation was applied.

Further droplet vitrification protocols with only slight modifications

Kim et al. 2006
Yoon et al. 2006

12.8.5 Gentiana *Encapsulation Vitrification*

By D Tanaka based on Tanaka et al. 2004

Checklist for alginate bead-vitrification

Day 1: Preparation and preculture

1. Scalpel and forceps
2. Sterile filter papers
3. Preculture medium: ½ strength MS (Murashige and Skoog 1962) with 0.3 M sucrose and 8 g/L agar
4. Cold-acclimated plants for shoot tips

Day 2: Cryopreservation

1. Scalpel and forceps
2. Alginate solution: liquid calcium-free MS medium with 2.0 M glycerol and 0.4 M sucrose and 3% Na-alginate (low viscosity)
3. Calcium chloride (0.1 M $CaCl_2$) solution: liquid MS with 2.0 M glycerol and 0.4 M sucrose (10 mL for each set of shoot tips)
4. Pipettes for making beads, beakers for alginate, beakers for $CaCl_2$
5. Cryotubes (2.0 mL) and markers
6. PVS2: 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), and 15% (w/v) dimethyl sulfoxide (DMSO) in sterile liquid MS basal medium (pH 5.8)
7. Rinsing solution: MS with 1 M sucrose
8. Recovery medium: ½ strength MS with 8 g/L agar

Prepare in advance

1. Three week-old *in-vitro* plants are cold acclimated at 5°C for 20 days with an 8 h photoperiod at 26 μM m^{-2} s^{-1}.
2. Excise shoot apices with two–three pairs of leaf primordia (2.0 mm long and 1.0 mm in diameter). Use homogeneous specimens in terms of size, cellular composition, physiological state, and growth response.
3. PVS2
4. You will need a separate beaker of $CaCl_2$ solution, beaker of alginate solution and at least one sterile pipette per genotype.

The procedure

This is a 3 day procedure once the cold acclimation is accomplished.

Cryopreservation

Day 1

Preculture shoot apices excised from cold-acclimated plants on sterile filter papers on preculture medium at 5°C for 1 day with an 8 h photoperiod (26 µM m^{-2} s^{-1}). Cold acclimation for 10–50 days is needed to obtain high recovery (70–80%).

Day 2

1. Suspend shoot apices in alginate solution in a small sterile beaker.
2. Pick up one apex and some alginate with the pipette. Drip alginate with shoot apex into a 200 mL beaker of 0.1 M $CaCl_2$ solution (bead size about 3 mm in diameter).
3. Place beads on a rotary shaker (100 rpm) for 90 min at 25°C to allow completion of polymerization.
4. Place beads in 2.0 mL cryotubes (ten beads per tube), add PVS2 and hold 120 min at 25°C. After treatment plunge cryotubes in LN. The highest regrowth was with 80–140 min PVS2 treatment.
5. Warm cryotubes rapidly in 37°C water for 1–2 min, and then replace PVS2 with 1.0 M sucrose MS and incubate for 20 min at 25 °C. Transfer beads containing shoot apices onto solidified ½-strength MS medium.

Day 3

After 1 day, shoot apices are cut out of the beads and cultured on solidified ½-strength MS medium.

Table 12.3 Regrowth of shoot apices of ten cultivars/lines of *Gentiana* species cryopreserved with the alginate bead-vitrification procedure.

Species (cultivar)	Regrowth (% ± SE)
G. scabra (Yamagata 302)	93.3 ± 4.1
G. triflora × *G. scabra* (Yamagata 302)	86.7 ± 4.1
G. triflora (Kudoshiro 2)	83.3 ± 8.2
G. pneumonanthe (G-148)	80.0 ± 0.0
G. scabra (8-208)	80.0 ± 7.1
G. triflora × *G. scabra* (Marchen)	73.3 ± 8.2
G. triflora (9–99)	73.3 ± 4.1
G. triflora (Inasu 2)	63.3 ± 10.8
G. triflora (N 38)	60.0 ± 7.1
G. triflora (Kudoshiro)	43.3 ± 8.2
Average	73.7 ± 3.0

12.8.6 *Controlled Rate Cooling of* **Hypericum perforatum** ***L.***

By E Cellárová based on Urbanová et al. 2006

Preparation of plant material (3–4 weeks in advance)

Prepare in advance

1. Seeds of *Hypericum perforatum* L.
2. Aqueous solution of $AgNO_3$ (1%)
3. Sterile redistilled/deionized water
4. MS (Murashige and Skoog 1962) hormone-free medium, 0.7% agar
5. Sterile beakers, funnel, filter paper, tweezers

The procedure

1. Sterilize the seeds: 15 min in 1% aqueous solution of $AgNO_3$.
2. Place seeds on a filter paper in a funnel and wash 3 times with sterile water.
3. Culture seeds on solid MS medium at 23°C, 40% relative humidity, 16/8 h photoperiod (15 – 30 µE m^{-2} s^{-1}) for 3–4 weeks until the plants develop 6–8 leaf pairs.

`

Preculture (3 day procedure)

Prepare in advance

1. Sterile scissors, tweezers, Petri dishes
2. Sterile bacterial filters, syringe, and bacterial filter holders for sterilization of ABA solution
3. Preculture medium: liquid MS medium (MS with 0.5 mg/L BA) with 0.076 µM ABA. Add the filter-sterilized ABA solution to the medium before it is solidified (about 35–40°C).

The procedure

1. Prepare the preculture medium.
2. Isolate the shoot tips from 3–4 weeks-old seedlings and place on preculture medium.
3. Culture for 3 days at 23°C, 40% relative humidity, 16/8 h photoperiod (15–30 µE m^{-2} s^{-1}).

Cryoprotection, cooling and cryostorage
Prepare in advance

1. Filter-sterilized cryoprotective solution (10% glycerol, 20% sucrose, 10% DMSO)
2. Sterile 1.8 mL cryotubes
3. Sterile tweezers
4. Beakers with ice
5. Dewars with LN; one for freezing and the other for cryostorage
6. Programmable freezer

The procedure

1. Place the precultured shoot tips into cryotubes with 1 mL of cryoprotective solution (ten shoot tips per cryotube).
2. Place the cryotubes on ice and let them equilibrate for 1 h.
3. In the meantime set the program of the programmable freezer starting from 0°C as follows: from 0°C to –10°C at 0.5°C/min, then at 1.0°C/min to –40°C; 20 min holding period at –40°C.
4. Immerse the cryotubes into LN.

Rewarming
Prepare in advance

1. Water bath at 40°C
2. Liquid and solid MS medium with 0.5 mg/L BA

The procedure

1. Warm the cryotubes for 1–2 min in a 40°C water bath.
2. Remove cryoprotectant, replace with liquid MS medium for 1 h.
3. Place shoot tips on solid MS medium and culture them in the dark for 14 days, under half-intensity irradiation for 7 days, followed by standard growth conditions.
4. Regrowth can be determined by ability to differentiate multiple shoots 4–6 weeks after cryostorage.

Possible Problems

Manipulation with seedlings in the laminar flow cabinet should be as fast as possible. The seedlings are hyperhydric and the shoot tips are very small. Meristems are easily damaged by the dry environment of the laminar flow cabinet.

12.8.7 Encapsulation Vitrification of Sweet Potato (Ipomoea)

By D Hirai based on Matsumoto et al. 1995

Checklist for Encapsulation Vitrification

Day 1: Dissect shoot tips, make beads, and incubate

1. Tools, sterile plastic dishes or sterile Petri dishes
2. Alginate solution and calcium chloride solution
3. Sterile 50 mL tubes or sterile 100 mL beakers for calcium solution
4. Sterile pipettes (2 mL) for making beads
5. Pre-incubation medium in 100 mL flask
6. Nodal segments without leaves, consisting of a petiole and stem, (5–10 mm) cultured for 10 to 14 days to induce axillary buds (Fig. 12.2)

Day 2: Preculture beads

1. Tools, sterile pipettes (2 mL) for draining
2. Preculture medium

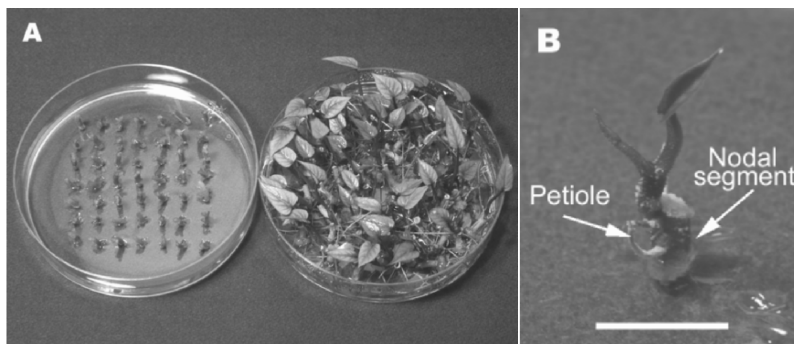

Fig. 12.2 Nodal segment culture and induced axillary buds. A: Nodal segments on day 0 (left) and 14 days after plating. B: Axillary bud developed from nodal segment. White bar indicates 1 cm. Material: *Ipomoea batatas* (L.) Lam. cv. 'Beniazuma'

Day 3: Osmoprotect, Dehydrate, and Cryopreserve beads

1. Tools, sterile pipettes (2 mL) for draining
2. Osmoprotection solution, PVS2, rinsing solution
3. Cryotubes (2 mL), Parafilm, markers
4. Recovery medium 1

Day 10: Change medium: Tools, recovery medium 2

Prepare in advance

1. Nodal segments: Nodal segments on solid MS (Murashige and Skoog 1962) with 30 g/L sucrose, 1 g/L casamino acid, 2 g/L gellan-gum and 0.5 mg/L BA at 25°C
2. Alginate solution: Liquid MS without calcium chloride and with 20 g/L Na-alginate and 0.4 M sucrose (in a 15 mL plastic tube)
3. Calcium chloride solution: Liquid MS with 0.1 M calcium chloride, 0.4 M sucrose (in a 50 mL plastic tube)
4. Pre-incubation medium: Liquid MS with 30 g/L sucrose and 1 g/L casamino acid
5. Preculture medium: Liquid MS with 0.3 M sucrose and 1 g/L casamino acid
6. Osmoprotection solution: Liquid MS with 2 M glycerol and 1.6 M sucrose (in a 500 mL bottle)
7. PVS2: Liquid MS with 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (in a 500 mL bottle)
8. Rinsing solution: Liquid MS medium with 1.2 M sucrose (in a 50 mL plastic tube)
9. Recovery medium 1: MS medium with 30 g/L sucrose, 1 g/L casamino acid, 2 g/L gellan-gum, 0.5 mg/L BA and 1 mg/L GA_3
10. Recovery medium 2: Recovery medium 1 without BA
11. Sterile pipettes for forming beads and sterile 100 mL flasks for pre-incubation, preculture, osmoprotection and dehydration

The procedure

1. Dissect shoot tips into alginate solution in plastic dish or Petri dish.
2. Pull up alginate solution and one shoot tip with a sterile pipette and drip into calcium solution to make a bead. Avoid air bubbles in alginate solution.
3. Leave the beads in the solution for 20 min to firm up.
4. Place beads in pre-incubation medium in 100 mL flasks and shake gently at 25°C for 1 day (start pre-incubation at 5 pm). You need about 1 mL medium per bead.
5. Drain pre-incubation medium and add preculture medium and shake gently at 25°C for 16 h (start preculture at 5 pm.

6. Drain preculture medium and add osmoprotection solution, shake gently at 25°C for 4 h (from 9 am to 1 pm). Drain osmoprotection solution, add PVS2 and shake gently for 1 h (from 1 to 2 pm).
7. Place about 10 beads and 1 mL PVS2 in a cryotube and seal tightly with Parafilm. Place cryotubes on cane and submerge in LN.
8. To rewarm stir cryotubes in 40°C water for 2 min. Drain PVS2 immediately and replace twice at 10 min intervals with 1 mL rinsing solution.
9. Place beads on recovery medium 1. After 1 week place beads on recovery medium 2 to grow (Fig. 12.3). Three cultivars tested had 82–95% shoot formation after recovery.

Possible problems

1. Making beads just after dissecting makes this protocol simpler than the ordinary encapsulation vitrification.
2. By dissecting leaves from nodal segments you have uniform axillary buds for cryopreservation.
3. When you drop shoot tips and alginate solution into calcium solution, you need to adjust the height from the surface of calcium solution to make completely round beads.
4. To keep the same cooling rate/warming rate, you need to keep the volume of beads and PVS2 same in every cryotube.

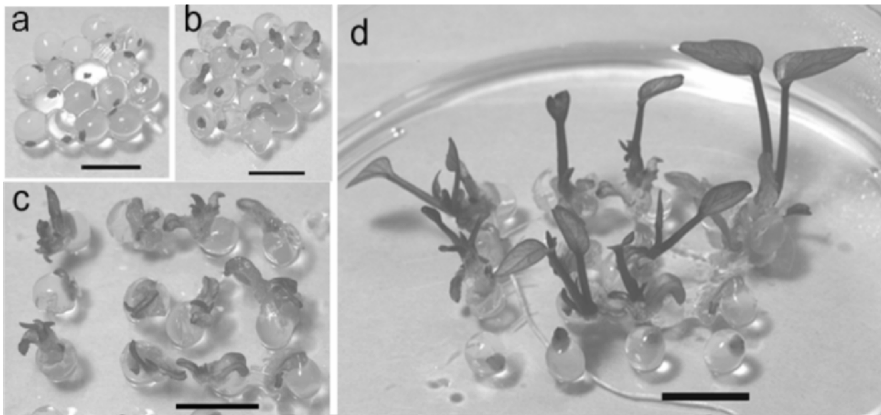

Fig. 12.3 Recovery growth of cryopreserved shoot tips by encapsulation vitrification. a, b, c, d: 2, 7, 14, 21 days after plating, respectively. Black bars indicate 1 cm. Material: *Ipomoea batatas* (L.) Lam. cv. Beniazuma (Matsumoto et al. 1995)

12.8.8 *Vitrification for* Limonium *Shoot Tips*

By T Matsumoto based on Sakai et al. 1990; Matsumoto et al. 1997, 1998

Day 1: Items needed to dissect shoot tips and preculture

1. Tools
2. Preculture medium: ½ strength MS (Murashige and Skoog 1962) (1/2 $K4NO_3$ and NH_4NO_3) with 0.3 M sucrose and 2 g/L gellan-gum

Day 2: Vitrification and recovery

1. Sterile Pasteur pipette for removing solutions
2. Cryotubes (about 2 mL)
3. Osmoprotective LS solution (2 M glycerol + 0.4 M sucrose in liquid MS medium)
4. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M sucrose in liquid MS medium
5. Rinsing solution: Liquid 1.2 M sucrose MS medium
6. Recovery medium: ½ MS medium with 0.1 mg/L BA, 0.01 mg/L NAA, 3% sucrose and 2 g/L gellan-gum
7. Dry sterile filter papers
8. Tools and sterile Petri dishes

Prepare in advance

1. Dissect shoot tips (about 1 × 1 mm) from 30 to 40 day-old *in vitro* plantlets (about 30 mm long) onto preculture medium.
2. Preculture meristems under continuous light for 16 h at 25°C.

The procedure

1. Place ten precultured meristems in a cryotube.
2. Add the osmoprotective LS solution for 20 min at 25°C.
3. Remove osmoprotective LS solution using Pasteur pipette.
4. Add PVS2 and hold for 15 min at 25°C or 50 min at 0°C (in crushed ice).
5. Remove PVS2 and add fresh PVS2 after 5 min at 25°C or 30 min at 0°C.

6. Exchange 1 mL of fresh PVS2 1 min before of the end of PVS2 treatment then plunge cryotubes into LN.
7. Warm cryotubes in 40°C water for 1 min with stirring.
8. Drain PVS2 immediately and add rinsing solution for 20 min at 25°C.
9. Transfer meristems onto a sterilized filter paper disc over recovery medium and culture under white fluorescent light (50 μM s^{-1} m^{-2}) with a 16 h photoperiod at 25°C.
10. After 1 day transfer the meristems onto a fresh paper disc onto a new dish of recovery medium and culture under the same conditions.
11. Regrowth is rated after 6–8 weeks (Fig. 12.4).

Possible problems

1. Meristems in cryotube are easily injured during draining and adding solutions using the Pasteur pipette. You need careful technique.
2. PVS2 is toxic to the plant material especially at 25°C. You need to finish the PVS2 treatment on time.

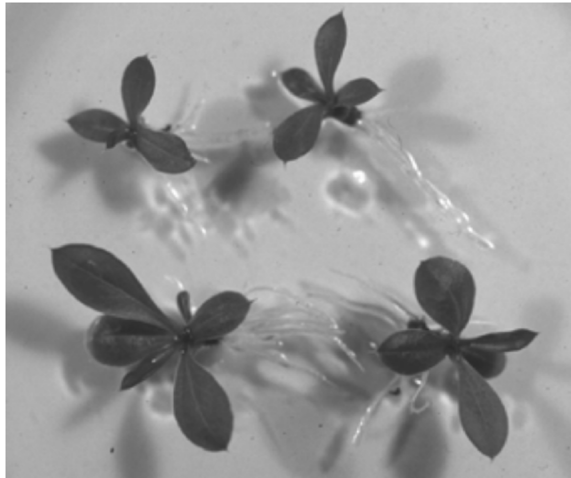

Fig. 12.4 Shoots formed from *Limonium* meristems vitrified in liquid nitrogen (LN), 60 days after plating. (From Matsumoto et al. 1998)

12.8.9 Encapsulation Vitrification of Mint Shoot Tips

By D Hirai based on Matsumoto et al. 1995

Items needed to dissect shoot tips and make beads

1. Tools, sterile Petri dishes
2. Alginate solution and calcium chloride solution
3. Sterile 50 mL plastic tubes or 100 mL beakers for calcium solution
4. Sterile pipettes (2 mL) for making beads
5. Cold acclimated nodal segments
6. Preconditioning and recovery medium: MS (Murashige and Skoog 1962) medium with 30 g/L sucrose, 1 g/L casamino acid, 2 g/L gellan-gum in plastic dishes

Items needed to osmoprotect, dehydrate, and cryopreserve beads

1. Osmoprotection solution
2. PVS2 at 0°C
3. Sterile 100 mL flasks
4. Tools and sterile pipettes
5. Rinsing solution
6. Cryotubes (2 mL), Parafilm, markers
7. Recovery medium

Prepare in advance

1. Nodal segments, consisting of a pair of leaves and 8–10 mm-long stem, incubated on preconditioning medium at 25°C for 1 day to induce axillary buds, then cold acclimated for 3 weeks (Fig. 12.5).

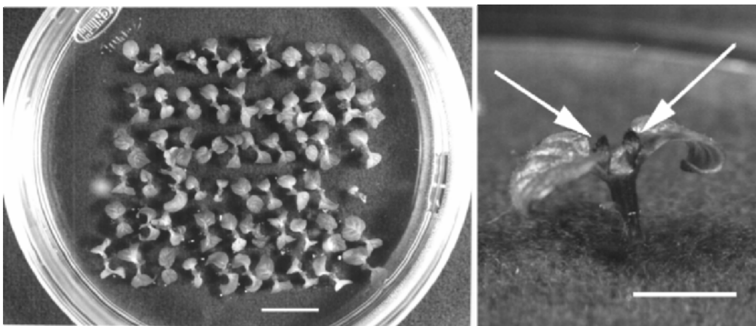

Fig. 12.5 Nodal segment culture (left) and induced axillary buds (right). White bars indicate 1 cm. Material: *M. spicata* L. cv. 'Spearmint common' (From Matsutmoto et al. 1995)

2. Alginate solution: Liquid MS without calcium and with 20 g/L Na-alginate and 0.4 M sucrose (in a 15 mL-plastic tube)
3. Calcium chloride solution: Liquid MS with 0.1 M calcium chloride, 0.4 M sucrose (in a 50 mL-plastic tube or a 500 mL bottle)
4. Osmoprotection solution: Liquid MS medium with 2 M glycerol and 0.4 M sucrose (in a 500 mL bottle)
5. PVS2: Liquid MS with 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (in a 500 mL bottle), filter sterilized
6. Rinsing solution: Liquid 1.2 M sucrose MS (in a 50 mL plastic tube)
7. Recovery medium
8. Sterile pipettes for forming beads and sterile 100 mL flasks

The procedure

1. Dissect shoot tips onto alginate solution in plastic dish or Petri dish. You need about 0.1 mL alginate solution for a shoot tip. At least ten shoot tips are necessary for a treatment.
2. Pull up alginate solution and a shoot tip with a sterile pipette and drop them into calcium solution to make a bead. Avoid air bubbles.
3. Leave the beads in the solution for 20 min to firm up.
4. Place beads in osmoprotection solution in 100 mL flasks on a shaker and shake gently at 25°C for 1 h.
5. Drain and add cool PVS2 and shake gently at 0°C for 3 h.
6. Place about 10 beads and 1 mL PVS2 in a cryotube and seal tightly with Parafilm. Place cryotubes on cane and submerge in LN.
7. To rewarm: Stir cryotubes in 40°C water for 2 min. Immediately drain PVS2 and replace twice at 10 min intervals with 1 ml rinsing solution.
8. Place beads on recovery to grow. Note regrowth at 6–8 weeks (Fig. 12.6; Table 12.4)

Notes

1. Osmoprotection is to increase the tolerance of shoot tips for osmotic dehydration by PVS2.
2. When you drop shoot tips and alginate solution into calcium solution, you need to adjust the height from the surface of calcium solution to make completely round beads.

3. When you need shoot tips without cold acclimation; dissect shoot tips from nodal segments incubated at 25°C for 3–5 days and use liquid MS medium with 2 M glycerol and 0.8 M sucrose as osmoprotection solution.
4. To keep the same cooling/warming rate, you need to keep the same volumes of beads and PVS2 in every cryotube.

Table 7.4 Shoot formation of cold-acclimated or non-acclimated shoot tips from three cultivars of mint cryopreserved by encapsulation-vitrification. Encapsulated shoot tips osmoprotected with MS liquid medium containing 2 M glycerol + 0.4 M sucrose (cold-acclimated shoot tips) or 2 M glycerol + 0.8 M sucrose (non-acclimated shoot tips) were dehydrated with PVS2 for 3 h at 0°C prior to LN.

Cultivars	Shoot formation (% ± standard errors)	
	Cold-acclimated	Non-acclimated
Spearmint common	87.0 ± 8.9	63.0 ± 8.5
Shubi	72.6 ± 11.3	54.0 ± 13.2
Ezo	97.0 ± 3.0	89.7 ± 5.2

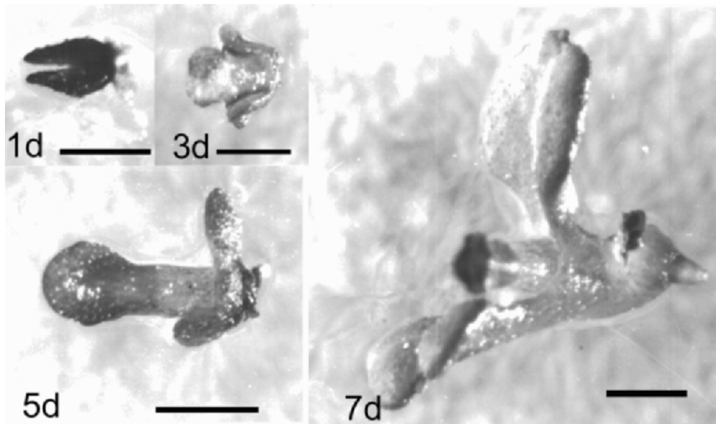

Fig. 12.6 Recovery growth of shoot tips cryopreserved by encapsulation vitrification 1, 3, 5, 7 days after plating. Black bars indicate 1 mm. Material: *M. spicata* L. cv. 'Common Spearmint' (From Matsumoto et al. 1995)

12.8.10 Vitrification of Innala (Solenostemon rotundifolius)

By T Niino based on Niino et al. 2000

Day 1: Items needed to dissect shoot tips and preculture

1. Tools
2. Preculture medium: MS (Murashige and Skoog 1962) medium with 0.3 M sucrose and 8.0 g/L agar at pH 5.8

Day 2: Vitrification and recovery

1. Sterile pipette tips for removing solutions
2. Modified osmoprotective LS solution (2 M glycerol + 0.4 M sucrose + 0.1 M $CaCl_2$ in liquid MS medium)
3. Cryotubes (about 2 mL)
4. Modified PVS2: (30% [W/V] glycerol + 15% [W/V] EG + 15% [W/V] DMSO + 0.4 M sucrose + 0.1 M $CaCl_2$ in liquid MS medium)
5. Rinsing solution: Liquid 1.0 M sucrose MS
6. Recovery medium: MS with 0.2 mg/L BA, 2.5% sucrose, 8.0 g/L agar at pH 5.8
7. Dry filter papers, tools, sterile Petri dishes

Prepare in advance

1. Preconditioning: To obtain lateral buds to be cryopreserved, nodal segments from *in vitro* grown shoots were cultured on MS medium with 0.1 M sucrose, 8.0 g/L agar at pH 5.8 for 3 weeks under a 16 h photoperiod 25°C
2. Preculture: Nodal segments (0.5–1.0 mm) with two lateral buds were dissected from the shoots and precultured on MS medium with 0.3 M sucrose and 8.0 g/L agar under an 8 h photoperiod at 5°C for 2 days.

The lateral buds of nodal segments to be cryopreserved should be globular stage buds (less than 0.1 mm in size), with meristem domes fully covered by the outer leaf primordia.

The procedure

1. Nodal segments are precultured for 2 days at 5°C.
2. Ten segments are transferred to 2 mL cryotube.
3. Osmoprotect with 1 mL modified osmoprotective LS solution in the cryotube for 20 min at 25°C.
4. Remove LS and add 1 mL modified PVS2 for 18 min at 25°C.
5. During the PVS2 treatment, the modified PVS2 is replaced once with fresh solution.
6. Just before immersion into LN, the modified PVS2 is replaced by 0.5 mL of fresh solution.
7. After 18 min of modified PVS2 treatment at 25°C, the cryotube is immersed into LN and stored.
8. Rewarm cryotube in 35°C water for 1 min with stirring. Then drain the modified PVS2 immediately and add rinsing solution for 20 min at 25°C.
9. Transfer buds onto MS medium for regrowth.

Notes

1. Exposure time to modified PVS2 is most important factor for producing high regrowth.
2. The preconditioning of nodal segments enables the production of a large numbers of uniform lateral buds. The correct stage of lateral buds should be selected.

12.8.11 Vitrification for Wasabi (Eutrema wasabi) *Shoot Tips*

By T Matsumoto based on Sakai et al. 1990; Matsumoto et al. 1994, 1995

Day 1: Items needed to dissect shoot tips and preculture

1. Tools
2. Preculture medium: modified MS (Murashige and Skoog 1962) (1/2 concentrations of KNO_3 and NH_4NO_3) with 0.3 M sucrose and 0.2% gellan gum

Day 2: Vitrification and recovery

1. Sterile Pasteur pipette for removing solutions
2. Cryotubes (about 2 mL)
3. Osmoprotective LS solution: 2 M glycerol + 0.4 M sucrose in liquid MS medium.
4. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M sucrose in liquid MS medium
5. Rinsing solution: Liquid 1.2 M sucrose MS medium
6. Recovery medium: solidified modified MS medium (1/2 concentrations of KNO_3 and NH_4NO_3) with 3% sucrose and 0.1 mg/L BA and 3% gellan gum
7. Filter papers
8. Tools and sterile Petri dishes

Prepare in advance

Shoot tips (about 1 × 1 mm) derived from 30 to 40 day-old *in vitro* plantlets (about 30 mm long) precultured on solidified MS with 0.3 M sucrose and maintained under continuous light for 16 h at 20°C.

The procedure

1. Preculture dissected meristems.
2. Place 10 precultured meristems in a cryotube and fill the tube with osmoprotective LS solution for 20 min at 25°C.
3. Drain osmoprotective LS solution using Pasteur pipette.
4. Add PVS2 for 10 min at 25°C; or 50–80 min at 0°C (on crushed ice). Drain PVS2 and add fresh PVS2 after 5 min at 25°C or 30 min at 0°C. Remove PVS2 and add 1 mL of fresh PVS2 1 min before of the end of PVS2 dehydration treatment.
5. After PVS2 treatment immerse cryotubes into LN.

6. Warm cryotubes in 40°C water for 1 min with stirring. Drain PVS2 immediately and fill tube with rinsing solution for 20 min at 25°C.
7. Transfer meristems onto a sterilized filter paper disc over recovery medium and culture under white fluorescent light (50 μM s^{-1} m^{-2}) with a 16 h photoperiod at 20°C.
8. After 1 day transfer the meristems onto a fresh paper disc over new recovery medium and culture under the same conditions.
9. Regrowth is noted at 6–8 weeks (Table 12.5; Fig. 12.7).

Notes

1. It is easy to injure meristems in a cryotube when draining and adding solutions using a Pasteur pipette. You need careful technique.
2. PVS2 is toxic to plant material, especially at 25°C. You need to finish the PVS2 treatment on time.

Table 12.5 Regrowth of wasabi meristems from four cultivars cooled to –196°C by vitrification (Matsumoto et al. 1994)

Cultivars	Regrowth (% ± standard error)
Shimane No.3	92.2 ± 1.7
Iwami	78.5 ± 2.9
Sanbe	81.8 ± 2.7
Rakan No.2	84.9 ± 2.6

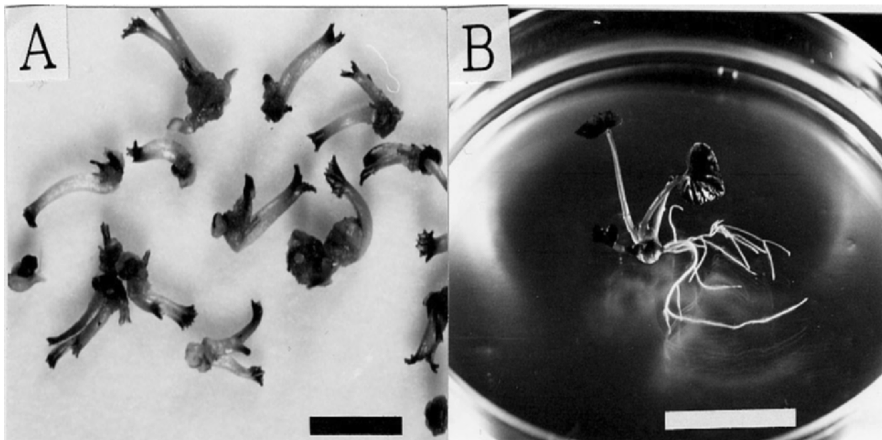

Fig. 12.7 *Eutrema* shoots developed from meristems cooled to –196°C by the vitrification technique 30 days (A) and 60 days (B) after reculture. (From Matsumoto et al. 1994)

12.8.12 Pelargonium *Encapsulation Dehydration*

By A Gallard based on Dumet et al. 2002

Day 1: Media preparation and sterilization (see solutions list)

1. Beakers 250 mL with 100 mL of preculture medium
2. One beaker of 250 mL with 100 mL of $CaCl_2$ solution
3. One bottle of 100 mL with 50 mL of 3% alginate solution
4. One sterile Petri dish with semisolid waiting medium (next page)
5. Glass boxes (10 cm diameter) with 40 g silica gel, regenerated at 80°C for 24 h.

Day 2: Alginate beads polymerization and preculture

1. Scalpels and forceps
2. Sterile Petri dishes
3. Shaker
4. Plants from the greenhouse

Day 4: Evaporative dehydration, cryopreservation and recovery

1. Forceps, silica gel boxes
2. Sterile filter papers (Whatman n°1 and Whatman n°3)
3. Cryotubes, aluminum canes for cryotubes
4. Liquid preculture medium, M0 medium for recovery

The procedure

Day 1: Cryopreservation

1. Excised apices are placed on a Petri dish of waiting medium.
2. Suspend the apices in alginate solution in the sterile Petri dish.
3. Draw up one apex and some alginate in a pipette and drip into 250 mL beaker of $CaCl_2$ solution. Hold for 10 min.
4. Transfer beads into 250 mL beaker of preculture solution (approximately 60 beads per beaker). Shake at 100 rpm for 43 h
5. Sterilize silica gel boxes in a Pasteur oven at 110°C for 4 h.

Day 3

1. Drain beads and dry on filter paper, then place on filter paper (Whatman 3) and put into silica gel boxes (20 beads per box).
2. The whole silica gel box and beads are placed at 28°C for 3 h (the final water content of beads must be around 0.25 g/g DW).
3. The dried beads are put into cryotubes and plunged into LN.
4. To warm add liquid preculture medium to cryotubes for 1 min.
5. Transfer beads containing apices onto M0 medium for recovery.

Media and Solutions

Based on MS (Murashige and Skoog 1962), Vitamins after Morel (1950)

Preculture solution

Macroelements MS	10%
Microelements MS	0.1%
Morel vitamins	0.2%
MES	0.5%
Sucrose	0.75 M
pH = 5.8	

$CaCl_2$ solution

Macroelements MS without calcium	10%
Microelements MS	0.1%
Morel vitamins	0.2%
Sucrose	30 g/L
Calcium chloride	14.7 g/L
pH = 5.8	

Alginate solution

Macroelements MS without calcium	10%
Microelements MS	0.1%
Morel vitamins	0.2%
Sucrose	30 g/L
Alginate (2% low viscosity)	30 g/L

Waiting medium

Macro and micro MS (Duchefa)	4.4 g/L
Morel vitamins	0.2%
Sucrose	30 g/L
Agarose LSM	6 g/L
pH = 6.12	

M0 medium

Macroelements MS	10%
Microelements MS	0.1%
Morel vitamins	0.2%
MES	0.5%
Sucrose	30 g/L
Active charcoal	2 g/L
AIA	0.5 mg/L
Ferric citrate	5 mL/L
Agarose LSM	6 g/L
pH = 5.8	

12.8.13 Encapsulation Dehydration of Hop Shoot Tips

By MA Revilla and H Fernández, based on Martínez D and Revilla MA 1998; Martinez et al. 1999; Revilla and Martinez 2002.

Prepare in advance

1. MS (Murashige and Skoog 1962) medium with 0.75% agar without hormones
2. Plants are grown on MS at 25°C for 16 h photoperiod at 40 µmol $m^{-2}s^{-1}$ with subcultures at 4 weeks.
3. Shoots are cold acclimated in the dark at 4°C for 1–2 weeks at the end of a culture period
4. Alginate solution: Liquid MS without calcium, with 3% low viscosity alginate and 0.5 M sucrose (in a flask)
5. Calcium chloride solution: Liquid MS with 100 mM calcium chloride for forming beads (in a flask)
6. Sterile 250 mL beakers for forming beads in calcium chloride.
7. Agar plates for holding shoot tips temporally
8. Sterile Pasteur pipettes (2 mL) for forming beads
9. Sterile sieves or tea strainers for removing beads from solutions.
10. Preculture medium: 0.75 M sucrose MS medium with 0.75% agar
11. Petri dishes with 30 g of silica gel (previously activated at 120°C for 2 days) covered with sterile filter paper for draining beads
12. Regrowth medium: MS with 1 mg/L BAP and 0.1 mg/L GA_3, 0.75% agar at pH 5.8

Items needed to dissect shoot tips and make beads

1. Flow cabinet, sterile sharp forceps and scalpel
2. Materials for dissection (sterile tile and filter papers)
3. Alginic acid (salt disodium) of low viscosity
4. Calcium chloride dolution, sterile 250 mL beakers
5. Cryotubes (2 mL) and markers
6. Cryocanes and a Dewar flask with LN
7. Sterile strainers, Petri dishes

The procedure

This is a 4 day procedure once the cold acclimation has been accomplished.

1. Dissect apical and axillary shoot tips (0.5–2.0 mm in length) including the meristematic dome with one or two pairs of foliar primordia and place them onto the agar plates.
2. Suspend the shoot tips in alginate solution in a tube.

3. Using a pipette pick up a shoot tip and some alginate and drop into a 250 mL beaker of calcium chloride solution to make beads. Avoid air bubbles in the alginate. Hold in the solution for 30 min.
4. Place beads on preculture medium for 2 days at 25°C in the dark.
5. Drain beads briefly on sterile filter paper to absorb excess moisture.
6. Place beads on top of a filter paper in an open sterile Petri dish (9 cm) containing 30 g of silica gel and dry in the air current of a laminar flow hood for 4 h (to 16% water content).
7. Place the beads in cryotubes, insert onto cryocanes, plunge in LN.
8. Rewarm at room temperature for 15 min. Transfer to Petri dishes of regrowth medium for 30 days at 25°C and 16 h photoperiod.
9. Emerging shoots are removed from the beads and cultured in MS medium without hormones (Fig. 12.8).

Results: Several cultivars were assayed and shoot recoveries from 50% to 90% were achieved for the following hop cultivars: First Gold, Fuggle, Challenger, J-78, Taurus, Chinook, Nugget, and H-7

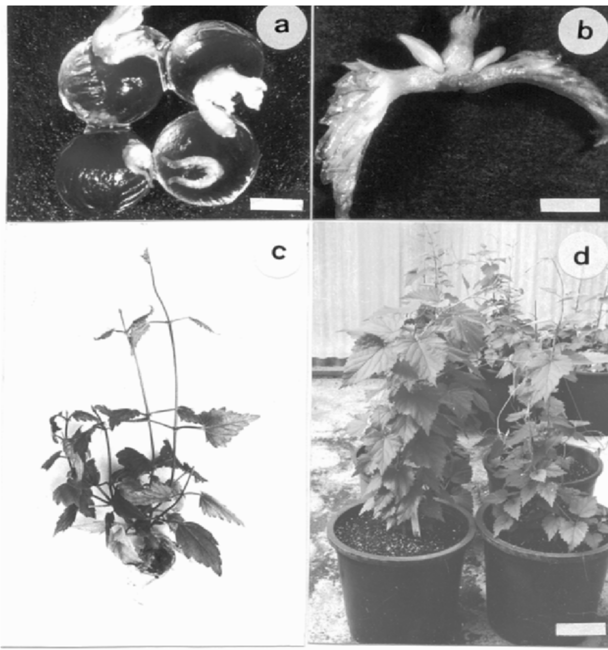

Fig. 12.8 Recovery of hop shoot-tips cryopreserved in liquid nitrogen (LN): **a.** shoot tips in alginate beads (bar 2 mm), **b.** shoot development (bar 2 mm), **c.** rooted plants and **d.** plants in the greenhouse (bar 10 cm), after 7, 30, 60, and 90 days of culture

12.8.14 Controlled Rate Cooling of **Humulus** *and* **Mentha**

By B Reed based on Reed et al. 2003; Uchendu and Reed 2007. This protocol can be applied to a wide range of germplasm from either genus.

Preparation of Supplies and Plant Materials

1. Forceps with very fine tip and scalpels with very sharp, fine blade.
2. Sterilized moist paper towel in Petri dish for dissecting shoot tips.
3. DMSO preculture medium. MS (Murashige and Skoog 1962) basal medium with 3% sucrose and 0.8% agar. Autoclave in a flask and while still warm add DMSO (5%) 50 mL per liter. Pour plates in the laminar flow hood. Wrap and store in the refrigerator.
4. Liquid MS medium in a 250 mL flask, at ~ 4°C
5. Cryotubes (1.2 mL) and cryogenic markers
6. Ice-bath with a plastic tray for holding cryotubes at 0°C
7. Plastic sterile pipettes (1.5–2 mL)
8. Filter sterilizer
9. PGD cryoprotectant: 10% each polyethylene glycol (MW 10,000), glucose, and DMSO in liquid MS medium.
10. Cryotube holder frozen in a container or in an ice tray
11. Thermometer and two 500 mL plastic beakers
12. Sterile dry filter paper in Petri-dishes
13. Cell wells or plates of recovery medium for each accession
14. Recovery medium: MS medium without auxin and 1 g/L less agar
15. Three weeks after last subculture plantlets are cold acclimatized with alternating temperatures and photoperiod [22°C with 8 h light (10 μM m^{-2} s^{-1}) and –1°C 16 h dark] for 2–5 weeks.

Preparation of PGD cryoprotectant

1. In a 50 mL graduated cylinder add 30 mL of liquid MS medium and small stir bar. Add 5 mL DMSO (reagent grade, do not use DMSO older than 1 year).
2. Slowly add 5 g glucose (dextrose) while stirring. Very slowly add 5 g polyethylene glycol while stirring and let stir until dissolved. Bring to volume (50 mL) with liquid MS medium, cover with Parafilm to mix.
3. Filter-sterilize cryoprotectant and place in the freezer until ready for use (at least 30 min but not much longer or it will freeze).
4. While the cryoprotectant is cooling you have 30 min to transfer the shoot tips to the cryotubes.

The procedure

Day 1: Dissect shoot tips (0.8–1.0 mm) and plate them on 5% DMSO preculture medium for 48 h in cold acclimation conditions.

Day 3

1. Dispense 0.25 mL cold MS medium to each 1.2 mL cryotube on ice.
2. Transfer shoot tips to the cryotube, 25 shoot tips per tube. Leave 5 or more shoot tips as non-freezing controls.
3. Add PGD cryoprotectant dropwise up to 1.2 mL over 30 min. Keep the cryotubes at 0°C for 30 min. Remove cryoprotectant to 1 mL mark and gently close the tube. Over tightening will cause leaking.
4. Cool to –40°C at 0.1°C/min in a programmable freezer and with exotherm at –9°C. To induce the exotherm of the cryoprotectant and the shoot tips, the freezer cools at 99.99°C/min to –50°C when the chamber reaches –9°C (you might shake the cryotube holder and make sure the cryoprotectant solution is frozen) and then warms at 20°C/min to –15°C, and resumes cooling down at 0.1°C/min to –40°C.
5. Plunge the cryotubes in liquid nitrogen.
6. Rewarm for 1 min in 45°C water with stirring, then move to 25°C water for 2 min.
7. Remove PGD and rinse with liquid MS. Pipette shoot tips onto dry sterile filter paper.
8. Transfer shoot tips to recovery medium and move them to normal growth room conditions under low light. Recover plants on growth medium without auxin to avoid callus formation
9. Shoots will begin to develop into plantlets in 3–4 weeks.

Notes

1. PGD is relatively non toxic so all rewarming can be completed before shoot tips are rinsed.
2. Controls can be rinsed and plated at the time of the exotherm, or after the freezer has finished.
3. The recovery medium is critical to the formation of callus, so auxin should be eliminated from the medium.

Chapter 13
Cryopreservation of Temperate Berry Crops

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13.1 Introduction

Preservation of clonal genotypes of most fruit crops requires vegetative propagation, so cryopreservation is an ideal method for long-term germplasm storage (Engelmann 2004). Some of the first and most successful experiments with the cryopreservation of apical meristems began with a temperate berry crop, strawberry (Kartha et al. 1980; Sakai et al. 1978). These initial studies showed that slow cooling of <1 mm shoot tips in cryoprotectant solutions was a feasible option for long-term storage of clonally propagated plants. During the last two decades, cryopreservation methods were developed for many species of fruit crops (Reed 2001).

13.2 Cryopreservation Techniques

Most of the existing cryopreservation techniques are effective for temperate berry crops. The availability of many techniques provides options for germplasm curators who wish to use cryopreservation to back up plant collections or for researchers who need to hold important plant materials for future use. Initially controlled rate cooling was the only available technique for the cryopreservation of plant tissues (Kartha et al. 1980; Sakai et al. 1978).

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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It is still an important protocol with a myriad of modifications (Chap. 5). It has the advantage that the cryoprotectants used are of low toxicity, many samples can be processed at one time and the procedure is mechanized to provide a standardized cooling protocol. Controlled rate cooling is very successful for temperate and some subtropical crops. Acclimation of *in vitro* plants to either cold temperatures or high sucrose concentrations is needed to develop freezing tolerance; however, once this is established shoot tips can be stored successfully. For *in vitro* grown shoot tips, a very slow cooling rate (0.1°C/min to –40°C) produces high regrowth for many species including: *Vaccinium, Pyrus, Malus*, *Morus*, *Fragaria* and *Vitis* (Chang et al. 1992; Plessis et al. 1993; Reed 1989, 1990, 1992, 1993; Reed and Hummer 1995). Cryoprotectants, most commonly DMSO or mixtures containing DMSO, are commonly used. Highly concentrated vitrification solutions (PVS2) are now used for some controlled rate cooling methods (Brison et al. 1995).

Vitrification techniques are also successful with temperate berry crops (Chap. 3). Plantlets require conditioning treatments for this technique as well, usually cold acclimation or preculture with sucrose. Most plants are sensitive to the highly viscous vitrification solutions and thus require osmotic conditioning solutions or other preculture treatments. Vitrification using plant vitrification solution number two (PVS2) (Sakai et al. 1991) is commonly used.

Encapsulation dehydration of shoot tips is successful for many plant types (Chap. 4). In a few cases plants do not adapt well to encapsulation or to the high sucrose concentrations required, but for most this is a very successful technique. Encapsulation dehydration (ED) (Dereuddre et al. 1990) is also successfully used for most temperate berry crops.

Difficult genotypes may be successfully cryopreserved by combining methods. Encapsulated grape axillary-shoot tips were cooled at 0.1°C/min to –100°C before LN exposure; shoot formation increased significantly over encapsulation alone (Plessis et al. 1993). Encapsulation vitrification was developed to overcome some of the toxic effects of the PVS2 vitrification solution and the difficulty of air dehydrating some genotypes (Hirai et al. 1998; Sakai et al. 2000).

13.3 Cryopreservation

13.3.1 Actinidia

Jian and Sun (1989) studied cryopreservation of dormant Chinese gooseberry, also called fuzzy kiwifruit or hardy kiwifruit (*Actinidia chinensis* Planch.) stem segments. Surface-sterilized winter shoots were cut into four

segments and cryoprotected with combinations of cryoprotectants. Shoot segments were cooled at 1.0°C/min to –40°C and held at –40°C for 2 h before immersion in liquid nitrogen (LN). Following fast warming 75% of segments produced callus and adventitious shoots were produced from the callus. Bachiri et al. (2001) developed an optimized ED protocol for *in-vitro* grown *Actinidia* hybrids with 85–95% regrowth of several genotypes (Table 13.1) (For protocol see 13.5.1). By gradually increasing the sucrose concentration of the incubation medium and drying over silica gel to 20% moisture content, regrowth was obtained for most of the genotypes tested. Preliminary studies with PVS2 vitrification were not successful due to the sensitivity of the shoots to highly osmotic solutions. The recovery medium was also very important with a softer agar concentration and changes in the plant growth regulators that contributed to the good recovery. An additional ED protocol for *A. deliciosa* cultivars Tomuri M and Tomuri F and *A. chinensis* var *chinensis* was developed using extended culture, cold acclimation and serial culture of encapsulated shoot tips on solid medium with increasing sucrose concentrations (Wu et al. 2001). The conditioned beads were dehydrated to 26% moisture and slowly cooled to –40°C before LN exposure. Regrowth of 22–56% was noted for three accessions. There was little or no recovery from samples that were rapidly cooled.

Table 13.1 Regrowth of *Actinidia* species and hybrids following cryopreservation by encapsulation dehydration. 14 Days preconditioning, progressive sucrose concentration increase to 0.75 M and 4 days incubation in 0.75 M liquid sucrose medium (Bachiri et al. 2001)

Species or hybrid (ploidy)	% Regrowth after cryopreservation
A. deliciosa (cv. Hayward, 6 X)	83±4.2
A. deliciosa (cv. Tomuri, 6X)	68±6.1
A. chinensis (2X)	76±7.1
A. chinensis (4X)	≤10
A. arguta x A. deliciosa (6X)	85±8
A. chinensis x A. eriantha (2X)	58±7.8
A. chinensis x A. callosa (2X)	65±7.2
A. eriantha x A. callosa (2X)	75±4.3
A. chrysantha x A. arguta (4X)	≤10

13.3.2 Fragaria

Sakai et al. (1978) first reported that apices of field-grown strawberry runners *Fragaria x ananassa* Duch. cv. Hokowase slowly precooled to –20 to –30°C and plunged into liquid nitrogen, developed normal shoots *in vitro*

after warming. Regrowth ranged from 60 to 80% for apices slowly cooled in the presence of 12–16% DMSO and then plunged in LN. *In vitro* grown meristems of *F. x ananassa* 'Redcoat' precultured on medium with 5% DMSO, cryoprotected with 5% DMSO and cooled at 0.84°C/min or with 5% glycerol and cooled at 0.94°C/min to –40°C before LN had 35–55% regrowth (Kartha et al. 1980). Fifty-six *Fragaria* species and cultivars were tested for possible cryopreservation by controlled rate cooling at the USDA-ARS National Clonal Germplasm Repository-Corvallis (Reed and Hummer 1995). *In vitro* plantlets were cold acclimated, 0.8 mm meristems pretreated on 5% DMSO medium for 2 days, then cooled at 0.8°C/min to –40°C in the cryoprotectant PGD (a combination of 10% polyethylene glycol, 10% glucose and 10% DMSO) and plunged into LN. Regrowth was dependent upon genotype; 25 of the 56 genotypes had greater than 50% regrowth. A slower cooling rate (0.3°C/min) improved regrowth (Protocol 13.5.3). No off-types were found during field evaluations of 15 cultivars regrown from cryopreserved meristems at the NCGR-Corvallis (Reed and Hummer 1995). The use of glucose in lieu of sucrose in cryoprotection was also noted by Vysotskaya et al. (1999b) (Protocol 13.5.4). The first test of encapsulation dehydration successfully preserved axillary buds of several cultivars after 2 months of cold acclimation (Navatel and Capron 1997). An encapsulation–dehydration protocol using a loading solution in the beads is the basis of storage of germplasm in Spain (Clavero-Ramirez et al. 2005; Clavero-Ramirez et al. 2004).

Encapsulation vitrification developed by Hirai et al. (1998) showed that 2-week cold acclimation followed by encapsulation and PVS2 vitrification produced twice the recovery (95%) of encapsulation dehydration alone (40%). Vitrification of strawberry produced 93% success using cold-acclimated shoot tips, precultured on sucrose and glycerol, treated with loading solution and PVS2 at room temperature for 50 min and rapid warming (Niino et al. 2003) (Protocol 13.5.2). The PVS2 vitrification protocol provided the basis for a strawberry germplasm storage system in Japan and is also used at the USDA-ARS National Center for Genetic Resources Conservation (NCGRP), Fort Collins, Colorado, USA.

13.3.3 Ribes

Sakai and Nishiyama (1978) first demonstrated that winter-dormant *Ribes* buds (gooseberry cv. Oregon Champion and red currant cv. London Market) could be cooled at 5°C/day to –40°C, plunged into LN, and slowly warmed with 100% regrowth. The first *in vitro* studies showed that controlled rate cooling, vitrification and encapsulation–dehydration techniques

were successful for cryopreserving apical meristems from *in vitro* grown *Ribes* (Reed et al. 2000, 2001, 2005 Reed and Hummer 2002; Reed and Yu 1995). *R. diacantha* Pall. meristems had moderate to high regrowth with each of the modifications tested and most methods resulted in regrowth for some *R. aureum* Pursh meristems. *R. rubrum* L. cv. Cherry meristems had moderate (60%) regrowth with encapsulation dehydration (3 h dehydration) and very poor results with the other techniques. Vitrification with PVS2 was successful for several *Ribes* genotypes when preculture treatments were instituted (Luo and Reed 1997). Differential scanning calorimetry analysis of *Ribes* species showed that vitrification with PVS2 produced stable glasses while ED did not (Benson et al. 1996). Conditioning shoots with either cold acclimation or sucrose pregrowth provided good recovery for many genotypes (Dumet et al. 2000b) Further studies of sucrose pretreatment of shoot tips to replace cold acclimation, found very successful regrowth for a wide range of black currant (*R. nigrum* L.) genotypes following sucrose pretreatment and encapsulation dehydration, while gooseberry genotypes responded poorly to the technique (Dumet et al. 2000a; Reed et al. 2005) (Table 13.2). Germplasm storage of *Ribes* at NCGRP uses the ED technique with cold acclimation (Protocol 13.5.5).

Table 13.2 Regrowth of *Ribes* shoot tips following three critical steps in the encapsulation-dehydration process: osmotic desiccation in sucrose for 22 h, evaporative desiccation under laminar flow for 4 h and exposure to liquid nitrogen for 24 h.

***Ribes* taxon**	**Cultivar/ accession number**[y]	**Regrowth of shoot tips (%)**[z]		
		Osmotic desiccation	**Evaporative desiccation**	**Liquid nitrogen**
R. cereum Douglas	237.001	0	20	10
R. diacantha Pallas	31.001	100	90	10
R. nigrum L	Baldwin/746.001	NA[x]	80	60
	Ben More	NA	NA	70
	Ben Tron	NA	100	83
	Consort/307.001	100	88	90
	Crusader/121.001	NA	70	60
	Kerry/377.001	100	100	92
	Malling Jet/376.001	100	90	60
	Ojebyn/998.001	100	100	91
	Sopiernik/426.001	100	100	57
	Tenah/410.001	40	87	50
	Topsy/5.001	80	100	70
	Willoughby/385.001	100	50	60
R. odoratum Wendl.f.	Crandal 216.001	NA	60	60

R. rubrum L	Gloire des Sablons/ 314.001	100	30	40
	Portal Ruby/269.001	100	83	60
	White Dutch/387.001	100	90	81
R. sanguineum Pursh	King Edward VII/ 761.001	100	30	28
R. species	355.001	NA	100	80
R. uva-crispa L	Golda/205.001	100	57	0
R. viscosissimum Pursh	281.001	100	71	84

All data taken after 8-week recovery on RIB medium (Reed et al. 2005). [z]Shoot tips producing shoots in 4 weeks. Based on five shoot tips of each genotype for osmotic dehydration and 10 each for evaporative desiccation and liquid nitrogen exposure. [y]Identifying accession number from the National Clonal Germplasm Repository, Corvallis, OR. [x]NA – No data available

Mean regrowth at each step± SD. 88.8 ± 28.3 76 ± 25.6 58.9 ± 26.9

13.3.4 Rubus

The first cryopreservation of *Rubus* was with winter dormant buds of raspberry (*Rubus idaeus* L. cv. Latham) that were cooled at 5°C/day to –40°C, immersed in LN and slowly warmed with 100% regrowth (Sakai and Nishiyama 1978). The first shoot tips of actively growing *Rubus in vitro* grown plants cryopreserved used the combination cryoprotectant PGD (Reed and Lagerstedt 1987). This cryoprotectant solution of 10% each polyethylene glycol, glucose and DMSO (Finkle and Ulrich 1979) and controlled rate cooling at 0.5°C/min to –35°C before LN produced good results with *in vitro* grown *Rubus* meristems.

Temperate fruit crops naturally prepare for dormancy during exposure to the colder temperatures of fall. During this time there is a substantial increase in the concentrations of sugars and proteins in the cells. Based on this natural phenomenon, the first successful use of cold acclimation for cryopreservation demonstrated that even short-term acclimation greatly increased recovery from cryopreservation. Alternating-temperature cold acclimation (22°C 8-h day/–1°C night) more than doubled regrowth of cryopreserved *Rubus* meristems (Reed 1988; Reed and Lagerstedt 1987). Non-acclimated meristems of two genotypes were recovered at 18% and 41% while cold-acclimated plants reached 51–67% regrowth following LN. An additional study showed that 1 week of cold acclimation was effective for most genotypes tested, and a combination of 50 µM ABA and

cold acclimation significantly improved regrowth for one *Rubus* genotype (Reed 1993).

Exclusion of auxin from the recovery medium produced greatly improved recovery of shoot tips for a range of *Rubus* species (Chang and Reed 1999) (Protocol 13.5.7). A comparison of recovery media found better regrowth for meristems grown on Murashige and Skoog (1962) based medium rather than on Anderson's (1980) medium (Reed 1993). Extending cold acclimation from 1 week to as long as 10 weeks was needed to increase recovery of some Rubus shoot tips to >80% following controlled rate cooling and was genotype dependent (Chang and Reed 1999, 2000).

Recovery of seven raspberry genotypes following encapsulation dehydration averaged 50% recovery when dried to 17% moisture while regrowth of meristems following encapsulation vitrification was 68% with a 90 min osmotic loading solution exposure and 180 min PVS2 (Wang et al. 2005) (Protocol 13.5.6). Encapsulation dehydration and PVS2 vitrification were both successful for 25 raspberry and blackberry genotypes (Gupta and Reed 2006). Encapsulation–dehydration regrowth following drying to 20% moisture ranged from 60 to 100% (Table 13.3) while that for vitrification with 20 min osmotic loading solution and 20 min PVS2 at 25°C was 40–75%. Three genotypes compared directly with the two techniques averaged 71% regrowth from PVS2 vitrification and 92% from encapsulation dehydration. In general, all three of the main techniques are directly applicable to a wide range of *Rubus* germplasm.

Table 13.3 Regrowth of shoot apices of 18 *Rubus* genotypes cryopreserved using encapsulation dehydration with 6 h desiccation and rapid warming. Results are for 30 shoot tips removed following each step of the process (Gupta and Reed 2006)

Taxon & cultivar	U.S. plant introduction number	Regrowth at 4 weeks (%)			
		Encapsulation	Osmotic dehydration	Air dehydration	LN
Blackberry					
R. caesius (wild)	324058	100	100	80	80
R. caucasicus (seedling selection)	553143	100	100	80	80
R. cissoides Bush Lawyer (wild)	553163	100	100	100	80
R. drejeri (wild)	553186	100	100	100	100
R. hybrid 'Cherokee'	553247	100	100	80	80
R. hybrid 'Dirksen Thornless'	553251	100	100	80	95

R. hybrid 'Ebony King'	553251	100	100	80	95
R. hybrid 'Olallie'	553255	100	100	100	90
R. hybrid 'Ashton Cross'	553278	100	100	100	90
R. hybrid OSU & USDA selection	553281	100	100	100	90
R. hybrid 'Chester Thornless'	553322	100	100	80	90
R. hybrid 'Loch Ness'	638182	100	100	80	70
R. hybrid 'Black Diamond'	638257	100	100	100	95
Raspberry					
R. idaeus 'Skeena'	553374	100	100	80	60
R. idaeus 'Washington'	553380	100	100	100	100
R. idaeus 'Willamette'	553362	100	100	100	80
R. idaeus 'Comet'	553556	100	100	100	100
R. occidentalis 'Munger'	553740	100	100	100	75

13.3.5 Vaccinium

Reed (1989) cryopreserved *in vitro* grown *Vaccinium* meristems by controlled rate cooling with the cryoprotectant PGD (10% each polyethylene glycol, glucose and DMSO). *V. corymbosum* L. regrowth increased from 6 to 58% following three or more weeks of alternating-temperature cold acclimation (22°C day/–1°C night) but *V. ovatum* Pursh. and *V. uliginosum* L. improved only slightly. Cooling meristems at 0.1°C/min produced the best regrowth. Cold acclimation requirements and optimum cooling rates varied among species. Work is ongoing to test additional techniques with a range of germplasm (Uchendu and Reed personal communication).

13.3.6 Vitis

Early studies with grape worked mainly with dormant buds (Dereuddre et al. 1993; Leddet et al. 1993). Plessis et al. (1993; 1991) successfully

encapsulated in alginate beads, precultured in sucrose-enriched medium, dehydrated at room temperature and slowly cooled from 20°C to –80°C, then immersed in LN with 20% regrowth. Regrowth of 30% was noted using low prefreezing temperatures (–100°C) and slow cooling rates (0.1°C/min). Plessis et al. (1993) tested 'Chardonnay' grape with encapsulation dehydration with moderate success. The first vitrification study of grape was completed with *V. vinifera* 'Cabernet Sauvignon' (Matsumoto and Sakai 2000) (Protocol 13.5.9). Recovery reached 80% following a 3-day preculture on 0.3 M sucrose, treatment with osmotic loading solution for 20 min at 25°C, 50% PVS2 for 30 min and PVS2 for 50 min at 0°C. This technique was applied to ten other *Vitis* accessions with average recovery of 64% (Matsumoto and Sakai 2003) (Table 13.4, Fig. 13.1). Wang et al. (2000) tested encapsulation dehydration with two grape cultivars with moderate success (Protocol 13.5.8). Successful recovery of plants required 4 days of preculture on increasing sucrose concentrations followed by dehydration to 15–18% moisture content and rapid warming. Wang et al. (2003) were able to eliminate grapevine virus A from 97% of 1–1.5 mm shoot tips through cryopreservation using ED and VIT protocols. Cryopreservation is known to aid in the elimination of some viruses by selectively killing virus infected cells while the uninfected meristematic cells survive (Helliot et al. 2002; Wang et al. 2003).

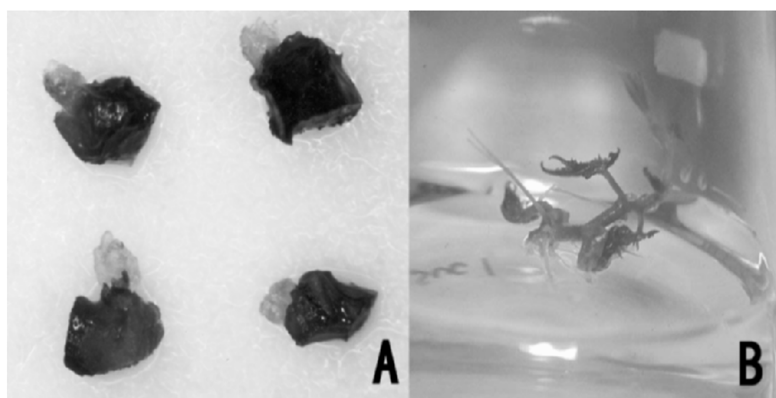

Fig. 13.1 Plantlet developed from meristems of grape cooled to –196°C by vitrification 20 days (A) and 100 days (B) after reculture (Matsumoto and Sakai 2003)

Table 13.4 Regrowth of grape cultivars and species cryopreserved by vitrification with PVS2 (Matsumoto and Sakai 2003)

Cryopreserved grape cultivars	
Cultivars/species	**Recovery (%SE)**
Wine grape variety (*V. vinifera*)	
Cabernet Sauvignon	85.0 ± 5.0
Merlot	86.7 ± 3.3
Cabernet Franc	33.3 ± 4.2
Chardonnay	73.3 ± 3.3
Table grape variety (*V. vinifera*)	
Muscat of Alexandria	76.7 ± 6.7
Rizamat	46.7 ± 8.8
Dried grape variety (*V. vinifera*)	
Thompson Seedless	56.7 ± 3.3
Root stock variety	
Teleki 5BB (*V. berlandi x V. riparia*)	30.0 ± 5.8
Teleli 5C (*V. berlandi x V. riparia*)	63.3 ± 3.3
1202 (*V. mourvedre x V. rupestris*)	75.0 ± 5.0
Wild grape	
V. coigneae	75.0 ± 5.0

13.4 Conclusions

Several major areas are critical for continued improvement in cryopreservation recovery. The choice of plant materials is an important consideration for any cryopreservation technique. It is very important to pay careful attention to the *in vitro* culture of plants destined for cryopreservation. Plantlets used for cryopreservation should be free of internal contaminants, show vigor but not excessive growth and lack physiological problems such as hyperhydricity (the physiological disorder causing water logging of tissues). Changes in the physiological status of a plant may produce varying recovery following cryopreservation (Reed 1993). More emphasis needs to be placed on the physiological condition of plants prior to cryopreservation. Genotypes vary widely in their response to cryopreservation, as well as to various cryopreservation techniques, but these differences can often be accommodated with small changes to the culture technique or pretreatments (Reed et al. 2003).

Research in cryopreservation techniques has greatly advanced in the last decade. These storage technologies are now available for many important temperate fruit and nut crops and are being developed for many more. *In vitro* and cryopreserved collections of important germplasm are now established in several countries (Reed 2001). *In vitro* collections are increasingly used for virus elimination, backup storage and distribution of clonal crops. Cryopreservation is now practical for secure backup collections for clonal crops formerly only preserved as field collections.

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13.5 Protocols

13.5.1 Encapsulation Dehydration of Kiwifruit (Actinidia)

Based on Bachiri et al. 2001

Plant Materials and growth medium

1. Two weeks prior to cryopreservation place single node cuttings onto fresh standard growth medium and grow for 2 weeks.
2. Excise shoot tips (0.75–1.5 mm) and place on basal MS (Murashige and Skoog 1962) medium overnight in the dark.
3. Growth medium: MS with 30 g/l sucrose, 6.5 g agar, pH 5.8, 2 mg/l benzyl amino purine (BAP), 0.05 mg/l naphthalene acetic acid for *A. deliciosa* and 1 mg/l BAP and 1 mg/l zeatin for other genotypes.

Items needed to make beads

1. Tools, sterile Petri dishes, sterile filter papers
2. Alginate solution: calcium-free MS liquid medium with alginic acid (3% low viscosity)
3. Sterile 5 ml pipettes for bead formation
4. 50 ml beaker or Petri dish for mixing meristems with alginate
5. Calcium chloride solution: MS liquid medium with 100 mM $CaCl_2$
6. Sterile 250 ml beakers for calcium chloride
7. Sterile strainers for removing beads from solutions
8. Pretreated shoot tips
9. You will need a separate 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile Petri dish and pipette per treatment (or genotype)
10. Preculture solution: 0.3, 0.5 and 0.75 M sucrose in MS medium in 100 ml flasks (1 flask per genotype), gyratory shaker

Dehydration and cryopreservation

1. Sterile strainers, sterile 250 ml beaker
2. Sterile dry filter paper for draining beads
3. Sterile Petri dishes with 40 g silica gel for drying beads
4. Tools and sterile Petri dishes
5. Cryotubes and markers
6. Recovery medium with 5.5 g/l agar (Standard medium without NAA)

The procedure

1. Dissect meristems from nodal cuttings onto regular agar plates and hold in the dark overnight.
2. Suspend meristems in alginate solution in a small sterile beaker or Petri dish. Using a sterile pipette, drop meristems in alginate into a 250 ml beaker of calcium chloride solution to make beads. Avoid air bubbles in the alginate.
3. Leave the beads in the solution for 20 min to firm up. Pour solution through sterile strainer to remove beads.
4. Place beads in 0.3 M sucrose in 125 ml flasks on a shaker for 1 day, transfer to 0.5 M sucrose for 1 day, then to 0.75 M sucrose for 2–4 days.
5. Drain beads using sterile strainer and briefly place on sterile filter paper in Petri dish to absorb excess moisture.
6. Place 20 beads in a sterile Petri dish with 40 g silica gel for ~4–5 h (22.5–19.5% moisture content). They should not touch each other or they will not dry properly. There should be no extra moisture in the dish (absorb with sterile filter paper if necessary).
7. Place beads in cryovials and submerge in liquid nitrogen.
8. Warm at room temperature in an empty Petri dish for 20 min before plating on recovery medium. Use a softer than normal recovery medium (5.5 g/l agar).
9. Place in the dark for 3 days before transfer to standard growth room conditions.

Notes

Controls should include all of the following during initial testing as some plants are sensitive to certain steps. Loss of many shoot tips at steps 1-4 requires investigation before further use of the technique. Some plants are very sensitive to the 0.75 M sucrose and the gradual increase in sucrose concentration is critical.

1. Dissection
2. Encapsulated shoot tips
3. Encapsulated shoot tips after sucrose treatment
4. Dried beads to 19–23% moisture content
5. LN exposed beads

A. deliciosa stock cultures require BAP plus NAA but for cryopreservation the recovery medium should exclude NAA or callus formation will result. See Table 13.1 for results.

13.5.2 Vitrification of Strawberry (Fragaria) *Shoot Tips*

By T Niino based on Niino et al. 2003

Basic requirements

Day 1: Items needed to dissect shoot tips and preculture

1. Tools
2. Preculture medium: solidified Murashige and Skoog (1962) (MS) medium with 0.3 M sucrose and 2 M glycerol in Petri dish

Day 2: Vitrification and recovery

1. Sterile pipette tips for removing solutions
2. Osmotic loading solution: 2 M glycerol + 0.4 M sucrose in liquid MS medium
3. Cryotubes (about 2 ml), liquid nitrogen
4. PVS2: 30% W/V glycerol + 15% W/V ethylene glycol + 15% W/V DMSO + 0.4 M sucrose in liquid MS medium
5. Rinsing solution: 1.0 M sucrose in liquid MS medium
6. Recovery medium: MS with 0.2 mg/l benzyladenine (BA), 1 g/l polyvinyl pyrrolidone (PVP), 2.5% sucrose, 8.0 g/l agar, pH 5.8
7. Sterile dry filter papers, tools and sterile Petri dishes

Prepare in advance

1. *In vitro* grown shoots are cultured on MS medium with 0.2 mg/l BA, 2.5% sucrose, 8.0 g/l agar at pH 5.8, 16 h photoperiod at 25°C
2. After the last subculture, 2-week-old normal shoots are cold-acclimated at 5°C, 8 h photoperiod for 20–30 days.
3. Shoot tips (1.0–1.5 mm in length) with 1–2 leaf primordia and 2 young leaf bases are dissected from the cold-acclimated shoots onto preculture medium and grown at 5°C with an 8 h photoperiod for 1 day.

The procedure

1. Precultured shoot tips (10) are transferred to 2 ml cryotube.
2. Add 1 ml osmotic loading solution for 20 min at 25°C.
3. Remove solution and hold with 1 ml PVS2 for 50 min at 25°C.
4. PVS2 is replaced once with fresh PVS2 solution during the 50 min and just before immersion into LN the PVS2 solution is again replaced with 0.5 ml of fresh PVS2 solution.
5. After 50 min of PVS2 treatment at 25°C, cryotube is immersed into LN.

6. For rewarming, cryotubes are rapidly warmed in 35°C water for 1 min with stirring. PVS2 is drained and replaced twice with 1.0 M sucrose solution at 25°C. Shoot tips are blotted and transferred onto the recovery MS medium.

Possible problems

1. Exposure time to PVS2 solutions is most important for producing high regrowth.
2. PVP and BA in the recovery medium increased the regrowth significantly compared with standard MS medium.

13.5.3 Strawberry (Fragaria) *and* Rubus *Controlled Rate Cooling*

By B Reed and Y Chang based on Reed and Hummer 1995; Chang and Reed 1999

Supplies

1. 2–3 forceps with very fine tips and 2–3 stainless scalpels
2. Sterilized moist paper towel in Petri dish for dissecting shoot tips
3. 1.2 ml cryotubes and permanent markers
4. Ice-bath with a plastic tray for holding cryotubes at 0°C
5. Plastic sterile pipettes (1.5 ml)
6. Filter flask for sterilizing cryoprotectant
7. Cryotube holder frozen in a container (10 × 20 cm) for maintaining the cryoprotectant at low temperature during the procedure
8. Thermometer and two 500 ml plastic beakers
9. Sterilized liquid MS medium in a 250 ml flask
10. Sterilized dry filter paper in Petri dish

Media

1. DMSO preculture medium: MS (Murashige and Skoog, 1962) medium with 3% sucrose and 0.8% agar. Autoclave in a flask and while still warm add DMSO (5%) 50 ml. Pour plates in the laminar flow hood.
2. Recovery medium: Welled culture plates (24 wells) or Petri plates with standard medium but without auxin and less agar (0.7%)
3. Strawberry NCGR-FRA medium: MS medium with 4.4 µM N^6 benzyladenine (BA), 3% sucrose and 0.6% agar
4. *Rubus* NCGR-RUB a modified Murashige and Skoog 1962 (MS) medium with doubled EDTA-Fe, 4.4 µM BA, 0.49 µM indole-3-butyric acid (IBA), 3% sucrose, 0.7% agar

Plant materials

1. Three weeks after the last subculture, cold acclimatize plantlets with alternating temperatures and photoperiod (22°C with 8-h light [10 µE m^{-1} s^{-1}] and –1°C 16-h dark) for 4 weeks.
2. Preculture: Meristems (< 1 mm) taken from these plants are grown for 2 days on a firmer medium (1 g more agar than normal) with 5% DMSO added.

Preparation of PGD cryoprotectant (a mixture of 10% each of polyethylene glycol [MW 10,000], glucose and dimethyl sulfoxide [DMSO] in liquid MS medium).

1. In a 50 ml graduated cylinder add 30 ml of liquid MS medium (no hormones) and small stir bar. Add 5 ml DMSO (reagent grade, do not use DMSO older than 1 year).
2. Slowly add 5 g glucose (dextrose) while stirring. Very slowly add 5 g polyethylene glycol while stirring and let stir until dissolved. Bring to volume (50 ml) with liquid MS medium, cover with Parafilm to mix.
3. Filter-sterilize cryoprotectant through a filter flask and place the flask in the freezer until ready for use (at least 30 min but not much longer or it will freeze).
4. While the cryoprotectant is cooling you have 30 min to transfer the shoot tips to the cryotubes.

The procedure

1. Two days before cooling: Dissect shoot tips (0.8–1.0 mm) and plate them on 5% DMSO pretreatment medium, hold for 48 h in cold acclimation conditions.
2. Day of cooling: Label the cryotubes. Dispense cold 0.25 ml MS medium to each 1.2 ml cryotube using a sterile plastic pipette and put them in a rack in an ice bath.
3. Use forceps or a sterile pick to collect the shoot tips from the DMSO pretreatment plates and transfer them to the cryotube with 0.25 ml MS medium at 0°C and 25 shoot tips per tube. Place dissection controls directly on recovery medium.
4. Add the PGD cryoprotectant dropwise up to 1.2 ml over 30 min on ice. Keep the cryotubes at 0°C for an additional 30 min for the cryoprotectant to equilibrate. Remove cryoprotectant down to the 1 ml mark and gently close the tube. Over tightening will cause leaking.
5. Program freezer
6. Cooling rate by genus:
7. *Fragaria* 0.3°C/min to –40°C
8. *Pyrus, Lolium*, *Humulus* 0.1°C/min to –40°C
9. *Rubus* 0.5°C/min to –35°C

10. Cool to –40°C at 0.3°C/min in a programmable freezer and with seeding and the resulting exotherm at –9°C. To induce the exotherm the programmable freezer cools at 99.99°C/min to –50°C after the chamber temperature reaches –9°C (you may need to shake the cryotube holder and make sure the cryoprotectant solution is frozen) and then warms at 20°C/min to –15°C, and resumes cooling down at 0.3°C/min to –40°C.
11. Plunge the cryotubes into liquid nitrogen.
12. Warm cryotubes in a 500 ml beaker with 45°C water for 1 min with stirring, then move to 25°C water for 2 min.
13. Rinse the shoot tips with liquid MS medium then dry by pipetting onto sterile filter paper. Transfer to recovery medium and move them to normal growth room conditions.
14. Shoots will begin to develop into plantlets in 4–6 weeks.
15. Good results were seen for most genotypes of *Rubus* (Table 13.5) and also for *Fragaria.*

Table 13.5 *Rubus* species and cultivars cryopreserved by controlled rate cooling in PGD at 0.5°C/min to –35°C and plunged in LN (Reed unpublished)

Species or cultivar	NCGR identifying number	Plant introduction number	% Regrowth	Fruit type
Hillemeyer	252.001	553275	56±9	Blackberry
Merton Thornless	254.001	553276	51±8	Blackberry
ORUS 1362	459.002	553305	33±25	Blackberry
R. caesius	1776.001	618497	60±7	Blackberry
R. grabowski	48.001	379534	40±23	Blackberry
R. ulmifolius	813.001	204915	51±6	Blackberry
Silvan	633.001	553308	74.5±6	Blackberry
Logan Thornless	253.001	553258	67±9	Hybrid berry
R. leucodermis BLJ-12–2	647.001	553680	28±6	Black raspberry
Heritage	285.001	553382	67±10	Raspberry
Mandarin	743.001	553493	33±15	Raspberry
R. hirstus	266.002	553230	10±3	Raspberry
R. parviflorus	52.001	553786	45.9±5	Raspberry
R. parvifolius	776.001	553820	84±6	Raspberry
R. spectabilis Red	256.001	553987	66±14	Raspberry

13.5.4 Controlled Rate Cooling of Strawberry

By Olga Vysotskaya and A S Popov based on Vysotskaya et al. 1999a; 1999b

Day 1: Preparation and preculture

1. Scalpel and forceps
2. Sterile Petri dishes (d = 30–40 mm or 90 mm)
3. Preculture medium: Liquid modified MS (Vysotskaya et al. 1999a; Vysotskaya et al. 1999b) with 5–6% sucrose or glucose, BA (1.0 mg/l), thidiazuron (0.2 mg/l), 5% DMSO
4. Cold acclimated plants for isolation of shoot tips
5. Binocular microscope, laminar box, tools, markers

Day 2: Cryopreservation

1. Cryotubes (2.0 ml) with 1.5 ml of cryoprotectant (7% DMSO and 6% sucrose or glucose in double distilled water) containing ice crystals
2. 5 mm × 15 mm filter paper strips
3. Sterile pipettes
4. Recovery medium: MS with 3% glucose, BA (0.5 mg/l),TDZ (0.1 mg/l) and 0.7% agarose (Sigma) in Petri dishes (d = 30–40 mm)
5. Binocular microscope
6. Cryobath: Container with liquid nitrogen and sample holder
7. Programmable freezer, Dewar for storage

Day 3: Plant recovery

1. Recovery medium
2. Water bath (40°C)

Prepare in advance

1. 3–4 week-old plantlets should be cold hardened at 4–8°C for 15–90 days in darkness.
2. Cryoprotectant: 7% DMSO in sterile MS with 5% sucrose

Cryopreservation: This is a 3-day procedure once the cold hardening is accomplished.

Day 1

1. Excise shoot apices (1.0–2.0 mm long and 1.0–2.0 mm diameter).
2. Preculture shoot apices for 16–19 h in Petri dishes (d = 30–40) on sterile filter papers placed on the surface of preculture medium at 4–8°C in darkness.

Day 2

1. Transfer apices to sterile filter papers crossed on the surface of cryoprotectant in cryotubes containing ice crystals (in tube holder in liquid nitrogen cryobath).
2. Put cryotubes into the programmable freezer precooled to –10°C, and cool at 0.3°C/min from –10°C to –40°C then at 10°C/min from –40°C to –70°C, then directly immerse in liquid nitrogen.
3. Store immersed in liquid nitrogen (–196°C).

Day 3
Rewarming

1. Warm cryotubes rapidly in 40°C water for 1–1.5 min, then place into ethyl alcohol (96%) for 10–30 s.
2. Remove paper with shoot apices out of cryotubes and place on the recovery medium and culture for 19–24 h at 20°C.
3. After 19–24 h shoot apices should be transferred to fresh recovery medium and at 20°C, 4–5 with a 16 h photoperiod.

Notes

Shoot require cold hardening and results may vary due to the physiological state of meristems. Proper cold-hardening of plantlets for more than 7 and less than 90 days can ensure background for high post-cryogenic survival of strawberry and red raspberry meristems. This cryopreservation procedure can be used to establish strawberry and red raspberry germplasm cryobanks (Table 13.6).

Table 13.6 Regrowth of strawberry meristems after cryopreservation by controlled rate cooling

Cultivar	Regrowth %	Cultivar	Regrowth %
Alaya Zorka	21.0	Red Star	35.5
Amulet	57.3	Redgauntlet	72.0
Feslival'naya	12.3	Roxana	29.4
Jesco	51.8	Talka	59.8
Kokinskaya pozd'naya	85.5	Tribute	66.9
Mont Everest	31.0	Tristar	61.3
Pandora	60.0	Vola	72.8
Purpurovaya	49.0	Zenit	56.8
Rapella	36.7		

13.5.5 Encapsulation Dehydration of Ribes

By B Reed based on Reed et al. 2005

Pretreatment A is for black currants only: Seven days in advance of cryopreservation cut 1 cm shoot tips from 3-week-old black currant plantlets and place on 0.75 M sucrose agar plates for 7 days in the growth room. This technique provides 90–100% regrowth from black currant shoot tips. It is not effective for red currants or gooseberries. Sucrose preconditioning plates for black currant: MS medium with 256.73 g/l sucrose, 3.5 g/l agar and 1.75 g/l Gelrite.

Pretreatment B is useful for all Ribes: Cold acclimate 3-week-old *in vitro* plantlets for 2–4 weeks with alternating temperatures (8 h at 22°C with low light (10 µM $m^{-2}s^{-1}$) and 16 hr at –1°C dark). This preconditioning treatment results in 60–100% regrowth of shoot tips.

The procedure takes parts of 2 days

Day 1: Items needed to dissect shoot tips and make beads

1. Tools, sterile Petri dishes, sterile filter papers
2. Agar medium to hold shoot tips until beads are made
3. Alginate solution: Murashige and Skoog 1962 (MS) liquid medium with no calcium with 256.73 g/l sucrose and 30 g/l alginic acid (3% low viscosity). Stir slowly and heat to dissolve, autoclave in advance. Usually 250–500 ml is sufficient.
4. Sterile 2 ml plastic pipettes or pipette tips for bead formation
5. 50 ml sterile beakers and Petri dishes
6. Calcium chloride solution: MS medium with 14.6 g/l $CaCl_2$
7. Sterile 250 ml beakers for calcium chloride
8. Sterile tea strainers for removing beads from solutions
9. MS medium with 0.75 M sucrose in 125 ml flasks
10. Preconditioned plants (A or B) for shoot tips
11. You will need a separate flask of 0.75 M sucrose, 250 ml beaker of calcium chloride medium, 100 ml beaker of alginate solution and at least one sterile Petri dish and pipette per treatment (or genotype).

Day 2: Dry beads and cryopreserve

1. Sterile strainers, sterile 250 ml beaker
2. Dry filter paper for draining beads
3. Sterile Petri dishes for drying beads (one for each 25 beads)
4. Tools and sterile Petri dishes
5. Liquid MS medium for rehydrating beads (10–25 ml)

6. Cryotubes and markers, information for labels
7. Recovery medium in 24 cell plates for each accession: NCGR-RIB growth medium (MS with 20 g/l glucose, 0.5 mg/l benzyladenine, 0.1 mg/l indole 3 butyric acid (IBA), 2.5g/l agar and 1.0 g/l Gelrite)

The procedure

Day 1

1. Dissect 1 mm meristems onto regular agar plates.
2. Place meristems in alginate solution in a small dish. Using a sterile pipette pick up one meristem and some alginate. Hold the pipette above the 250 ml beaker of calcium chloride solution and drip alginate with shoot tips to make beads. Avoid air bubbles in the alginate. Adjust the height above the solution so the beads are round.
3. Leave beads in the solution for 20 min to firm up. Pour solution through sterile strainer to drain beads. Separate out empty beads.
4. Place beads in 0.75 M sucrose in 125 ml flasks on a shaker for 18–22 h.

Day 2

1. Drain beads using sterile strainer and briefly place on sterile filter paper in Petri dish to absorb excess moisture.
2. Place beads in rows in open sterile Petri dishes and dry in the air flow for ~5 h (20% moisture content). They should not touch each other or they will not dry properly. There should be no extra moisture in the dish (absorb with sterile filter paper if necessary). Or use sealed Petri dishes with silica gel.
3. Moisture determination: Determine the fresh weight of 10 beads replicated three times, each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
4. Place beads in cryovials and submerge in liquid nitrogen one at a time, hold under the surface for 30 s then release. Or place vials on cane and submerge.
5. Warm for 1 min in 45°C water and 2 min in 25°C water then add liquid MS medium to the tube for 10 min to rehydrate, place beads on a softer recovery medium (1 g/l less agar than normal).
6. Place under low light (10 μM $m^{-2}s^{-1}$) for 1 week and move to higher intensity (40 μM $m^{-2}s^{-1}$) light and monitor for regrowth for another 3 weeks. Determine viability by the formation of normal shoots and multiple sets of leaves by week 4 of recovery. It is not necessary to remove the shoots from the beads if they are rehydrated.

Results

Recovery of all accessions was good (60–100%) using cold acclimation as a pretreatment (Reed and Yu 1995). All black currant accessions responded positively to the sucrose pretreatment protocol (Fig. 13.2: Table 13.2). The two cultivars of red currants and four species of gooseberries tested exhibited extremely varied, but generally poor, responses to the procedure (Table 13.2). PVS2 vitrification procedures also work well for some *Ribes* but controlled rate cooling gives poor results (Reed and Yu 1995; Luo and Reed 1997).

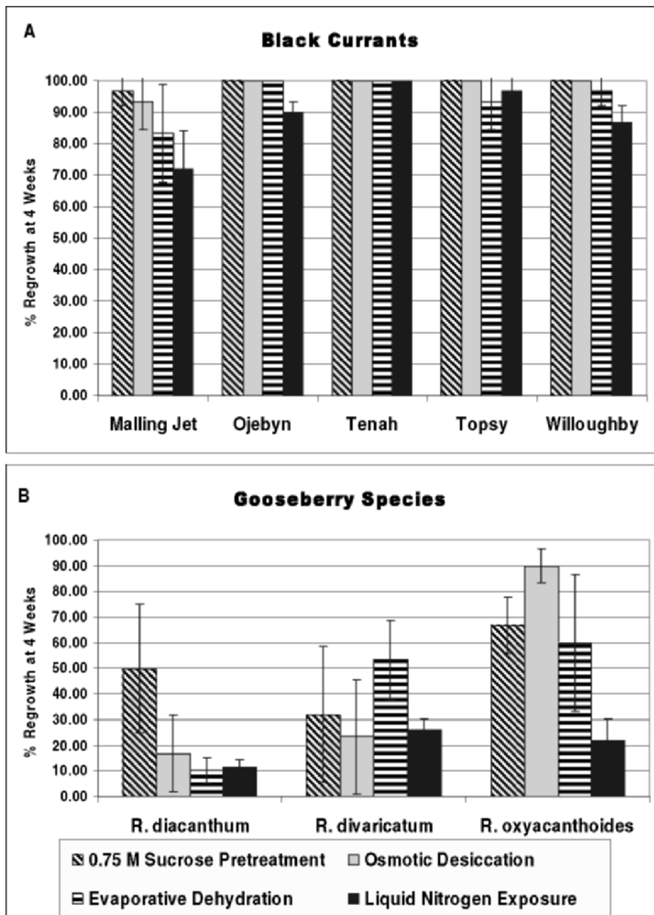

Fig. 13.2 Recovery of *Ribes* shoot tips following each of the steps in the modified encapsulation–dehydration cryopreservation protocol. (A) Black currant (*R. nigrum*) cultivars. (B) Gooseberry species. Means ± SD (Reed et al. 2005)

13.5.6 *Encapsulation Dehydration and Encapsulation Vitrification of Raspberry* (Rubus idaeus)

By Qiaochun Wang and Jari P.T. Valkonen, based on Wang et al. 2005

Basic requirements

1. Cryotubes (2 ml)
2. Erlenmeyer flasks (250 ml) and culture jars (8.5 × 8.5 cm)
3. Liquid nitrogen
4. Sterile pipettes (1 ml for making beads, 5 ml for solutions)
5. Petri dishes (9 cm in diameter), filter papers (9 cm in diameter)
6. Styrofoam container (for holding liquid nitrogen)
7. Water bath at 40°C

Preparation of media and solutions

1. Murashige and Skoog 1962 (MS) with (per liter) 40 mg Fe-EDTA, 30 g sucrose, 0.5 mg benzyl amino purine (BAP), 0.05 mg indole 3 butyric acid (IBA), 1.2 g Gelrite and 3.5 g agar, pH = 5.4
2. Pretreatment medium: MS with 2.5 g/l activated charcoal (AC) 30 ml per Petri dish
3. Modified calcium chloride solution: liquid MS medium with 100 mM calcium chloride, 2 M glycerol and 0.4 M sucrose, pH = 5.4
4. Modified alginate solution (2.5%): liquid MS medium without calcium with 25 g/l sodium alginate, 2 M glycerol and 0.4 M sucrose, pH = 5.4
5. Preculture medium: MS with 0.25, 0.5 or 0.75 M sucrose
6. Osmotic loading solution (LS): liquid MS with 0.4 M sucrose and 2 M glycerol, pH = 5.4
7. PVS2: 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) DMSO and 15% ethylene glycol, 0.4 M sucrose in liquid MS, pH 5.4
8. Rinsing solution: liquid MS with 1.0 M sucrose, pH 5.4
9. Recovery medium: MS medium
10. Stock cultures are grown in jars (8.5 × 8.5 cm) containing 60 ml of MS medium and maintained at a temperature of 24±2°C and under a 16-h photoperiod (50 μmol $s^{-1}m^{-2}$) provided by cool-white fluorescent tubes. Subculture is carried out every 4 weeks.

Procedure

The encapsulation–dehydration protocol

Day 1: Shoot tips (1 mm) are excised from 4-week-old stock cultures and placed on pretreatment medium for 3 days to overcome browning (Note 1).

Day 4

1. Suspend 10 shoot tips in 1.5 ml alginate solution in a Petri dish. Pick up one shoot tip and alginate with a sterile pipette (1 ml) and drop into 60 ml of 100 mM calcium chloride solution in a 250 ml Erlenmeyer, leave for 20 min.
2. Drain off liquid and place beads on sterile filter papers in a Petri dish for a few seconds to absorb excess moisture.

Day 4–7: Culture 20 beads each in a Petri dish containing 30 ml of 0.25 M sucrose preculture medium for 1 day, transfer to 0.5 M sucrose for 1 day, and then to 0.75 M sucrose for 1 day.

Day 8

1. Place 10 beads on each sterile filter paper placed in a Petri dish and dry in the laminar flow bench to reach about 17% water content (Note 2). (about 3 h)
2. Moisture determination: Determine the fresh weight of 10 beads replicated three times, each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
3. Place 10 beads into each 2 ml cryotube, plunge in liquid nitrogen.
4. To rewarm quickly move cryotube to 40°C water bath for 3 min.
5. Culture 20 beads on 30 ml recovery medium in a Petri dish.
6. The cultures are kept in the dark for 2 days, and then transferred to light conditions for regrowth. Subculture is done every 4 weeks. Regrowth is defined by shoot tips that grow to shoots in about 6 weeks.

The encapsulation–vitrification protocol

Day 1–7

1. Excise and pretreat shoot tips as for encapsulation dehydration.
2. Form beads and preculture on sucrose medium as above.

Day 8

1. Transfer 10 beads into each Petri dish containing 10 ml of osmotic loading solution (LS) and incubate for 90 min.
2. Drain and dry on sterile filter papers for a few seconds.

3. Transfer 10 beads into each Petri dish with 10 ml of PVS2 and incubate for 3 h at room temperature (Note 2, Table 13.7).
4. Drain and surface dry on sterile filter papers for a few seconds.
5. Transfer 10 beads into each 2 ml cryotube, directly immerse cryotube into liquid nitrogen.
6. To rewarm, quickly move cryotube to 40°C water bath for 3 min.
7. Transfer 10 beads in each Petri dish with 10 ml of rinsing solution and hold for 20 min.
8. Surface dry on sterile filter papers for a few seconds.
9. Culture 20 beads in Petri dish with 30 ml recovery medium.
10. The cultures are kept in the dark for 2 days then transferred to light conditions for regrowth. Subculture is done every 4 weeks. Regrowth is defined by shoot tips that regenerate to shoots in about 6 weeks.

Table 13.7 Cryopreservation of *in vitro* grown shoot tips of raspberry (*Rubus idaeus)* by encapsulation vitrification. Means ±SE are shown (Wang et al. 2005)

Cultivars/genotypes	Regrowth (%)
TTA-508	75±8
Z-13	65±6
TTA-422	60±6
Jatsi	85±8
Muskoka	55±8
Maurin Makea	85±6
Ottawa	50±6
Average	68

Note 1. For many raspberry cultivars, shoot tips turn brown shortly after excision from the stock cultures. Stabilization of shoot tips by pretreating on pretreatment medium with charcoal is a necessary step to ensure high efficiency of cryopreservation. Shoot tips turn green after 2 or 3 days of culture on pretreatment medium.

Note 2. The optimal duration of dehydration by air drying or by vitrification solution may vary greatly with different cultivars. Working on seven raspberry cultivars, the highest (85%) and lowest (55%) regrowth were obtained with cultivars Maurin Makea and Muskoka when a 3-h-vitrification was employed (Wang et al. 2005, Table 13.7). To achieve a maximum regrowth for a given cultivar, duration of dehydration has to be optimized.

13.5.7 Encapsulation Dehydration of Grapevine (Vitis)

By Qiaochun Wang based on Wang et al. 2000

For *Vitis*, preculture is a necessary step to induce tolerance to dehydration. *Vitis* is sensitive to high sucrose concentration and requires a stepwise preculture with increasing sucrose concentrations. An optimal duration of dehydration by air drying may vary greatly with different species and cultivars. For example, with dehydration by air drying, the highest regrowth (60%) of the LN33 hybrid was obtained at 8 h of dehydration, while dehydration for 6 h gave the highest regrowth (40%) for *Vitis vinifera* cv. Superior (Wang et al. 2000).

Basic requirements

1. Cryotubes (2 ml)
2. Liquid nitrogen
3. Pipettes (1 ml for making beads and 5 ml for solutions)
4. Petri dishes (9 cm in diameter)
5. Sterile filter papers (9 cm in diameter)
6. Styrofoam container (for holding liquid nitrogen)
7. Test tubes (2.5 × 12.5 cm) for maintenance of stock cultures
8. Water bath

Preparation of media and solutions

1. Half-strength Murashige and Skoog (MS) with 30 g/l sucrose and 2.4 g/l Gelrite, pH = 5.8
2. Modified calcium chloride solution: liquid ½ strength MS with 100 mM calcium chloride, 2 M glycerol, 0.4 M sucrose, pH = 5.8
3. Modified alginate solution: liquid ½ strength MS medium without calcium containing 2.5% sodium alginate (low viscosity), 2 M glycerol and 0.4 M sucrose, pH = 5.8
4. Preculture medium: ½ strength MS with 0.25, 0.5, 0.75 or 1.0 M sucrose and solidified with 2.4 g/l Gelrite, pH = 5.8
5. Recovery medium: ½ strength MS with 1 mg/l BAP and 0.1 mg/l NAA, pH = 5.8

The encapsulation–dehydration protocol

Stock cultures are grown in test tubes (2.5 × 12.5 cm) with 15 ml of ½ strength MS at 24±2°C, 16-h photoperiod (45 μmol $m^{-2}s^{-1}$) provided by cool-white fluorescent tubes. Subculture at 4 weeks.

Day 1–5

1. Excise shoot tips (1 mm in size) from 4-week stock cultures.
2. Place 10 shoot tips at a time in 1.5 ml of 2.5% alginate solution in a Petri dish. Pick up one shoot tip and some alginate and drip into the calcium chloride solution in a 250 ml Erlenmeyer, hold for 20 min to form beads, each with one shoot tip.
3. Surface dry on sterile filter papers for a few seconds.
4. Preculture 20 beads in each Petri dish containing 30 ml of 0.25 M sucrose preculture medium for 1 day, transfer to 0.5 M sucrose for 1 day, transfer to 0.75 M sucrose for 1 day, and then to 1.0 M sucrose for 1 day. Culture conditions are the same as for maintenance of the stock cultures.
5. Place 10 beads on sterile filter paper placed in a Petri dish and dry in the laminar flow to about 15–18% water content.
6. Moisture determination: Determine the fresh weight of 10 beads replicated three times, each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
7. Transfer 10 beads into each 2 ml cryotube, directly immerse cryotube into liquid nitrogen.
8. To rewarm, quickly move cryotube from liquid nitrogen to 40°C water bath for 3 min.
9. Post culture 20 beads on each Petri dish with 30 ml of recovery medium. The cultures are kept in the dark for 2 days and then transferred to light conditions for regrowth. Subculture is done every 4 weeks. Regrowth is defined by shoot tips that regenerate to shoots in about 6 weeks of post culture.

13.5.8 Vitrification of Grape (Vitis) *Shoot Tips*

By T Matsumoto based on Matsumoto and Sakai 2003

Checklist for vitrification

Day 1: Items needed to dissect shoot tips and preculture

1. Modified Murashige and Skoog (1962 MS) (half strength of KNO_3 and NH_4NO_3) with 0.3 M sucrose, 0.8% agar for preculture (25 ml in plastic Petri dish)
2. 5- to 6-month old *in vitro* plantlets, transferred to new medium each month without cutting

Day 2: Vitrification (2-step method) and recovery

1. Sterile Pasteur pipettes for removing solutions
2. Cryotubes (about 2 ml)
3. Osmotic LS: 2 M glycerol, 0.4 M sucrose, in liquid MS
4. PVS2: 30% w/v glycerol + 15% w/v ethylene glycol + 15% w/v DMSO + 0.4 M sucrose in liquid MS medium
5. Half strength PVS2: PVS2 diluted 1:1 with MS medium
6. Rinsing solution: 1.2 M sucrose in liquid ½ strength MS medium
7. Recovery medium: Modified MS with 3% sucrose and BA 1 mg/l
8. Dry filter papers, tools, sterile Petri dishes, LN

Prepare 3 days in advance

Dissect red-colored axillary buds from stock plants. Preculture shoot tips (about 1 × 1 mm) for 3 day on MS with 0.3 M sucrose and 0.8% agar, maintained under continuous light at 25°C.

The procedure

1. Place 10 precultured meristems in a cryotube and add osmotic loading solution. Hold for 20 min at 25°C.
2. Remove solution with a Pasteur pipette and add half strength PVS2 for 30 min at 0°C (on crushed ice).
3. Drain, replace with PVS2 for 50 min at 0°C. Exchange 1 ml of fresh PVS2 1 min before the 50 min are over. Immerse tubes in LN.
4. Warm cryotubes in 40°C water for 1 min with stirring, then drain PVS2 and add rinsing solution for 20 min at 25°C.

Transfer meristems onto a filter paper disc over recovery medium and culture in standard conditions. After 1 day transfer the meristems onto a fresh paper disc and new medium (Results in Table 13.4 and Fig. 13.1).

Chapter 14

Cryopreservation of *In Vitro* Tissues of Deciduous Forest Trees

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14.1 Global Forest Resources

The Food and Agriculture Organization of the United Nations' (FAO's) international definition of a forest, as presented in the Global Forest Resources Assessment 2000 (GFRA-2000), sets the canopy cover requirement of 10% as the threshold value between forest and other types of land. According to the latest preliminary information of GFRA published in 2005 by the FAO, there are around 3.9 billion ha of forest in the world making up about one third of the total land area. Between 1990 and 2000 the loss of forest land was 8.9 million ha per year and between 2000 and 2005, a little less than 7.3 million ha per year (GFRA-2005; Finnish Statistical Yearbook of Forestry 2005). The area of natural forests, which accounts for 36% (i.e. 1423 million ha) of the total forest area is decreasing around 6% per year (GFRA-2005).

Today forests are managed for many different purposes. Approximately one third are used to produce construction timber, pulp, paper and other forest products. Maintaining biodiversity was the priority in 11% (i.e. 443 million ha) of the forests, and the area of these forests has increased by 96 million ha since 1990. Around 9% of the global forests are intended for

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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different protection purposes, such as soil, groundwater or coast protection, to prevent desertification or to control avalanches (GFRA-2005). However, in Africa 545 million m^3 of wood is annually consumed for fuel which is more than six times the amount of wood consumed for all the other purposes (FAO 2005).

14.2 Characteristics of Forest Trees

Economically important forest tree species include gymnosperms, especially pines and spruces, and several families of angiosperms. Compared with agricultural plants, forest trees are less domesticated and even the seed produced in breeding programs is genetically diverse. Many forest trees are characterized by long rotation times and regeneration intervals as well as by large size. Many species are wind pollinated but there are also some deciduous species with scattered distribution, and often limited migration of pollen or seed among the fragments. Due to the green canopy of forests (one third of the total land area), the importance of global forests as a carbon sink was recently emphasized in several reports (Schulze et al. 2000; Myneni et al. 2001).

The increasing global need for food and fiber results in new demands for the efficiency of wood production and wood products (Fenning and Gershenzon 2002). The decreasing trend in global forest area, and the over 8000 tree species that are threatened with extinction (http://www.globaltrees.org/), emphasize the importance of the immediate development of long-range conservation strategies for forest tree species. In this review, we will consider the conservation strategies represented for forest tree species and especially the role and applicability of cryopreservation among the strategies.

14.3 Conservation Strategies for Forest Tree Resources

14.3.1 General Conservation Principles of Forest Trees

All forest areas, both natural and managed, have a role in forest biodiversity and conservation. The increased efficiency of fiber production on intensively managed plantations might decrease pressure on natural forests, such as rain forests, as sources of fiber (Sedjo 1999; Campbell et al. 2003; Walter 2004). However, this will remain unrealized unless progress with *in vitro* propagation is extended to the high-value tropical species that are

extensively harvested (Merkle and Nairn 2005). For fuel-wood production, exotic fast-growing species such as *Eucalyptus* species could be transplanted to protect native species (Merkle and Nairn 2005). However, forest plantations with exotic trees may affect soils, making them poorer when compared with plantations with indigenous species (Lemenih et al. 2004). In Europe, the importance of forest genetic resources for both economic and social welfare is widely acknowledged. European Forest Genetic Resources Programme (EUFORGEN, http://ipgri-pa.grinfo.net/index.php?itemid=194) is a collaborative mechanism among European countries that promote the conservation and sustainable use of forest genetic resources.

Generally, the conservation strategies of forest trees are based on *in situ* or *ex situ* conservation. The principal method for genetic conservation of widely distributed and wind-pollinated species is the establishment of gene-reserve forests. For this type of species, a single stand contains a considerable proportion of the genetic diversity within species, and with *in situ* conservation it is possible to ensure continuous evolution of the species. Whenever feasible, the goal is to find suitable stands which represent the indigenous gene pool and which are large enough to ensure pollination within the selected stand. This principle was followed in the gene conservation of silver birch (*Betula pendula* Roth) in Finland. *Ex situ* conservation practices are generally applied to deciduous species with scattered distribution and often limited migration of pollen or seed among the fragments. These collections usually represent random samples of genetic diversity. It is emphasized by Rusanen et al. (2004) that if these samples, grafts or seedlings, are preserved in field collections, they can also be used to produce genetically diverse seed. Clonal field repositories have played and continue to play an important role as a means of woody plant conservation. These repositories typically need large land areas, have high running costs and, especially in the case of fruit trees, huge amounts of accessions might be needed. The samples in these collections are vulnerable to environmental factors and pests. Diseases may also be easily distributed within the local collections. For instance *Prunus* species are easily infected by pollen-transmitted viruses, and *Prunus* field collections, even virus-tested and virus-free, can be maintained uncontaminated for only a few years. *Ulmus* species are greatly endangered due to the recurrence of Dutch elm disease (DED), caused by the fungus *Ophiostoma novo-ulmi* Brasier (reviewed by Harvengt et al. 2004).

Other *ex situ* conservation strategies such as storing desiccated seeds at a low temperature are also applied to forest trees. In many gymnosperms the seeds are orthodox and can be conserved for extended periods at a low temperature (Chap. 19). Many angiosperm forest species have non-orthodox seeds (Chap. 18) with limited conservability, and some of them, such as

silver birch, have orthodox seed but their viability decreases during storing. *In vitro* collections are also used, especially in fruit trees, as a backup for field collections. In some cases *in vitro* propagation methods were developed to enable the *ex situ* conservation of an endangered species such as *Eucalyptus impensa* Brooker and Hopper (Bunn 2005).

14.3.2 Why Cryopreserve Forest Tree Germplasm?

As the complementary system for the existing *in situ* and *ex situ* conservation strategies of forest trees, cryopreservation provides an additional or duplicate way to conserve germplasm. Sometimes, it may be difficult to find diverse enough natural stands for *in situ* conservation, which furthermore emphasizes the role of cryopreservation as a backup method. In addition to general germplasm conservation at the species level, there are also other reasons to conserve genetic resources of forest trees. There are some specific forms, cultivars or individuals with specific important phenotype characteristics that should be conserved vegetatively. Cryopreservation of meristems may also help to eliminate virus diseases. Tree breeding programs may benefit if the breeding material will be safely conserved during progeny testing. There are also several kinds of research material, such as easy-to-transform genotypes that need to be safely stored, or genetically modified tree lines for which, due to regulatory reasons, the only option for safe storage for longer periods of time may be cryopreservation. In the case of embryogenic cultures of conifers, the growth and embryogenic potential may vary over the course of time or they may be lost after some months of subculturing, underlining the role of cryopreservation (reviewed by Häggman et al. 2000, 2006). Last but not least, climate change will affect distribution of trees, pose new or different ecological threats, and possibly increase the conservation need in the future.

14.4 Cryopreservation Protocols

In deciduous forest trees, the cryopreservation protocols applied to *in vitro* meristematic tissues are vitrification, encapsulation dehydration or controlled rate cooling. Most of the protocols were developed for fruit trees but in this context we will present the cases in which cryopreservation is applied to non-fruit forest tree species that are important in timber or pulp production. Background information on cryopreservation technology is found in Section 1 of this volume.

14.4.1 Controlled Rate Cooling

Controlled rate cooling is commonly used to cryopreserve woody temperate plants (Reed 1988, 1990). To our knowledge, the only case in which the controlled rate cooling method was successfully used in preservation of an important timber and pulp species is silver birch (*Betula pendula* Roth) (Ryynänen 1996, 1998), which is both economically and ecologically an important hardwood species with a long tree-breeding history in the Nordic countries. On average, the cryopreservation success of individual genotypes by the slow cooling method was high, varying from 30 to 80% depending on the genotype (Ryynänen and Aronen 2005b) (Protocol 14.7.1). Interestingly, the recovery success was enhanced when ammonium ions in the growth medium were substituted by KNO_3 during cold acclimation and recovery cultivation of silver birch shoot tips (Ryynänen and Häggman 1999, 2001). The genetic fidelity of the regenerated genotypes after cryopreservation has proven unchanged when followed by phenotypical observations, RAPD markers, chromosome analyses and transgene integration and expression (Ryynänen et al. 2002; Ryynänen and Aronen 2005a).

14.4.2 Vitrification

Recently, the vitrification method (Sakai et al. 1990) was applied to *in vitro* axillary buds of silver birch. The optimized method, which included the preconditioning of buds on Woody Plant Medium (Lloyd and McCown 1980) with 0.7 M sucrose for 24 h and treatment with PVS2 for 120 min, proved to be successful with slightly higher recovery than in controlled cooling. It varied from 86 to 55% depending on the genotype (Ryynänen and Aronen 2005b) (Protocol 14.7.2). Both controlled rate cooling and vitrification are appropriate for silver birch.

The species of genus *Populus* (including aspen, poplar and cottonwood species) belong to one of the most exploited groups of forest trees. Genus *Populus* is also a model for tree biology. It is easily transformed, at least some of the species are easily *in vitro* propagated, its physiology is relatively well characterized, it has a relatively small, compact nuclear genome, and the draft sequence of the whole genome of *P. trichocarpa* ‘Trichobel’, was released in 2004 (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) allowing more advanced functional genomic studies. Thus, it is not surprising that due to the importance of the genus in research, cryopreservation protocols utilizing *in vitro* tissues are needed for conservation and storage purposes, and they are even considered as the bottle neck in *Populus*

functional genomics research (Tsai and Hubscher 2004). The vitrification protocol was applied to *in vitro* shoot tips of white poplar (*P. alba* L.) (Caccavale et al. 1998, Lambardi et al. 2000), resulting in about 60 % recovery of rooted shoots after cryostorage (Protocol 14.7.3). Jokipii et al. (2004) compared controlled rate cooling and vitrification of *in vitro* apical segments and buds of hybrid aspen (*P. tremula* x *tremuloides*) and found that controlled rate cooling gave only 1% regrowth but vitrification had 3–75% depending on the genotype. Shoot tips of *Robinia pseudoacacia* were successfully cryopreserved with PVS2 following 3 days of preculture on increasing concentrations of sucrose (Verleysen et al. 2005) (Protocol 14.7.4).

14.4.3 Encapsulation Dehydration

Application of the encapsulation–dehydration technique (Fabre and Dereuddre 1990) was successful for species belonging to the genera *Morus*, *Eucalyptus*, *Melia* and *Robinia*. In all cases, the number of genotypes or cultivars included was limited, and further investigations are needed for an evaluation of the applicability of the procedure at the species level.

Genus *Morus* is distributed in a wide area of tropical, sub-tropical, temperate and sub-arctic zones. Originally, mulberry was cultivated for sericulture (for feeding silk worms) but today it is cultivated for various purposes, such as fruit production, medicinal use, paper production, mushroom production and animal feed (Machii et al. 2000). The encapsulation–dehydration cryopreservation protocol was applied to one specific mulberry cultivar, Kenmochi (*Morus bombysis* Koidz), with a 65% recovery success, as reported by Niino and Sakai in 1992.

Eucalyptus is a diverse genus of trees including more than 700 species which are native to Australia but a few of which can also be found in New Guinea, Indonesia and the Philippines. The encapsulation–dehydration method was successfully applied to the *in vitro* axillary buds of *Eucalyptus* species (Poissonier et al. 1992; Blakesley and Kiernan 2001; Páques et al. 2002). Poissonier et al. (1992) optimized the encapsulation–dehydration procedure for *E. gunnii*. Pácques et al. (2002) applied the method of Poissonier et al. (1992) and compared the cryopreservability of several clones. The results emphasized the importance of sucrose concentration and the progressive addition of sucrose. A rapid increase in the sucrose concentration resulted in a higher percentage of shoot tip recovery in all species tested, compared with a slow sucrose increase, i.e. 2 days was better than 7 days (Pácques et al. 2002). The authors also showed that the recovery was genotype dependent, so that when the optimized procedures were used, the

recovery ranged from 12 to 63%. Blakesley and Kiernan (2001) reported that the recovery varied between 44 and 96% with encapsulation–dehydration in a *Eucalyptus* hybrid (*E. grandis* W. Hill ex Maiden x *E. camaldulensis* Dehnnh). For this clone, the incorporation of glycerol as a cryoprotectant remarkably increased the average recovery success.

Black locust (*Robinia pseudoacacia* L.), a deciduous shrub or tree belonging to the family of Fabaceae is an important resource for fuel wood, honey, furniture and parquets, and an alternative for tropical wood plantations in the Netherlands and France (Verleysen et al. 2005). Cryopreservation of black locust is possible both via vitrification and encapsulation dehydration. In the encapsulation–dehydration protocol, encapsulation was required before preculture with high (0.7 M) constant sucrose concentrations to result in an 87% recovery after cryostorage (Protocol 14.7.5).

Paradise tree (*Melia azedarach* L var. gigantean, clone "El Dorado") belongs to the Meliaceae family and it is native to the Asiatic Middle East. It is an important forest tree in Argentina, with good growth and excellent adaptability to a wide range of soil and climate conditions, and it is considered valuable in reforestation programs (Scocchi et al. 2004). The encapsulation-dehydration approach that resulted in the highest shoot proliferation rate after cryopreservation was achieved when encapsulated apical tips were precultured on gradually increasing sucrose concentrations (0.5 M sucrose followed by 0.75 M and subsequently 1 M sucrose), desiccated for 5 h with silica gel and followed with either a rapid or a slow cooling rate. Shoot proliferation after rewarming ranged from 43 to 60%. The genetic fidelity of the material was evaluated and confirmed by isoenzyme analyses and RAPD markers (Scocchi et al. 2004).

14.5 Long-term Storage and Genetic Fidelity

In the case of long-living forest trees, monitoring the genetic fidelity of cryopreserved specimens is particularly important. There is often a need for long-term storage, e.g. during progeny testing or when the maintenance of large archive collections with hundreds or thousands of accessions is too expensive. Occasional mutations or genome rearrangements may not be observed in young plants but may be expressed later in mature trees (reviewed by Häggman et al. 2000, 2001). Comparative studies that include the natural mutation rate would prove of interest.

Somaclonal variation caused by genetic or epigenetic reasons is a well-known phenomenon when an *in vitro* step, and especially a prolonged one with numerous subcultures, is involved in plant multiplication or regeneration.

Due to the ultra-low temperature of liquid nitrogen, it is proposed that biological material could be stored and maintained for an indefinite amount of time with guaranteed genetic stability (Ashwood-Smith and Friedmann 1979; Kartha 1985). Ionizing radiation and the resulting molecular damage can still occur at liquid nitrogen temperatures (Grout 1990).

Specific attention was paid to the mutagenic potential of the most popular penetrating cryoprotectant DMSO, which was found to interact with chromatin and nucleic acids, to inhibit DNA synthesis, to alter the secondary structure of DNA and RNA and to cause mitotic irregularities (reviewed by Häggman et al. 2000). Finkle et al. reported in 1985 that a 2–10% solution of DMSO may generate genetic or epigenetic changes in callus and suspension cultures of higher plants. This was also shown as an increase in intraclonal variation in RAPD profiles of the embryogenic cultures of *Abies cephalonica* that were treated with DMSO (Aronen et al. 1999).

There is at present no evidence of morphological or genetic alterations in forest trees caused by cryopreservation. This may be due to the limited number of reports in which genetic stability was followed. This may also be due to the fact that even for the most important woody tree species (fruit trees or timber or pulp species) there exist only a few with appropriate genetic marker systems (e.g. microsatellites) available. So far, genetic markers (such as random amplified polymorphic DNAs, i.e. RAPD markers) were used together with morphological observations, transgene integration and expression analysis, isoenzyme markers or chromosome analyses (Ryynänen et al. 2002; Jokipii et al. 2004; Scocchi et al. 2004; Ryynänen and Aronen 2005a, b).

Populus species are examples of a forest tree species with large-scale EST (expressed sequence tags) libraries available (Sterky et al. 1998; Bhalerao et al. 2003; www.poppel.fysbot.umu.se). Also, the draft sequence of the whole genome of *P. trichocarpa* 'Trichobel', was released in 2004 (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). In the future, new sequencing techniques will certainly revolutionize both the speed and accuracy of genome sequencing (e.g. Margulies et al. 2005), increase the available sequence data for forest tree species and improve the possibilities for genetic stability analyses. Although there is no evidence of alterations caused by cryopreservation in forest tree germplasm, the need for long storage times and the mutagenic potential of some cryoprotective compounds, emphasize the need to develop protocols for regular observations of genetic stability of the cryopreserved germplasm.

14.6 Conclusions

The number of forest trees for which cryopreservation protocols are developed is still limited. The protocols are usually applied or optimized for only a limited number of genotypes, and the applicability of the protocol generally at the species level remains to be determined. This is an important question because forest trees are still less domesticated than agricultural crops, and have a wide genetic diversity. For this kind of forest tree species, cryopreservation cannot be the one and only conservation method but it may be an important tool together with other *in situ* and *ex situ* conservation strategies. The future prognoses of climate change, the increasing global need for food and fiber, the already decreasing trend in forest area, and the over 8,000 tree species threatened with extinction emphasize the immediate need for the development of long-range conservation strategies for forest-tree species. These future and present threats also emphasize the need for diverse conservation strategies. In this context, cryopreservation as a cost-effective, low labor- and space-demanding alternative will have an important role.

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14.7 Protocols

14.7.1 Vitrification of Populus alba *Shoot Tips*

By M. Lambardi based on Lambardi et al. 2000

Checklist for vitrification approach
Day 1: Items needed for sugar pretreatment

1. Forceps/Tools
2. Preculture medium: MS (Murashige and Skoog 1962) medium, with 0.09 M sucrose, 7% agar, hormone-free

Day 3: Items needed for cryopreservation

1. Forceps/Tools
2. Sterile pipettes or syringes for solutions (one for each)
3. Cryovials and markers
4. Cryobox or cryocane to hold the cryovials during immersion in LN
5. Nalgene Labtop Cooler (0°C model)
6. Filter sterilizing equipment for PVS2
7. Osmoprotectant
8. PVS2
9. Rinsing solution
10. Regrowth medium for recovery after warming
11. Rooting medium

Prepare in advance

1. *In vitro*-grown plants, cold acclimated for 3 weeks at 5°C, 8 h photoperiod
2. Preculture medium
3. Nalgene Labtop Cooler (0°C model) placed at –20°C to cool overnight
4. Osmoprotectant: 2M glycerol + 0.4 M sucrose in liquid MS medium
5. PVS2: 30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% DMSO (w/v), in MS medium with 0.4 M sucrose; pH 5.8
6. Rinsing solution: liquid MS medium + 1.2 M sucrose
7. Regrowth medium: MS medium, without NH_4NO_3 and with 0.09 M sucrose, 1.5 μM BA and 0.5 μM GA_3.
8. Rooting medium: MS medium, with 3 μM IBA

The procedure

1. Excise shoot tips (1–2 mm in length and 1–1.5 mm base diameter), consisting of the apical meristem and 4–5 leaf primordia and young leaves, from axillary buds of *in vitro* plants.
2. Preculture shoot tips for 2 days at 5°C under a 8 h photoperiod (30 μmol m^{-2} s^{-1}).
3. Place 1 ml osmoprotectant solution to a 2-ml cryovial, add 10–12 shoot tips and hold for 20 min at 25°C.
4. Remove solution with a Pasteur pipette and add PVS2 at 0°C. Maintain cryovials on ice or in Nalgene Labtop Cooler for 60 min.
5. Replace the PVS2 with 0.6 ml of fresh PVS2.
6. Plunge the cryovials directly into LN.
7. Warm the shoot tips in 40°C water bath for 50 s, following initial 5–10 s at room temperature (warming rate of about 180°C/minute).
8. Remove PVS2 and add rinsing solution at 25°C for 20 min.
9. Plate the shoot tips on regrowth medium and incubate for 3 weeks at 22°C under 16 h photoperiod (60 μmol m^{-2} s^{-1}).
10. After 3 weeks, transfer the growing shoot tips onto a gelled, hormone-free MS medium, with 0.1% activated charcoal, for shoot development.
11. Root the shoots (longer than 1 cm) on rooting medium.

14.7.2 *Controlled Rate Cooling of* In Vitro *Meristems of Silver Birch*

By Leena Ryynänen based on Ryynänen 1996, 1998; Ryynänen and Häggman 2001; Ryynänen and Aronen 2005a

Items needed before preculture

1. Stereomicroscope
2. Sterile forceps and scalpels
3. Sterile Petri dishes
4. Ice and cold plates

Prepare before preculture

1. Preconditioning medium: modified WPM (Lloyd and McCown 1980) with 100 μM abscisic acid (ABA), 0.09 M sucrose and all the inorganic nitrogen [5 mM NH_4NO_3 and 2 mM Ca $(N0_3)_2$] of the medium substituted by 10 mM KNO_3, 0.7% agar
2. Cold acclimate *in vitro* donor shoots: cultivate the donor shoots on in glass jars under conditions of 8/16 h light/dark photoperiod (SD), with a light intensity of 23 μE m^{-2} s^{-1} at a temperature of 5°C for 4 weeks
3. Preculture medium: modified WPM preculture medium as above, but with 5% dimethyl sulfoxide in Petri dishes

Preculture procedure

Dissect axillary buds or apices (2–5 mm) from cold-acclimated shoots onto preculture medium in Petri dishes. Avoid warming the shoot material and meristems during working. Preculture the meristems under cold-acclimation conditions for 3 days.

Cryopreservation

Items needed

1. 2 ml cryovials and markers
2. Controlled rate freezer and drawers/canes
3. Liquid nitrogen

Prepare before cryopreservation

1. Liquid WPM with 0.09 M sucrose and the same KNO_3- substitution of inorganic nitrogen source as above, but no growth regulators
2. PGD: 10% (w/v) polyethylene glycol (MW 8000), 10% (w/v) glucose and 10% (v/v) DMSO in water

Freezing procedure

1. Add 0.25 ml cold liquid WPM to the cryovials on ice. Transfer the meristems into the vials. Add 1 ml of PGD drop wise into the vials over a period of 30 min. Keep on ice for another 30 min.
2. Transfer the vials into drawers/canes, put the drawers/canes into the cooling chamber of the freezer, and cool at 0.17°C min^{-1} to –38°C (with an exotherm at –99°C).
3. After reaching –38°C submerge the drawers/canes in liquid nitrogen. Keep them in liquid nitrogen until needed.

Warming

Items needed

1. Water bath (37°C)
2. Sterile forceps, sterile filter paper, sterile Petri dishes
3. Shading gauze
4. Glass jars
5. Growth room with 16 h light: 8 h dark photoperiod, light intensity of 85–125 µE m^{-2} s^{-1}, temperature 22°C

Prepare before warming the meristems

1. Liquid modified WPM as with the preconditioning medium above
2. Solid WPM as above with 4.4 µM 6-benzylaminopurine (BA), 0.09 M sucrose in Petri dishes
3. Solid WPM with 4.4 µM BA, 0.09 M sucrose and 5 mM NH_4NO_3 and 2 mM $Ca(N0_3)_2$ in Petri dishes.

Warming procedure and cultivation of the meristems

1. Warm the samples in a water bath at 37°C for 3–4 min, and then keep on ice for 0.5 min, then at room temperature until cultivation.
2. Drain the PGD solution from the cryovials and replace with 1 ml liquid WPM for 30 min.
3. Change the liquid medium once and pour the material onto sterile filter paper in a Petri dish.
4. Plate the meristems onto solid WPM with 4.4 µM BA, 0.09 M sucrose in Petri dishes. Cultivate for 3 days. Decrease the light intensity by shading the Petri dishes with gauze for 1 week.
5. Transfer the meristems onto solid WPM with 4.4 µM BA, 0.09 M sucrose and 5 mM NH_4NO_3 and 2 mM $Ca(N0_3)_2$ as inorganic nitrogen source in Petri dishes.

6. Transfer the meristems onto fresh medium after 3 weeks. Estimate the regrowth of meristems after 6 weeks of cultivation: meristems with new shoots and leaves are classified as regrown meristems.
7. Transfer the meristems into glass jars for multiplication using the same WPM.

Notes

1. Using controlled rate cooling the recovery is somewhat lower and genotypic differences are greater compared to those of vitrified meristems of the same genotypes.
2. Recovery of cryopreserved silver birch is considerably promoted by cold acclimation on WPM with KNO_3-substitution of the nitrogen source. However some genotypes recover better with a slightly modified protocol, e.g. different ABA concentrations and duration of cold acclimation (Fig. 14.1).
3. No genetic instability was found when the regrown silver birch plants were compared to the original donor trees using RAPD assays together with chromosome analysis.

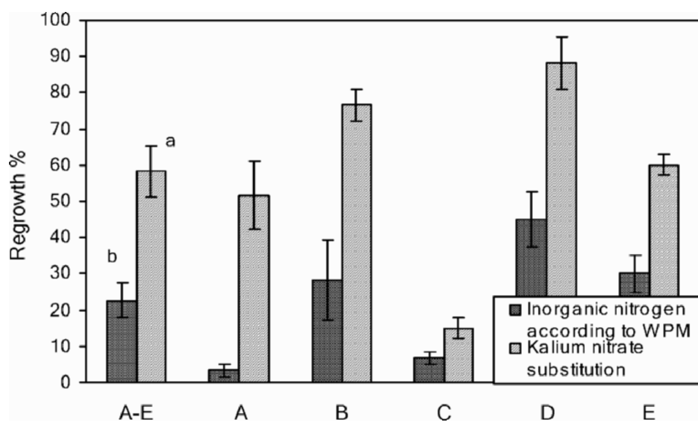

Fig. 14.1 Regrowth percentage (± SE) of apices of five silver birch genotypes (A, B, C, D, and E) and all the genotypes together (A–E) when cryopreserved according to the controlled rate cooling protocol and without KNO_3 (Kalium) substitution. Letters in A–E column indicates the significance ($P < 0.05$) of the effect of KNO_3 substitution on recovery, calculated over all the material (Ryynanen and Häggman 2001)

14.7.3 Vitrification of Axillary Buds of Silver Birch

By Leena Ryynänen based on Ryynänen and Aronen 2005a

Items needed before preculture

1. Stereomicroscope
2. Sterile forceps and scalpels
3. Sterile Petri dishes
4. Ice and cold packs

Prepare before preculture

1. Preconditioning: Cold acclimate *in vitro* donor shoots: cultivate the donor shoots on a modified WPM (Lloyd and McCown 1980) with 100 μM abscisic acid (ABA), 0.09 M sucrose and all the inorganic nitrogen [5 mM NH_4NO_3 and 2 mM $Ca(N0_3)_2$] of the medium, substituted by 10 mM KNO_3, 0.7% agar, in glass jars under conditions of 8/16 h light/dark photoperiod (23 μE m^{-2} s^{-1}) at 5°C for 4 weeks.
2. Preculture medium: Solid modified WPM medium with 100 μM ABA, 0.7 M sucrose and all the inorganic nitrogen [5 mM NH_4NO_3 and 2 mM $Ca(N0_3)_2$] of the medium substituted by 10 mM KNO_3.

Preculture procedure

1. Dissect axillary buds or apices (2–5 mm) from cold-acclimated shoots and transfer onto preculture medium in Petri dishes. Avoid warming the shoot material and meristems during working by placing them on cold packs.
2. Preculture the dissected meristems under the same cold-acclimation conditions as the shoot cultures for 1 day.

Cryopreservation
Items needed

1. 2 ml cryovials and markers
2. Liquid nitrogen container and drawers/canes

Prepare before freezing

1. Osmoprotective loading solution: liquid modified WPM with 2 M glycerol, 0.4 M sucrose, no growth regulators
2. PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (v/v) dimethyl sulfoxide (DMSO) in liquid WPM with 0.4 M

sucrose and with KNO_3-substitution of inorganic nitrogen source, but no growth regulators

Vitrification procedure

1. Transfer the meristems into the vials. Add 0.5 ml of osmoprotection solution at room temperature and hold for 20 min.
2. Remove the solution and transfer the vials to ice. Add 1 ml of cold PVS2 to the vials and hold for 120 min.
3. Replace the PVS2 with fresh solution, transfer the vials to drawers/canes, and submerge in liquid nitrogen.

Warming

Items needed

1. Water bath (37°C)
2. Sterile forceps, sterile filter paper, sterile Petri dishes
3. Shading gauze
4. Glass jars
5. Growth room with 16 h light : 8 h dark photoperiod, light intensity of 85–125 $\mu E\ m^{-2}\ s^{-1}$, temperature 22°C

Prepare before warming the meristems

1. Rinsing solution: Liquid WPM with 4.4 µM BA, 1.2 M sucrose and KNO_3-substitution of inorganic nitrogen source but no growth regulators
2. Solid WPM with 4.4 µM BA, 0.09 M sucrose and all the inorganic nitrogen of the medium substituted by 10 mM KNO_3, in Petri dishes
3. Solid WPM with 4.4 µM BA, 0.09 M sucrose and 5 mM NH_4NO_3 and 2 mM $Ca(N0_3)_2$ as inorganic nitrogen source in Petri dishes

Warming procedure and cultivation of the meristems

1. Warm the samples in a water bath at 37°C for 3–4 min, then keep on ice for 0.5 min, then move to room temperature until cultivation.
2. Drain the PVS2 from the cryovials and replace with 1 ml liquid WPM for 30 min.
3. Change the liquid medium once and pour the material onto sterile filter paper in a Petri dish.
4. Plate the meristems onto solid WPM with 4.4 µM BA, 0.09 M sucrose and all the inorganic nitrogen of the medium substituted by 10 mM KNO_3, in Petri dishes. Cultivate for 3 days.

5. Decrease the light intensity by shading the Petri dishes with gauze for 1 week.
6. Transfer meristems onto solid WPM with 4.4 µM BA, 0.09 M sucrose and 5 mM NH_4NO_3 and 2 mM $Ca(N0_3)_2$ as inorganic nitrogen source in Petri dishes.
7. Transfer meristems onto fresh medium after 3 weeks. Estimate the regrowth of meristems after 6 weeks: meristems with new shoots and leaves are classified as regrown.

Notes

1. After vitrification, recovery is better and genotypic differences are smaller than those of the same genotypes cryopreserved using the slow cooling protocol (Fig. 14.2).
2. Cold acclimation is essential for successful vitrification of silver birch. Some genotypes recover better with a slightly modified protocol, e.g. different ABA-concentrations and duration of cold acclimation.
3. Different silver birch genotypes have slightly different demands for preculture (e.g. sucrose concentration and duration) as well as for the dehydration time in PVS2.
4. No genetic instability was found when the regenerated silver birch plants were compared to the original donor trees using RAPDs.

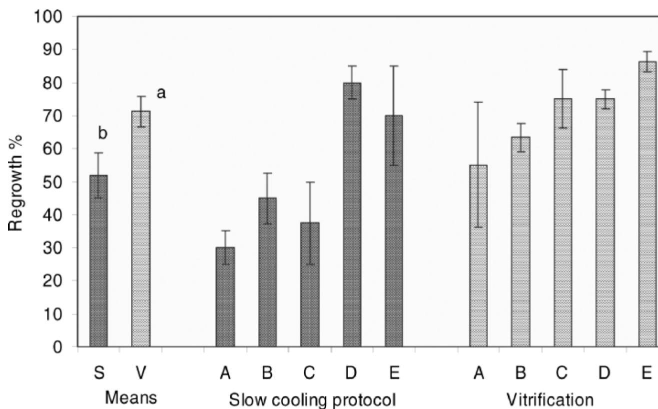

Fig. 14.2 Regrowth percentage (± SE) of axillary buds of five silver birch genotypes (A–E) and all genotypes together when cryopreserved by vitrification (V) and slow cooling (S) (Ryynänen and Aronen 2005a). Letters in S and V columns indicate the significance ($P < 0.05$) of differences in recovery, calculated over all the material

14.7.4 Vitrification of **Robinia pseudoacacia**

By Hans Verleysen based on Verleysen et al. 2005

Materials

1. Murashige and Skoog (1962) (MS) medium with 100 $mg.l^{-1}$ myo-inositol, 0.4 $mg{\cdot}l^{-1}$ thiamine-HCl, 100 $mg{\cdot}l^{-1}$ sequestrene (Sequestrene 138 Fe 100 SG, LIRO), 0.1 $mg{\cdot}l^{-1}$ meta-topoline, 0.058 M sucrose, MS-vitamins and 8 $g{\cdot}l^{-1}$ agar. pH 5.8 prior to autoclaving
2. Preculture medium: liquid medium with 0.3, 0.5 and 0.7 M sucrose
3. Sterile containers for preculture
4. Osmoprotective loading solution: 2 M glycerol + 0.4 M sucrose in liquid standard medium
5. PVS2: 30 % (w/v) glycerol, 15 % (w/v) DMSO and 15 % (w/v) ethylene glycol in liquid medium with 0.4 M sucrose (Sakai et al. 1990)
6. Rinsing solution: liquid medium with 1.2 M sucrose

Methods

Day 1–3

1. Dissection of shoot tips: Shoot tips from shoots cultured for 3 months without subculture are used. All leaves surrounding the meristem are removed, and it is isolated together with a small portion of stem tissue. The explant size is: length = 2.5 mm and width = 1 mm.
2. Preculture in liquid medium with sucrose: Preculture in Petri dishes after dissection. Day 1: 0.3 M; Day 2: 0.5 M; Day 3: 0.7 M

Day 4

1. Remove preculture medium, add osmoprotective loading solution and hold for 20 min at room temperature.
2. Remove solution, add PVS2 on ice and hold for 80 min.
3. Transfer explants to 2 ml cryovials with 1.8 ml fresh PVS2 solution. Seal with Teflon tape and plunge in liquid nitrogen.

Day 5

1. To rewarm, immerse cryovials in 38°C water for 2 min.
2. Remove PVS2 immediately and replace with rinsing solution, hold for 30 min. Plate on solid standard medium for regrowth in standard culture conditions.

14.7.5 Encapsulation Dehydration of **Robinia pseudoacacia**

By Hans Verleysen based on Verleysen et al. 2005

Materials

1. Murashige and Skoog (1962) (MS) medium with 100 mg.l^{-1} myo-inositol, 0.4 mg l^{-1} thiamine-HCl, 100 mg l^{-1} sequestrene (Sequestrene 138 Fe 100 SG, LIRO), 0.1 mg.l^{-1} meta-topoline, 0.058 M sucrose, and 8 g.l^{-1} agar. pH 5.8
2. Preculture medium: liquid MS with 0.7 M sucrose. Sterile containers should also be prepared for preculture purposes.
3. Alginate solution: 3 % (w/v) alginate in liquid MS with no $CaCl_2$ and with 0.4 M sucrose + 2 M glycerol
4. Calcium chloride solution: 0.1 M $CaCl_2$ in liquid 0.058M sucrose MS
5. Rehydration solution: Liquid MS with 1 M sucrose

Methods

Day 1–3

Dissection of shoot tips: *In vitro* apical shoot tips from shoots grown without subculture for 3 months are used. All leaves surrounding the meristem are removed, and it is isolated together with a small portion of stem tissue. Length = 2.5 mm and width = 1 mm. Preculture shoot tips in 0.7 M sucrose liquid culture medium for 3 days. Use approximately 50 ml liquid medium for 10–20 explants.

Day 4

1. Encapsulation: Mix the Na–alginate solution with the explants. Draw up a single shoot tip and some alginate in a sterile pipette and drop into the $CaCl_2$ solution. Leave the beads in the $CaCl_2$ for 15 min after the last bead is made.
2. Desiccation: Alginate beads are dried in open Petri dishes placed in the air current of a laminar flow bench. Optimal regrowth is obtained when beads with low water content are used (30%).
3. For moisture content (MC) determination: Determine the fresh weight in an aluminum foil boat then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
4. Cryopreservation: Beads were placed in cryovials (10 per vial) and plunged in liquid nitrogen.
5. Warming: Immerse vials in a 38°C water bath for 2 min.
6. Recovery: Add rehydration medium for 1 h, then transfer beads to solid standard medium for regrowth under standard conditions.

Chapter 15
Cryopreservation of Fruit and Ornamental Trees

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15.1 Introduction

Non-forest woody plants include many fruit trees and ornamental plants. They not only provide delicious fruit but are also important as horticultural landscape plants. Conservation of these germplasm resources is necessary for the continued development of agriculture. As most species and cultivars of woody plants are genetically heterozygous, their genetic integrity must be maintained through vegetative propagation. The conventional method for woody plants is conservation in the field. However for long-term preservation of these genetic resources the field genebank is not only costly, because of the land and constant maintenance, but also susceptible to insects, diseases and environmental stress (Engelmann 2000; Reed et al. 2005). *In-vitro* cultured plantlets can be mass propagated with limited space and under disease-free conditions. Most cultures can be held under reduced or minimum growth conditions for years without reculture. Thus *in-vitro* plantlets are suitable materials for short- or medium-term backup storage of plant germplasm (Engelmann 2003; Reed and Chang 1997).

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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Cryopreserved storage of plants in liquid nitrogen (LN) is the most promising approach to achieve long-term maintenance of woody plant germplasm (Sakai 1995). Cryopreservation can save labor and space, and it complements current germplasm storage methods, making it an important tool for long-term storage of vegetatively propagated plants (Engelmann 2000). Sakai (1960) first demonstrated that winter-hardy twigs survived after immersion in LN for 1 year. Since then many reports indicate that materials with meristems or embryogenic cultures of woody plants can be conserved successfully in LN (Kartha 1985, Sakai 1995, Reed and Chang 1997).

15.2 Plant Considerations

The ability of plants to withstand freezing and warming stress varies, so it is important to select and condition plants before cryopreservation. Parameters involved in the cryopreservation of woody plants can be broadly categorized as plant material, preconditioning, preculture, cooling, rewarming, recovery, regrowth, and viability testing.

15.2.1 Plant Material

An important requirement of plant germplasm conservation is to guarantee genetic stability of stored plants during prolonged preservation. For woody plants the progenies regenerated from meristems through vegetative propagation display genetic stability similar to other methods of plant propagation. Thus materials with meristematic tissues are ideal candidates for the cryopreservation of woody plants. Cryopreservation of woody plants is successful using dormant buds (Chap. 16) as well as *in-vitro* grown plantlets. The physiological condition of the plants is of paramount importance in either technique. Dormant buds must be deeply dormant and *in-vitro* shoots must be healthy, as well as cold acclimated, for successful cryopreservation. Preconditioning and preculture of plants is required for the successful regrowth of cryopreserved shoot tips.

15.2.2 Preconditioning

Adequate conditioning of shoot tips or mother plantlets is essential to induce a physiological status that allows the plant to withstand the stresses that occur during the cryopreservation process. Shoot cultures are generally divided and transferred to new growth medium at 3–4 week intervals.

The size of the plant and the time it has spent on the medium since the last division can greatly affect recovery following cryopreservation (Chang and Reed 2001; Reed 1996). The appropriate size of shoot tips suitable for cryopreservation is 0.8–1 mm and consists of the apical dome plus a few leaf primordia (Takagi 2000). Smaller apices tend to die or become lost during the process and larger ones are not properly cryoprotected.

15.2.2.1 Growth Medium

The optimal physiological status of shoot tips or mother plants is one of the essential factors to achieve high shoot regrowth. Plants may be held on the same medium for 3–4 weeks prior to preconditioning, or sometimes for several months. Studies on the cryopreservation of *Prunus in-vitro* shoot tips (Zhao et al. 1999) indicated that, while no difference was observed in the survival of cryopreserved shoot tips sampled from 30-day (60%) or 90-day old (62%) mother plants, the shoot tips from 30-day old mother plants remained green after cryopreservation and regrew faster than those from 90-d old mother plants. Not all kinds of materials respond this way. *Malus* plants cultured for 12 weeks without transfer had higher shoot regrowth following cryopreservation than those cultured for 6 weeks. Shoots excised from plants cultured without transfer for 6 weeks were faster growing and had higher meristem moisture contents (about 85%) than those cultured for 12 weeks (about 77%). Twelve-week old plantlets had nearly stopped growing, the water content was reduced and they could withstand cryopreservation better (Chang et al. 1992; Wu et al. 1999). Pear plantlets grown for long periods without transfer also had high post-cryopreservation recovery (Chang and Reed 2001).

15.2.2.2 Cold Acclimation

Cold acclimation (CA) preconditioning is used to trigger a plant's natural resistance mechanisms to cold weather and is most effective for temperate plants (Dereuddre et al. 1990b; Reed 1988, 1990). Many reports confirm that CA leads to improvements in recovery after cryopreservation for cold-tolerant species, as does preculture on medium with osmotic agents (Chang and Reed 2001; Engelmann 1997; Reed and Lagerstedt 1987; Reed 1988). Chang et al. (1992) found that CA of apple mother plantlets for 38 days at 5°C improved regrowth after cryopreservation significantly better than without CA. Further experiments demonstrated that CA not only improved the regrowth but also reduced the amount of callus during regrowth (Wu et al. 1999). CA of blackberry meristems at alternating temperatures for 2–4 weeks doubled the regrowth of meristems following cryopreservation

(Reed 1993; Chang and Reed 1999). There are several reports describing the amount of CA required for plants to reach a maximum freezing tolerance for cryopreservation (Chang and Reed 1999, 2000b, 2001). Many studies showed that CA requirements for successful cryopreservation varied among cultivars and species. Some pear cultivars were greatly improved by 1–15 weeks of CA while others were greatly improved after 1 week (Chang and Reed 2000a, b) (Fig. 10.1; Protocol 10.8.2). Blackberries required 2–10 weeks of CA, but 4 weeks provided adequate improvement for most genotypes (Chang and Reed 2000a). The type of CA was shown to be very important for reaching maximum cold tolerance in pears (Chang and Reed 2000b). Cold tolerance is expressed as LT_{50}; the temperature at which half of the tested plants die. The cold hardiness of shoots acclimated with alternating temperatures (22°C 8-h day/–1°C 16-h night) was –25°C while those acclimated with a constant 4°C were –15°C. Plants cultured at growth room temperature survived only to –10°C. Cryopreservation recovery was directly related to the cold hardiness of the pear shoots and alternating-temperature acclimation improved regrowth from <20% to 100% for both species tested. Constant-temperature acclimation improved cold hardiness and regrowth but not to the extent of the alternating temperatures. The photoperiod during CA did not significantly affect the recovery from cryopreservation (Chang and Reed 2000b).

CA is effective in increasing the freezing tolerance of most temperate plants and some subtropical plants. Tropical plants do not respond to CA, and sucrose preculture of shoot tips prior to freezing could increase their viability after cryopreservation. Temperate plants also respond to sucrose preconditioning. Pear shoot tips from plantlets grown on 5% or 7% sucrose medium for 3 weeks prior to CA had greatly increased recovery from cryopreservation (70%) compared to those grown on 3% sucrose medium (35%) (Chang and Reed 2001). In some instances sucrose preculture could replace the effect of the CA, increase cold-hardiness and impart desiccation tolerance (Stushnoff et al. 1998). Dumet's experiments on the cryopreservation of black current meristems using encapsulation dehydration showed that CA treatment of plantlets could be replaced by high sucrose conditioning of the shoots. In Ben Lomond, 95% of meristems from the 7-day 0.7 M sucrose-conditioned shoots regrew. Regrowth was similar to meristems from CA plantlets (Dumet et al. 2000).

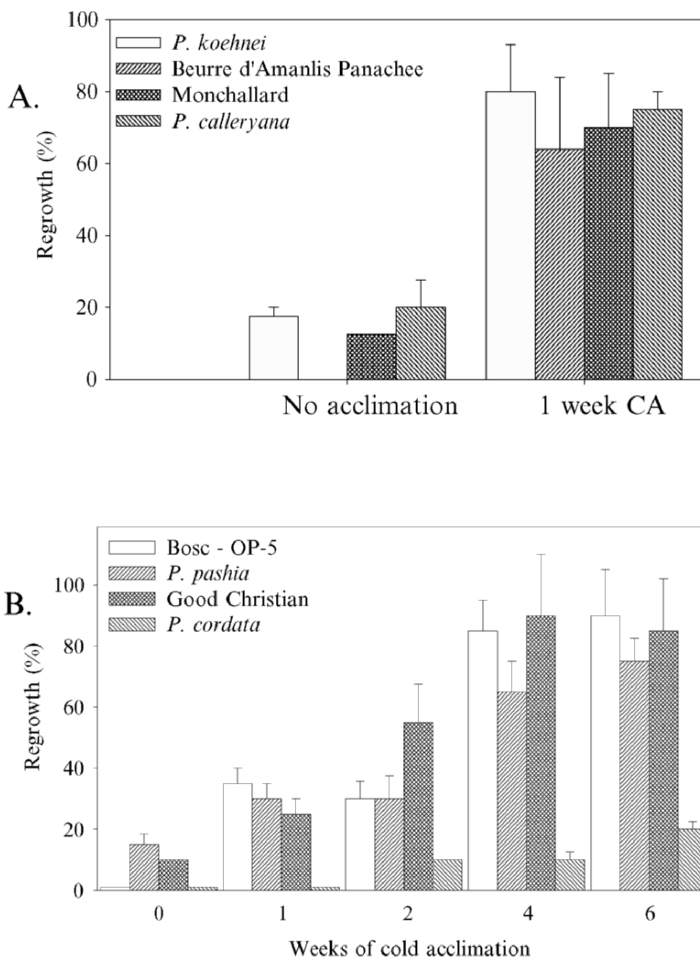

Fig. 15.1 Cryopreservation of pears by controlled rate cooling: A. Regrowth of control (no CA) and 1-week CA. CA conditions were 8 h light at 22°C and 16 h dark at –1°C. B. Effects of the length of CA on recovery of pear genotypes (From Chang and Reed 2000b) Lines indicate SD

15.2.2.3 Abscisic Acid

Abscisic acid (ABA) is generally very effective for inducing cold tolerance in plant cells, but it is often less effective for whole plant organs. *Pyrus* shoot tips treated with 50 μM ABA during CA had greatly increased regrowth following cryopreservation while ABA alone was not effective. (Chang and Reed 2001). The combination of alternating low-temperature acclimation and ABA significantly increased the cold hardiness of pear shoot tips and resulted in greatly increased regrowth following cryopreservation. Regrowth following CA increased from 15% without ABA to 75% with 50 μM ABA. The combination of alternating low-temperature CA and ABA significantly increased the cold hardiness (more negative LT_{50}) of pear shoot tips.

15.2.3 Preculture

DMSO is used as a preculture treatment after shoot tips are dissected and prior to cryoprotection. Preculture on medium with 5% DMSO for 48 h is commonly used with all controlled rate cooling and many vitrification protocols. Seventy to eighty percent of axillary apices excised from *Prunus in-vitro* cultured plants survived following vitrification when the preculture medium included 5% DMSO and 5% proline (Paulus et al. 1993). The same preculture medium was effective with controlled rate cooling or vitrification for two *Prunus* rootstocks with average shoot formation rate of 69% and 74% (Brison et al. 1995; de Boucaud et al. 2001) (Protocols 15.8.3; 15.8.4). One day preculture with 0.1 M sucrose increased regrowth of apple shoot tips after cryopreservation from 60% to 72.2%. The best results were observed when shoot tips were precultured in serial sucrose solutions of 0.1 M, 0.3 M and 0.7 M with daily transfers (Zhao et al. 1999). Excellent results were observed for the cryopreservation of pear and mulberry shoot tips with sucrose preculture (Niino et al. 1992a, b).

15.3 Cryopreservation

Shoot tips employed in cryopreservation generally contain high amounts of cellular water, and preconditioning and preculture of plants prior to cryopreservation are generally not enough to protect cells from injury. Cryogenic procedures such as controlled rate cooling, vitrification, and encapsulation dehydration achieve the required dehydration in different ways.

15.3.1 Controlled Rate Cooling

Controlled rate cooling protocols are used for the cryopreservation of many fruit trees. This technique allows crystallization of extracellular water to form ice outside the cell. The ice formation causes water to move from the cell cytoplasm to the extracellular ice, thus dehydrating the cells. The slower and longer the cooling period, the more dehydration occurs. Kuo and Lineberger (1985) tested cryopreservation for *in-vitro* grown shoot tips taken from proliferating cultures of apple (*Malus domestica* 'Jonathan'). Only callus was recovered after cooling at 1°C/min in 10% glycerol. About 85% of apple shoot tips survived after cryopreservation with 10% DMSO as a cryoprotectant (Chang et al. 1992). The best regrowth (98%) was observed when using 5% DMSO and 5% glycerol as cryoprotectants. Pear shoot tips cooled in the cryoprotectant PGD (10% each polyethylene glycol, glucose and DMSO in MS medium) at 0.1°C/min to –40°C followed by immersion in LN had 55% to 95% regrowth for the various genotypes (Reed 1990) (Protocol 15.8.2). Pear shoot tips were also compared by three methods (Zhao et al. 2004). Shoot tips of apple (Katano et al. 1984), and mulberry (Niino and Oka 1990; Yakuwa and Oka 1988) were successfully cryopreserved by controlled rate cooling. *Prunus* shoot tips cryopreserved by slow cooling recovered with 70–75% regrowth (Brison et al. 1995; Protocol 15.8.3).

15.3.2 Encapsulation Dehydration

Dereuddre et al. (1990a) developed the encapsulation-dehydration technique with *in-vitro* pear axillary shoot tips. Shoot tips from plantlets cold acclimated for two months were encased in alginate gel beads, precultured in a liquid medium with 0.75 M sucrose for 18 h, and dehydrated in the laminar-flow cabinet for 3 h. The beads were cooled rapidly in LN. After rewarming slowly in air at room temperature, high survival (80%) was obtained and 40% of the surviving shoot tips produced new plantlets. Niino and Sakai (1992) cryopreserved *in-vitro* grown pear, mulberry and apple shoot tips with a modified encapsulation-dehydration method. Plantlets were CA at 5°C for three weeks, shoot tips were excised and progressively precultured at 5°C by daily transfer of the shoot tips onto MS medium with 0.1, 0.4 and 0.7 M sucrose. Shoot tips were then encapsulated in alginate beads and treated with 1.0 M sucrose for 16 h at 5°C. After treatment the beads were dehydrated to 33% water content with silica gel before immersion in LN. Shoot regrowth was about 70%.

Experiments on the cryopreservation of almond shoot tips showed that with encapsulation-dehydration techniques the survival of shoot tips reached 62% after cryopreservation (Shatnawi et al. 1999). Several azalea cultivars were successfully cryopreserved using the encapsulation-dehydration protocol (Verleysen et al. 2003, 2004, 2005). Shoot tips (2.5 mm) from 3 month old plantlets were precultured on increasing amounts of sucrose before encapsulation and desiccation (Protocol 15.8.9). Beads were rapidly rewarmed, incubated in 1M sucrose and plated for regrowth. Apple cultivars were also cryopreserved using encapsulation dehydration (Zhao et al. 1999; Wu et al. 1999) (Table 15.1). Olive, *Olea europaea* cv. Arbequina, was successfully cryopreserved by dehydration alone (Martinez et al. 1999) (Protocol 10.8.8). Shoot tips were precultured in 0.75M sucrose, dehydrated in laminar flow for 2 h to 30% moisture content and plunged in LN. The shoots recovered at 30%. Other examples of cryopreservation of woody non-forest plants include walnut (de Boucaud and Brison 1995), *Malus* (Stushnoff and Seufferheld 1995) olive (Revilla et al. 2001), *Prunus* (Paulus et al. 1993; de Boucaud et al. 2001), *Ribes* (Reed and Hummer 2001), coffee (Dussert et al. 2001), and *Rosa* (Lynch 2001).

Table 15.1 Apple species and cultivars cryopreserved using the encapsulation-dehydration technique

Apple species/cultivars	Reference
M. domestica cv. Tsugaru	Zhao et al. 1999
cv. Orin	Zhao et al. 1999
cv. Red Fuji	Zhao et al. 1999
cv. Jonagold	Zhao et al. 1999
cv. HAC-9	Zhao et al. 1999
cv. Senshu	Zhao et al. 1999
cv. 278	Wu et al. 1999
cv. S-29	Wu et al. 1999
cv. Yatac	Wu et al. 1999
cv.CG-80	Wu et al. 1999
cv. Tsugaru	Wu et al. 1999
cv. Xianghong	Wu et al. 1999
cv. Red Star	Wu et al. 1999
M. robusta	Zhao et al. 1999

15.3.3 Vitrification

Niino et al. (1992) developed a successful vitrification method for apple and pear shoot tips. CA shoot tips were precultured at 5°C for 1 day on MS medium with 0.7 M sucrose, and transferred to PVS2. Resulting shoot

formation was 80%. PVS3 vitrification of apple was also successful (Zhao et al. 1995) (Protocol 15.8.1). The shoot tips of papaya (Ashmore et al. 2001) were successfully cryopreserved by using a modified PVS2 (Protocol 15.8.7) (Fig. 15.2). Zeng et al. (2004) found that 53.7% of papaya shoot tips survived and could regrow into plantlets following vitrification. Zhao et al. (1995) tested vitrification solutions PVS1, PVS2 and PVS3 on three apple cultivars with several exposure durations. Six genotypes of apple shoot tips were cryopreserved by a simplified vitrification technique using PVS3 (50% sucrose and 50% glycerol in water). The best results were obtained with shoot tips excised from CA plantlets precultured on solid MS medium with 0.7 M sucrose for 1 day, followed by dehydration with PVS3 for 80 min. Regrowth without callus formation was observed after rapid warming.

Prunus shoot tips of difficult to cryopreserve genotypes were successfully cryopreserved using vitrification on aluminum foil strips (de Boucaud et al. 2001; Protocol 15.8.4). Vitrification of chestnut shoot tips was obtained by Vidal et al. (2005). Cultures were CA at 4°C for 2 week before vitrification in PVS2 and rapid rewarming (Table 15.2; Protocol 15.8.6).

Table 15.2 Shoot recovery (% ± SE) of cryopreserved apices of six chestnut (*Castanea sativa*) genotypes from juvenile- and mature-tree origin. Assessment was made 8 weeks after cryopreservation by vitrification and plating on recovery medium. (From Vidal et al. 2005)

Genotype	Origin	Shoot recovery (%)
Castanea sativa 812	Juvenile	53.2 ± 1.6
818	Juvenile	53.3 ± 3.3
12	Juvenile	37.5 ± 9.8
LA1	Mature	35.4 ± 4.4
LA3	Mature	54.4 ± 8.1
Pr5	Mature	42.5 ± 3.8

15.3.4 Encapsulation Vitrification

Due to the high toxicity of vitrification solutions and the difficulties of manipulating many samples at the same time, several laboratories tested new vitrification solutions or vitrification techniques using a combination of encapsulation dehydration and vitrification. In-vitro grown ‘Gala’ apple shoot tips were successfully cryopreserved in a new vitrification solution PVSL (40% glycerol, 45% sucrose, 10% ethylene glycol and 10% DMSO). The average regrowth was 81% and the shoots showed the same regrowth as those with no treatment (Liu et al. 2004). Wang et al. (2002)

successfully cryopreserved the shoot tips of ‘Troyer’ citrange (× *Citron cirus* J. Ingram and H. Moore) by the encapsulation-vitrification technique. Excised shoot tips were precultured with increasing sucrose concentrations of 0.3, 0.5, 0.75 and 1 M for 4 days, encapsulated and treated with an osmoprotective loading solution of 2 M glycerol and 1 M sucrose for 60 min. The encapsulated shoot tips were then treated with PVS2 for 90 min. Regrowth was 100%.

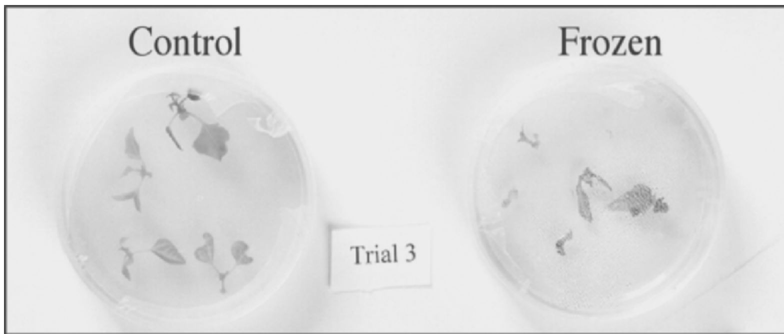

Fig. 15.2 Papaya (*Carica papaya*) shoot tips on recovery medium following vitrification with PVS2 on foil strips: control (left) and post LN samples (right) (From Ashmore et al. 2001) (Protocol 15.8.7)

15.3.5 Vitrification and Controlled Rate Cooling

Prunus rootstock and almond cultivars were cryopreserved using several methods (Brison et al. 1995; de Boucaud et al. 2001). Cryopreservation was performed in tubes with controlled rate cooling or rapid cooling on aluminum foil involved a vitrification solution of 25 % w/v glycerol, 15% w/v ethylene glycol, 12.5% w/v DMSO, 3% w/v polyethylene glycol 8000, 0.4 M sucrose in MS medium (Protocol 15.8.3; 15.8.4). Shoot tips were also successfully cryopreserved by encapsulation dehydration following the method of Derreudre et al. (1990a, b). A mixed method combining the two was also used. This consists of exposing the alginate-coated shoot tips to modified PVS2 and cooling them with controlled rate cooling (de Boucaud and Brison 1995).

15.4 Rewarming of Samples

Rewarming of cryopreserved shoot tips can be performed by immersing samples in a 37–45°C water bath. It is generally accepted that rapid warming is

required to retain viability of samples by avoiding ice recrystallization or devitrification within the cells which can occur if the rewarming rate is too slow (Withers 1980). Because of the technical difficulties in testing warming rates, the effect of warming of materials after cryopreservation has not been studied as extensively as cooling rates. Direct evidence on optimal warming conditions of plants is thus sparse. To avoid ice recrystallization and cell damage, it is important to keep all materials completely immersed in LN until rewarming (de Boucaud et al. 2001). Encapsulation-dehydration techniques that originally used room temperature warming now utilize rewarming in 40°C water for 1–2 min and yield improved results.

15.5 Recovery and Regrowth

Shoot tips cryopreserved with controlled rate cooling are generally rinsed with liquid MS (Murashige and Skoog 1962) medium and those from vitrification with 1.2 M sucrose MS medium. Those stored by encapsulation-dehydration techniques are commonly transferred onto the culture medium directly for regrowth. Sometimes the beads are rehydrated with liquid medium prior to plating (Gupta and Reed 2006). In some cases, shoot tips are removed from the beads for regrowth. Samples should be placed in optimal conditions to trigger rapid and direct growth. The culture medium is often modified from the normal medium, usually by changing growth regulators. Removing auxin from the recovery medium greatly decreased callus production and increased shoot formation of blackberry shoot tips following controlled cooling (Chang and Reed 1999). Recovery is commonly begun in the dark or with low light to decrease oxidative stress, and plantlets are moved into standard growth room conditions after one week. Any shoot tips regrowing as callus should be discarded to avoid variants.

15.6 Viability Assessment

Several methods are used to test the viability of cryopreserved cells, but these tests are not very useful for shoot tips. Final recovery data for shoot tips of woody plants should be based on direct shoot formation after 4–6 weeks of culture. Verleysen et al. (2004) evaluated several techniques for predicting viability of cryopreserved shoot tips and found that electrolyte leakage and visually analyzed TTC-staining were the best for predicting viability.

15.7 Conclusions

Cryopreservation protocols for woody-plant germplasm resources are well developed. The protocols now available can be performed in a reliable and practical manner and are in use for long-term storage at a number of facilities. Backup collections of the plant germplasm resources cryopreserved in LN are now established in several countries (Reed 2001; Reed et al. 2005). It is hoped that improved cryopreservation techniques will provide added security in the preservation of important woody plant germplasm formerly preserved only as field collections.

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15.8 Protocols

15.8.1 PVS3 Vitrification of Apple Shoot Tips

By Y Zhao based on Zhao et al. 1995

Items needed

1. Tools, pipettes, sterile Petri dishes and beakers
2. CA plants for dissection
3. Sterile cryotubes, LN

Prepare in advance

1. Preconditioning: micropropagated plants CA at 5°C for 3 weeks under a 16 h (19 $\mu mol \cdot m^{-2} s^{-1}$) photoperiod
2. Sterile Petri dishes, beakers, pipettes, cryotubes and tools
3. Preculture: MS (Murashige and Skoog 1962) medium with 0.7 M sucrose and 0.7% agar in Petri dishes
4. PVS3: 50% (w/v) sucrose and 50% (w/v) glycerol in MS medium. Heat slowly and boil to dissolve; autoclave.
5. Recovery medium: MS, 1.0 mg/l BA, 0.05 mg/l NAA, 200 mg/l casein hydrolysate, 0.7 g/l agar

The procedure

1. Dissect CA shoot tips onto preculture medium.
2. Preculture for 48 h at 5°C in CA incubator.
3. Transfer ten shoot tips into a 1.5 ml cryotube; add 1 ml PVS3 and equilibrate for 80 min. Adjust the timing based on your plant type.
4. Submerge the cryotubes in LN.
5. Warm the cryotube in a 37°C water bath for 3–4 min, then place shoot tips on recovery medium for regrowth. Regrowth is normally>60% (Table 15.3).

Table 15.3 Regrowth (%) of apple shoot tips cryopreserved using vitrification in PVS3. (From Zhao et al. 1995)

Cultivars	Regrowth (%)
Malus domestica cv. Tsugaru	60.0
M. domestica cv. Orin	80.5
M. domestica cv. Red Fuji	78.0
M. domestica cv. Jonagold	80.5
M. domestica cv. HAC-9	67.8
M. domestica cv. Senshu	80.0
M. robusta	65.0

15.8.2 Controlled Rate Cooling of In Vitro Pyrus *Shoot Tips*

By B Reed and Y Chang, based on Reed 1990; Chang and Reed 2000b, 2001

Items 1: shoot tip dissection and pretreatment

1. Tools
2. Sterile Petri dishes
3. CA plants [22°C 8-h day (3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light) and –1°C 16-h night], 2–4 weeks (up to 3 months for some genotypes)
4. Preculture medium: MS medium with 5% DMSO and 7 g/l agar
5. Liquid Murashige and Skoog 1962 (MS) medium

Items 2: cryoprotectants and slow cooling

1. Sterile cryotubes
2. Cryoprotectant PGD: 10% each polyethylene glycol (MW8000), glucose and DMSO in MS medium
3. Programmable freezer
4. Liquid MS solution for rinsing shoot tips
5. Recovery medium for shoot tip regrowth: MS medium with ½ the nitrogen stock solution, 1 mg/l benzyladenine and 6 g agar

You will need a separate Petri dish of preculture medium and recovery medium, about 2 ml cryoprotectant PGD, 10 ml liquid MS medium, several beakers, sterile Petri dishes and pipettes per treatment.

The procedure

This is a 2 day procedure once the CA is accomplished.

1. Dissect shoot tips onto preculture medium and hold for 48 h in CA.
2. Transfer the shoot tips to two drops (~0.1 ml) liquid medium in a 1.2 ml cryotube on ice and add ~1 ml of the cryoprotectant PGD drop by drop to the top of the tube over 30 min.
3. Equilibrate the cryotube at 4°C for 30 min and then the cryoprotectant is drawn down to 1 ml. Close cap but do not overtighten.
4. Place cryotubes in programmable freezer, cool at 0.1°C/min to –9 and initiate the exotherm. To initiate the exotherm the freezer cools at 99.99°C/min to –50°C after the chamber temperature reaches –9°C (you may need to shake the cryotube holder and make sure the cryoprotectant solution is frozen) and then warms at 20°C/min to –15°C, and resumes cooling down at 0.1°C/min to –40°C.

5. Submerge vials in LN.
6. Rinse the (non-frozen) control shoot tips at the exotherm or after submerging the other samples into LN.
7. Warm the cryotubes in a 45°C water bath for 1 min and then 1 min in a 22°C water bath. Fast warming is critical to recovery.
8. Rinse the shoot tips with liquid MS medium and place on recovery medium.

Notes

Over 100 pear genotypes have been stored with this technique. It is a good procedure if you have a large number of plant types or a large number of samples as it requires little technician time. Plants are generally not sensitive to the cryoprotectant treatment and controls can be rinsed at the exotherm or later without impacting regrowth. See Fig. 15.1 for data.

Transfer of vials from LN to warming bath or from Dewar to Dewar should be done rapidly. Even a few seconds at room temperature can greatly affect viability.

The following should be checked initially.

1. Dissection—regrowth should be 80–100%.
2. Cooling rate—slower is best in most cases (0.1°C/min).
3. Recovery medium—eliminate auxins if callusing is a problem.
4. CA is needed to achieve good results. Alternating temperatures are more effective than a single temperature and light is not needed but may help for longer CA periods. Two to 4 weeks should work for most genotypes.

15.8.3 *Controlled Rate Cooling of* Prunus *Shoot Tips in Modified PVS2*

By MT de Boucaud based on Brison et al. 1995

Items 1: shoot tip dissection and pretreatment

1. Tools
2. Sterile Petri dishes
3. Preconditioning: CA plants (23°C 16 h light and 4°C 8 h night, 6 days) in an incubator
4. Preculture: Murashige and Skoog 1962 (MS) medium with 5% DMSO and 2% proline and 0.7% agar
5. Modified PVS2: 12.5% DMSO 15% ethylene glycol, 25% glycerol, 3% polyethylene glycol (MW8000) and 0.4 M sucrose (w/v) in MS medium

Items 2: controlled rate cooling

1. Sterile cryotubes
2. Programmable freezer
3. Rinsing medium: Liquid ½ X MS medium with 1.2M sucrose
4. Recovery medium: MS basal medium (Murashige and Skoog, 1962) with 200 mg l^{-1} Sequestrene, 10 ml l^{-1} Morel and Wetmore vitamins (Morel and Wetmore, 1951), 30 gl^{-1} sucrose and 7 gl^{-1} agar, 4.4 mM 6-benzyladenine, 0.25 mM indole-3-butyric acid, and 0.29 mM gibberellic acid and pH 5.3
5. Ice bath

Prepare in advance

1. Preconditioning: Plants are CA with a 23°C 16-h light and 4°C, 8 h dark cycle for 6 days.
2. Sterile Petri dishes, pipettes, beakers, tools and cryotubes
3. Petri dishes of preculture medium
4. Rinsing medium
5. Modified PVS2 (MPVS2)
6. Ice
7. Recovery medium
8. You will need a separate flask of preculture medium, recovery medium, about 2 ml MPVS2 and 10 ml rinsing medium, and several beakers, sterile Petri dishes and pipettes per treatment.

The procedure
This is a 3 day procedure once the CA is accomplished.

Day 1: Dissect shoot tips onto preculture medium and hold for 48 h under CA conditions.

Day 2

1. Transfer the shoot tips to a cryotube with 1 ml MPVS2 on ice. Gently mix with shoot tips using a Pasteur pipette.
2. Equilibrate at room temperature for 30 min and then the MPVS2 is drawn down to 0.5 ml.
3. Place cryotubes in programmable freezer, cool at 1°C /min to –40°C and submerge in LN.

Day 3

1. Warm the cryotubes in 40°C water bath for 1 min.
2. Rinse the shoot tips twice with rinsing medium and place on recovery medium.
3. Take regrowth data at 6 weeks.
4. Two cultivars tested had 69% and 75% regrowth.

Possible problems
Plants are generally sensitive to PVS2 treatment and slow cooling, so the investigation should include all of the following controls before further use of the technique.

1. Dissection
2. Pretreatment
3. PVS2
4. Cooling rate
5. LN exposure

15.8.4 *Vitrification of* Prunus *Shoot Tips on Foil Strips*

By MT de Boucaud based on de Boucaud et al. 2002

Items needed

1. Sterile tools and pipettes
2. Sterile cryotubes 2 ml
3. Sterile Petri dishes
4. Sterile beakers
5. Gloves or forceps for handling cold cryotubes
6. Polystyrene box cleaned with alcohol to hold LN
7. CA plants for shoot tips
8. MS (Murashige and Skoog 1962) medium with 0.7% agar in Petri dishes (both 6 cm and 9 cm)
9. Preculture medium: Solid MS medium with both DMSO (5%) and proline (1–2 %) in small Petri dishes (6 cm)
10. Rinsing solution: MS medium with 1.2 M sucrose
11. Sterile rectangular (2 × 0.7 cm) aluminum foils (0.03 mm thick) cut to be introduced in cryotubes

Prepare in advance

1. Preconditioning: Plants are CA with a 23°C 16-h light and 4°C 8 h dark cycle for 6 days.
2. Excised shoot tips
3. Tools, pipettes, sterile Petri dishes, cryotubes
4. Sterile aluminum foil strips cut to fit cryotubes
5. Preculture medium
6. Standard solid MS medium
7. Modified PVS2 (MPVS2): 25% w/v glycerol, 15% w/v ethylene glycol, 12.5 % w/v DMSO, 3% w/v polyethylene glycol 8000, 0.4 M sucrose in MS medium
8. Rinsing solution: MS with 1.2 M sucrose medium
9. Sterile polystyrene box in the laminar flow cabinet, filled at the last moment with LN

The procedure

1. Excise shoot tips and preculture under CA conditions for 48 h.
2. Incubate shoot tips in MPVS2 for 75–120 min.
3. During incubation, place sterile aluminum foils in sterile Petri dishes (about six per dish) under laminar flow and make a bend in one corner to facilitate grasping foil. Place sterile opened cryotubes in other Petri dish (9 cm).

4. Near the end of the MPVS2 incubation prepare four–six droplets of MPVS2 on each foil with a shoot tip in each drop.
5. With one hand grasp each piece of foil with forceps and place it horizontally in polystyrene box filled with LN.
6. With other hand plunge a sterile cryotube in the LN of polystyrene box to fill it and introduce the foil. Close the cryotube with a bigger forceps.
7. Leave cryotubes a few minutes in the box or transfer them to a Dewar for longer storage.
8. For warming, keep the tubes submerged in LN, open tubes in LN with forceps, grasp aluminum foils with fine forceps and quickly plunge foils into 40°C rinsing solution.
9. Plate rinsed shoot tips on solid standard MS medium for regrowth.

Possible problems

1. Shoot tips can fall into the LN box if the foil is not held horizontally at the moment of plunging in LN.
2. It may be difficult to introduce frozen aluminum foils with shoot tips into the cryotubes if they are too large. Check the size in advance before starting.

This method succeeded on difficult forms of *Prunus* like cvs. Ogden, Torinel, Samisch, and Chasanov.

15.8.5 *Vitrification of* **Prunus domestica** *Shoot Tips*

By M Lambardi based on De Carlo et al. 2000

Checklist for vitrification

Day 1. Items needed for sugar pretreatment

1. Forceps/tools
2. Preculture medium: MS (Murashige and Skoog 1962) medium, hormone-free with 0.09 M sucrose and 0.7% agar

Day 3. Items needed for cryopreservation

1. Forceps, tools
2. Sterile pipettes or syringes for each solution
3. Cryovials and markers
4. Cryobox or cryocane to hold the cryovials
5. Nalgene Labtop Cooler (0°C model) or crushed ice
6. Osmoprotectant solution
7. PVS2
8. Rinsing solution
9. Regrowth medium for recovery after warming

Prepare in advance

1. CA *in vitro* plants for 3 weeks at 4°C, 8 h photoperiod
2. Preculture medium
3. Nalgene Labtop Cooler in a –20°C freezer to precool it
4. Osmoprotectant loading solution: 2M glycerol + 0.4 M sucrose in MS medium
5. PVS2: 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v), 0.4 M sucrose in MS medium pH 5.8
6. Rinsing solution: Liquid MS medium with 1.2 M sucrose
7. Regrowth medium: Quoirin and Le Poivre (1977) medium, lacking NH_4NO_3 and with 0.09 M sucrose, 1.5 µM benzyladenine and 0.5 µM gibberellic acid (GA_3), 7 g/l agar

The procedure

1. Excise 1.5–2 mm shoot tips from CA plantlets. Include the apical meristem and 4–5 leaflets from basal and axillary buds.
2. Transfer to preculture medium for 2 days in CA.
3. Transfer into a 2-ml cryovial (10–12 shoot tips per cryovial) and add osmoprotectant loading solution (approximately 1 ml). Hold for 30 min at 25°C.
4. Remove the osmoprotectant solution and add PVS2 for 90 min. During this period place the cryovials into the Nalgene Labtop Cooler or on ice to maintain the solution at 0°C.
5. After 90 min, remove the PVS2 and add 0.6 ml fresh PVS2.
6. Plunge the cryovials into LN.
7. Warm at room temperature for 5–10 s, and then plunge into a 40°C water bath for 50 sec (warming rate of about 180°C/min).
8. Drain off the PVS2 and rinse for 20 min at 25°C with rinsing solution.
9. Plate the shoot tips on regrowth medium and maintain at 23°C under a 16 h photoperiod (60 µmol m^{-2} s^{-1}).

15.8.6 Vitrification of Chestnut (Castanea sativa) *Shoot Tips*

By AM Vietez based on Vidal et al. 2005

Items needed for cryopreservation by vitrification

1. Tools
2. Sterile filter papers
3. CA terminal buds for dissection of shoot tips
4. Preculture: Gresshoff and Doy (1972) medium (GD), no hormones
5. Osmoprotectant loading solution in 100 ml flasks
6. PVS2 in 100 ml flasks
7. Cryovials and markers
8. Sterile pipettes for removing solutions from cryovials
9. Rinsing medium: (100 ml flasks)
10. Petri dishes with sterile filter papers for draining shoot tips
11. Recovery medium: GD with hormones

Prepare in advance

1. Chestnut shoot cultures maintained by subculture in GD with 0.2 mg/l BA, 0.7% agar and subcultured at 4–5 week intervals.
2. Preconditioning: CA terminal buds (1 cm long) from actively growing cultures and placed on GD medium with 0.05 mg/l BA and 7% agar at 4°C for 2 weeks under dim light
3. Preculture medium: 0.2 M sucrose GD medium, 0.7% agar, with no BA in Petri dishes
4. Osmoprotectant loading solution: 2 M glycerol + 0.4 M sucrose in GD medium, 50 ml in 100 ml flasks
5. PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO (w/v), and 0.4 M sucrose in GD liquid medium, filter sterilize
6. Rinsing medium: GD liquid medium with 1.2 M sucrose
7. Sterile filter paper discs for draining shoot tips
8. Recovery medium: GD with 7% agar, 0.5 mg/l benzyladenine, 0.5 mg/l indole-3-acetic acid and 0.2 mg/l zeatin, in Petri dishes

The procedure

1. Excise 1 cm terminal shoot buds from 3 to 4 week-old cultures.
2. Transfer buds to preconditioning medium and CA for 2 weeks at 3–4°C under dim light.
3. Dissect 0.5–1.0 mm shoot apices from terminal buds.
4. Preculture shoot apices for 48 h at 3–4°C.

5. Osmoprotection: Add 1 ml osmotic loading solution to 2 ml cryovials, add shoots (15 per vial) and hold for 20 min at room temperature.
6. Replace loading solution with ice-cold PVS2 for 120 min at 0°C.
7. Remove PVS2 and add 0.6 ml PVS2. Place vials on cane (or in a cryobox), and submerge rapidly in LN.
8. Warm cryovials rapidly for 2 min in a 40°C water bath.
9. Drain off the PVS2 and add rinsing solution twice for 10 min each.
10. Pour the contents of each cryovial onto sterilized filter paper discs. Pick up the top filter with the shoot apices and place on recovery medium.
11. At 24 h, 2 weeks and 4 weeks, transfer the apices to fresh recovery medium without paper discs. Assess shoot recovery 8 weeks after warming.

Notes

1. Controls (dissection of shoot tips, preculture in sucrose medium, loading and PVS2 treatments) without LN exposure should be included in initial experiments. Shoot tip dissection and growth in recovery medium may require further investigation before use of the procedure for some genotypes.
2. This protocol produced 38–54% shoot recovery among six chestnut clones (Table 15.2). Eight weeks after warming, the developing shoots are transferred to standard GD medium for further shoot growth. After two transfers of 1 month each, shoots can achieve 2–3 cm in length, and are thus suitable for both shoot multiplication and rooting.

15.8.7 Vitrification of Papaya (Carica papaya) *Shoot Tips*

By S Ashmore based on Ashmore et al. 2001

Checklist

Day 1: Items for shoot tip dissection and preculture

1. Sterile tools, including forceps and scalpels
2. Small sterile Petri dishes for preculture
3. Preculture medium
4. Dissecting microscope
5. Sterile Petri dishes or sterile filter paper for dissection
6. Apically dominant *in vitro* papaya plants for shoot tips

Day 2: Vitrification and cryopreservation

1. Liquid MS (Murashige and Skoog 1962) recovery medium
2. Sterile 250 ml bottle for preparing 20% PVS2
3. 20% PVS2: take 20 ml sterile 100% PVS2 and make up to 100 ml using 80 ml of sterile liquid recovery medium.
4. Tools and sterile Petri dishes
5. PVS2
6. Sterile foil strips (approx. 1×3.5 cm^2)
7. Rinsing medium
8. Sterile 250 ml beaker for warming process
9. Cryotubes if required
10. Small Petri dishes of solid recovery medium
11. Dewar of LN

Prepare in advance

1. Apically dominant *C. papaya* micropropagated using the method of Drew (1988)
2. Preculture medium: Liquid MS or DeFossard salts (DeFossard et al. 1974) containing 3% sucrose, 0.2 mg/l gibberellic acid (GA_3), 0.5mg/l indole-3-acetic acid (IAA) and 0.1mg/l N^6 benzyladenine (BA)
3. PVS2: 30% glycerol, 15% ethylene glycol, 15% DMSO (w/v), 0.4 M sucrose in liquid MS. Filter sterilize before use.
4. Twenty percent PVS2 in liquid medium
5. Rinsing medium: MS salts or DeFossard salts with 1.4 M sucrose, 0.2 mg/l GA_3, 0.5 mg/l IAA and 0.1 mg/l BA

6. Recovery medium: MS salts or DeFossard salts with 3% sucrose, 0.2 mg/l GA_3, 0.5 mg/l IAA and 0.1 mg/l BA and 7% agar in small Petri dishes
7. Sterile foil strips cut to fit the cryotubes
8. Sterile 250 ml bottles for preparation of 20% PVS2 and beakers for the warming step
9. Sterile Pasteur pipettes for removing 20% PVS2

This is a 2 day procedure

Day 1: Excision of shoot tips and overnight incubation

1. Dissect axillary buds from apically dominant plants. Each shoot tip should be 1–2 mm without expanded leaf primordia.
2. Place shoot tips in liquid preculture medium in a small Petri dish and incubate at 27°C until the following day (approx. 20 h).

Day 2: Vitrification, warming, and recovery steps

1. Remove preculture medium and add enough 20% PVS2 to cover the shoot tips; incubate for 60 min at room temperature (25°C).
2. Remove 20% PVS2 and replace with 100% PVS2 for 20 min at 25°C (10–60 min at either 0°C or 25°C may be appropriate, depending on the sensitivity of the genotype to PVS2).
3. Ensure that the time spent in PVS2 is carefully monitored as PVS2 solution is phytotoxic and a few minutes extra incubation may kill the shoot tips.
4. During this second incubation period, prepare sterile foil strips by placing four–five drops of 100% PVS2 onto each strip (Fig. 15.3).
5. A few minutes before the 20 min PVS2 treatment has ended, place a single shoot tip in each drop of 100% PVS2 on foil strips.
6. For each experiment it is important to include control shoot tips from each step prior to plunging.
7. Immediately plunge the foils directly into LN.
8. Where samples are to be stored for longer periods, foils should be placed in cryotubes and stored on canes in a large LN storage vessel. Alternatively, shoot tips can be treated with 100% PVS2 in cryotubes and frozen directly in LN in the cryotubes.
9. For warming, remove foils from tubes and plunge directly into approximately 100 ml of rinsing medium at room temperature. This should be done in the laminar flow hood.
10. Hold for 30 min gently swirling every 5–10 min to ensure dilution of remaining PVS2 prior to plating onto recovery medium.

11. Place onto solid recovery medium in small Petri dishes.
12. Remove any excess medium with a sterile Pasteur pipette.
13. Incubate at 27°C in the dark for the first 48 h, then transfer to a 16-h light/8-h dark regime (25 µM m^{-2} s^{-1}).

Possible problems

1. Even a minute or two over exposure to 100% PVS2 solution can result in loss of viability and too little exposure can result in tissue damage during the vitrification process.
2. Speed of both cooling and warming steps is critical to survival of tissue. Direct plunging and direct rewarming of shoot tips on foil strips allows a faster cooling or rewarming process than using cryotubes.

Fig. 15.3 Papaya shoot tips placed in drops of 100% PVS2 on foil strips prior to plunging in liquid nitrogen

15.8.8 Dehydration of Olive Shoot Tips

By M Angeles Revilla and Helena Fernández based on Martínez et al. 1999

Preparation of plants

1. Isolation of embryos from fruits: remove the fleshy mesocarp of fruits collected in October and break the endocarp using a vice.
2. Immerse seeds in water overnight and afterwards pull out embryos by making a longitudinal excision in the endosperm with a scalpel.
3. Sterilize embryos by immersion in 70% ethanol for 2 min, followed by immersion in commercial bleach diluted to 20%, wash three times with distilled water.
4. Culture on MS medium, 0.7% agar, pH 5.8, at 25°C and 16 h photoperiod (40 µmol m^{-2} s^{-1}).
5. Plantlets are suitable for use after 30–40 days.

Prepare in advance

1. *In-vitro* shoot cultures: Nodal segments from the plantlets regenerated from the embryos were cultured on MS (Murashige and Skoog 1962) or DKW medium (Driver and Kuniyuki 1984) with 1 mg/l N^6 benzyladenine (BA), 0.1 mg/l indole butyric acid (IBA), 0.7% agar, under the same conditions as above. Subcultures were made every 40 days.
2. Preculture medium: 0.75 M sucrose DKW medium with 0.7% agar in Petri dishes
3. Petri dishes with 30 g of silica gel (previously activated at 120°C for 2 days) covered with sterile filter paper for draining beads
4. Recovery medium: solid DKW medium with 1 mg/l BA and 0.1 mg/l GA_3, 0.7% agar, pH 5.8

Items needed to dissect shoot tips and make beads

1. Sterile sharp forceps and scalpel, laminar flow hood
2. Tile with filter papers wrapped with aluminium foil and sterilised
3. Stereo microscope
4. Cryotubes (2 ml) and marker
5. Cryocanes and a Dewar flask with LN

The procedure

This is a 3 day procedure once we have the *in-vitro* cultures.

1. Dissect shoot tips, the meristematic dome with one or two pairs of leaf primordia (2–5 mm), from the *in-vitro* shoots at the end of the culture period (40 days).
2. Preculture shoot tips for 2 days.
3. Transfer the shoot tips onto filter paper in an open sterile Petri dish containing 30 g of silica gel and dry in the air current of a laminar flow hood for 2 h (33% water content).
4. Place the shoot tips in cryovials, insert into the cryocanes and plunge into the Dewar of LN.
5. Rewarm cryovials at room temperature for 15 min and transfer shoot tips to Petri dishes containing recovery medium. Maintain for 30 days at 25°C and 16 h photoperiod and shoots recover after 40–50 days. Transfer to standard medium.
6. Maximum recovery of shoots in the var. Arbequina (the only variety assayed) was about 35%, but considering survival of the frozen samples in relation to the dehydration controls, from 60% to 70 % of shoots were recovered following these treatments.

15.8.9 Encapsulation Dehydration of Azalea (Rhododendron)

By Hans Verleysen based on Verleysen et al. 2003, 2004, 2005

Materials

1. Culture medium: Woody plant medium (Lloyd and McCown 1980) with WPM-salts, 5 mg·l^{-1} 2iP [6-(γ,γ-dimethylallylamino)-purine], 75 mg·l^{-1} Fe-sequestrene (Sequestrene 138 Fe 100 SG), 100 mg·l^{-1} myo-inositol, 0.058 M sucrose, WPM-vitamins and 6 g·l^{-1} agar; pH 5.4
2. Preculture medium: Liquid medium with 0.3, 0.45 and 0.6 M sucrose and sterile containers
3. Alginate solution: liquid medium without $CaCl_2$ and with 3% (w/v) alginate with 0.32 M sucrose and 1.62 M glycerol
4. Calcium chloride solution: Liquid medium with 0.1 M $CaCl_2$ with 0.32 M sucrose + 1.62 M glycerol
5. Rinsing solution: Liquid culture medium with 1 M sucrose
6. Sterile pipettes for making beads
7. Sterile beakers for calcium chloride solution

Methods

Days 1–3

1. Dissection of shoot tips: Dissect apical shoot tips from 3-month-old shoots. All leaves surrounding the meristem are removed, and it is isolated together with a small portion of stem tissue. The explant size is: length = 2.5 mm and width = 1 mm.
2. Preculture: preculture in liquid culture medium with increasing sucrose concentrations. Use approximately 50 ml liquid culture medium and 10–20 explants per container: One day in each solution: 0.3M sucrose, 0.45M sucrose, and 0.6M sucrose

Day 4

1. Encapsulation: Shoot tips are placed in the alginate solution in a small beaker or Petri dish. With a pipette, pick up a shoot tip and some alginate and drip it into the $CaCl_2$ solution to make a bead. Hold for 15 min in the $CaCl_2$ solution.

2. Desiccation: Alginate beads are dried in open Petri dishes placed in the air current of a laminar flow bench. Space beads evenly in the Petri dish so they are not touching. Optimal regrowth of azalea is obtained when beads are dried to low water contents (38.6%).
3. For moisture content (MC) determination: Determine the fresh weight of ten beads replicated three times, each in an aluminum foil boat, then dry in an oven set at 103°C for 24 h, cool in a desiccator and reweigh.
4. Cooling: alginate beads are placed in cryovials (2 ml, ten explants per cryovial) and plunged in LN.

Day 5

1. Rewarming: Plunge cryovials into 38°C water for 2 min.
2. Recovery: Add rinsing solution to the tubes and hold for 2 h.
3. Drain on filter paper and plant on solid culture medium under standard culture conditions.

Chapter 16
Cryopreservation of Dormant Buds

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16.1 General Status and Important Factors

Dormant vegetative buds from diverse species can be preserved using cryopreservation. Sakai (1960) provided one of the first studies showing that winter twigs of poplar (*Populus sieboldi*) and willow (*Salix koriyanagi*) could survive low temperatures if slowly cooled prior to immersion in liquid nitrogen. A later study demonstrated that this simple methodology was also applicable to twigs of several fruit species (Sakai and Nishiyama 1978). With rising interest in the preservation of genetic resources, methodologies were further developed for fruit, nut, forest and ornamental species that can cold acclimate. Although dormant buds from cold hardy herbaceous perennial species might also be useful for cryopreservation, there are few studies, with the exception of garlic, that addressed the use of cryopreservation to preserve dormant buds from herbaceous species. It should also be emphasized that in this chapter, we use the term "dormant" in a broad sense to include buds that are dormant due to either endogenous (endodormancy) or to a variety of environmental conditions (ecodormancy).

The methods for cryopreservation of dormant buds utilize techniques described for other systems, including controlled rate cooling, vitrification, and encapsulation dehydration. The main difference is that a dormant bud is used for these techniques as contrasted to an actively growing shoot tip.

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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The method used for cryopreservation depends on the species as well as on the cold-hardiness level of the collected material. For example, many apple species are quite cold hardy and nodal sections are used directly for cryopreservation using controlled rate cooling (Forsline et al. 1998; Towill and Bonnart 2005). Persimmon (*Diospyros kaki* Thunb.) is not as cold hardy and shoot tips excised from dormant buds are cryopreserved by vitrification (Matsumoto et al. 2001). Dormant buds or shoot tips from very cold-hardy species, but which are collected in a less cold-acclimated condition, may be processed by vitrification, encapsulation dehydration or encapsulation vitrification methods.

16.2 General Methodology

Branches or other parts of dormant plants are harvested, usually in mid winter. For most woody material, nodal sections of the branch or excised buds may then be used for cryopreservation. Buds or sections may be partially desiccated before subsequent treatments but not all protocols require desiccation. Excised and/or desiccated buds may be directly cooled or first treated with cryoprotectants and then cooled.

After storage at cryogenic temperatures, samples are rewarmed and then rehydrated, if initially desiccated, and/or rinsed to remove a cryoprotectant. Warming of desiccated sections or buds is usually done slowly in air, either at room temperature, or at approximately 4°C. Buds treated with cryoprotectants, either for controlled cooling or vitrification, are usually rapidly warmed in a water bath. Recovery of the plant after cryopreservation can occur by direct grafting of the bud or micrografting of the shoot tip dissected from the dormant bud (apple), by culture of the shoot tip on an appropriate medium (persimmon) or by direct rooting of the cryopreserved branch section (willow).

It is often desirable to collect materials from the field in the most cold-hardy state, but this state is not always easy to discern or easily tested. Different lines or species have different kinetics of acclimation and the degree of cold hardiness may vary from year to year due to temperature fluctuations. After collection, current season woody branches can be further acclimated by very gradual exposure to lower temperatures (Sakai 1966), but details of the success of post-harvest acclimation are sparse for most species. Since it is not always possible to process the samples immediately, storage of the dormant material at temperatures between –3 and –5°C usually retains cold hardiness and viability for several months. We have successfully cryopreserved apple from 6–8 month-old stored branches

(Towill unpublished), but have also observed that sweet cherry branches decline over a 4–6 month storage period.

The recovery method of choice for cryopreserved buds depends upon the information available for the species. For example, apple can be recovered by directly grafting a bud from a nodal stem section that has been cryopreserved (Protocol 16.4.1). Pear can be recovered by *in vitro* culture of a shoot tip dissected from a cryopreserved dormant vegetative bud (Protocol 16.4.2). The choice of the recovery system is dependent on the species. For example if *in vitro* culture is considered, suitable recovery medium must exist and this medium must be useful for a diverse array of genotypes or species. Another factor is contamination, which is usually very problematic for *in vitro* culture of excised shoot tips from dormant buds. Grafting success requires both a viable meristem and cambium, and thus the method requires a propagule containing a portion of the stem. Fortunately, grafting methods are well described for many fruit and nut species; however, methodologies for patch bud or micrografting of individual buds are not well developed for most species. Grafting buds from herbaceous perennials is usually not possible, so culture is often the only option.

16.3 Critical Factors for Successful Cryopreservation

The most important factor identified to date for successful cryopreservation of dormant buds is the extent of cold acclimation that the species can attain and the extent of acclimation that the material possesses when collected. The data we obtained with apple are illustrative. Species of *Malus* that are very cold hardy showed high percentages of regrowth after cryopreservation and species that are less hardy had less regrowth (Towill et al. 2004). All materials were collected from one locale and hence were exposed to similar cold-acclimating conditions. For a given line, materials harvested in a greater state of cold acclimation have shown better survival after cryopreservation; supporting the notion that the degree of cold acclimation is important. In addition, it is well known that cold acclimation of *in vitro* plants (for species which possess the ability to cold harden) improves the percentage of shoot tips surviving cryopreservation (Chang and Reed 1999, 2000, 2001). In the field, the extent of cold acclimation may vary considerably from year to year depending upon cultivar and environmental conditions, such as temperature, light, nutrition and disease.

Another factor that is important for optimizing recovery for some species is the extent of desiccation obtained before cooling. In apple, high survival can be obtained with nodal sections, where the moisture content is reduced from 45% when collected, to 25–30% prior to cooling (Forsline et al. 1998). Buds isolated from these sections also contained 25–30% moisture. Pear buds had the best survival when the moisture content was reduced to approximately 41% (Suzuki et al. 1997). The effect of optimal moisture content must be empirically determined for each species.

As with any cryopreservation protocol, controlled cooling rates are critical for success, but the rate used depends upon the cryopreservation method. Rates of about 0.25°C min^{-1} to 0.5°C min^{-1} are used with dissected shoot tips from dormant buds in the presence of a cryoprotectant. With larger pieces of tissue, for example 35 mm *Malus* nodal sections, slower cooling rates are used (i.e. 1°C h^{-1}). With controlled rate cooling the transfer temperature to liquid nitrogen is usually in the range of –30 to –40°C. If cryoprotectants are not used, the transfer temperature depends upon the extent of acclimation and the species. The use of slower cooling rates may obviate the need for desiccation. In apple, cooling at about 5°C day^{-1} with ambient moisture contents, gave about the same survival percentages as cooling at 1°C h^{-1} with samples first desiccated to 30% moisture (Towill and Bonnart 2005). Matsumoto et al. (2004) and Ai and Luo (2005) cryopreserved nodal sections of winter twigs of persimmon using a controlled rate cooling procedure and recovered plants *in vitro*. Shoot tips from dormant buds can also be preserved by vitrification, in which case rapid rates are needed.

The following protocols are representative of how dormant buds may be used for cryopreservation. Researchers used dormant buds for cryopreservation of diverse materials, for example butternut (*Juglans cinerea* L.) (Ellis unpublished), elm (*Ulmus*) (Harvengt et al. 2004) and Scots pine (*Pinus sylvestris* L.) (Hohtola 1995); however the percent survival was not always high. Often only one method is tested, and thus, in many cases further experimentation is necessary to obtain high survival and to develop methods that are applicable across a wide array of genotypes.

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16.4 Protocols

16.4.1 Cryopreservation of Apple (Malus domestica) *Dormant Buds*

By LE Towill based on Forsline et al. 1998

Materials needed

1. Polyolefin tubes (3M Corp.) 19–22 mm diameter, 20–30 cm long
2. Heat sealer
3. Trays or screens to dry samples
4. Controlled rate freezer (Cryomed, ThermoForma)
5. Vapor phase storage dewar
6. 4°C room for rewarming samples
7. Moist peat moss
8. Rootstocks for grafting

Collection and storage

1. Uniform diameter branches of current season's growth are collected in mid-winter as soon after January 1 each year when at least 72 consecutive hours of ambient temperatures <–5°C have occurred and after 3 days in which temperatures do not exceed 0°C.
2. Branches are wrapped in plastic bags and stored at –3 to –5°C until use.

Sample preparation

1. Branches are cut into 35 mm nodal sections with the bud in the center of the length. The extra internode length on each bud section is needed to obtain a graftable bud for future recovery. To obtain these lengths of scion pieces, it may be necessary to waste every other bud. Processing will be much more efficient if all pieces of each accession have uniform twig diameter.
2. Bud sections are spread on cafeteria trays and held unsealed in a freezer at –5°C to dehydrate. Desiccation periods for each accession are usually quite consistent if length of bud piece is uniform. Desiccation occurs more quickly as twig diameter decreases so groups of two 5-twig samples of each accession are prepared to monitor the level of desiccation. Individual twigs in both samples are weighed immediately after cutting. Individual twigs in one 5-twig sample are oven dried at 85°C to determine their moisture content (MC) on a fresh-weight basis:

$$MC = \frac{(\text{Fresh wt} - \text{Oven dry wt})}{\text{Fresh wt}} \times 100$$

The average MC of the five twigs becomes the predicted MC of the second 5-twig sample. A predicted oven-dry weight for each twig of the second 5-twig sample is calculated:

$$\text{Predicted Oven dry wt} = \text{Fresh wt} \times (1 - (\text{MC}/100))$$

The second 5-twig sample is kept on the cafeteria tray with the rest of the twigs of that cultivar at –5°C. Individual twigs of the second sample are weighed every 2–3 days and their current MC is calculated as such

$$MC_{\text{day x}} = \frac{(\text{Fresh wt}_{\text{day x}} - \text{Predicted oven dw})}{\text{Fresh wt}_{\text{day x}}} \times 100$$

3. When twigs are at about 30% moisture they are double wrapped in moisture-proof plastic to eliminate further desiccation and held at –5°C until LN treatment.

Cooling and warming

1. About 20 twigs are placed into each polyolefin tubes, heat sealed, placed at –5°C (cold room) and held for 30 min.
2. Tubes are cooled in a controlled rate freezer at 1°C /h from –5°C to –30°C and held at –30°C for 24 h.
3. Tubes are quickly removed from the cooling unit and placed within liquid nitrogen vapors (ca. –180°C).

Viability assessment

1. Tubes are removed from liquid nitrogen vapors and placed at 4°C for 24 h to thaw.
2. Bud sections are rehydrated for 15 days at 2°C covered in moist (not saturated) peat moss in moisture-tight plastic containers.
3. Two days before rehydration begins, apple seedling rootstocks (5.0–9.0 mm diameter) are potted into 64 × 255 mm 'Deepots'. Rootstocks are grown for 17 days at 24°C day/18C night. Supplemental lighting is supplied by high-pressure sodium lights for 15 h each day.
4. Rootstocks are grafted rehydrated buds using a modified chip bud. The bud sheath is sliced thinly to remove only phloem/bark tissue of the stem since the xylem generally appears to be injured from

LN exposure. This maximizes the contact of healthy tissues between stock and scion. Five rootstocks for each entry are double-budded 150–200 mm above soil level. Buds are inserted opposite each other on the rootstock and offset vertically by 25 mm. Buds are tied very firmly in place (this must be monitored closely) with 100 × 12 mm budding rubbers.

5. Budded rootstocks are placed in a greenhouse for 1–2 months. Budding rubber is removed after about 3–4 weeks. Seventeen days after grafting, tops are cut 50 mm above the grafted apical bud to reduce the effect of apical dominance. Emerging rootstock buds are removed to encourage the growth of the grafted buds. The top of the rootstock is removed to allow the grafted bud to develop.
6. Results vary with the cultivar (Table 16.1)

Table 16.1 Recovery of apples cryopreserved as dormant buds

Apple cultivar	Accession number	Recovery %
Novosibirski Sweet	1992	100
Starkrimson	94	97
Demir	202	73
Empire	157	45
Burgundy	150	40
Golden Delicious	3490	22
Ein Shemer	109	15
M. florentina	185	0

Notes

Shoot tips from dormant apple twigs have been cryopreserved by several different methods. Tyler and Stushnoff (1988a, b) presented a detailed study of moisture content and date of collection on cryopreservability using nodal sections. Katano et al. 1983 used undried sections and retrieved shoot tips in culture after liquid nitrogen exposure. Seufferheld et al. (1999) applied sugars and alginate to dormant sections of apple prior to desiccation and controlled rate cooling. This allowed them to cryopreserve materials that are more cold-tender. Wu et al. (2001) excised shoot tips from dormant materials and cryopreserved them with controlled rate cooling, vitrification and encapsulation–dehydration methods.

16.4.2 Cryopreservation of Pear and Mulberry Dormant Buds

By T Niino based on Oka et al. 1991; Suzuki et al. 1997 (Pear); Niino et al. 1992; Niino, 2000 (Mulberry)

Materials needed

1. Polyethylene bags or cryotubes
2. Deep freezers with adjustable temperatures
3. Suitable culture medium or grafting stock for recovery
4. Cold room
5. Recovery medium
   a. Pear: WPM (Lloyd and McCown 1980) medium with 1.0 mg l^{-1} N^6 benzyladenine (BA), 2.5 % (w/v) sucrose, 8 g l^{-1} agar at pH 5.6
   b. MS (Murashige and Skoog 1962) medium with 1.0 mg l^{-1} BA, 2.0 % (w/v) fructose, 8 g l^{-1} agar at pH 6.0

Collection and storage

1. Branches are collected in the winter when buds are still in a state of quiescence.
2. After harvest, the branches are covered with a polyethylene sheet and placed in a dark cool room (3–5°C) for less than 2 months.

Sample preparation

1. Axillary buds with about 5 mm (Pear) or 10 mm (Mulberry) of vascular tissue are removed from the branch.
2. The buds are dehydrated on paper at room temperature for 2 h such that the water content of the fresh bud is decreased (from ~60% [Pear] or ~50% [Mulberry] to ~40% MC.
3. About 20 buds are put into a polyethylene bag or cryotube and held at 0°C for 1 day.

Cooling and warming

Pear

1. The bags or cryotubes are cooled for 24 h at -5 °C, followed by 24 h at –10°C, 24 h at –15°C, 24 h at –20°C and 24 h at –30°C.
2. Following the 24 h at –30°C, the bags or cryovials are directly transferred into a deep freezer (–150°C) or in the vapor phase of liquid nitrogen.

3. For warming, the bag or cryotube is slowly warmed in 0°C water for at least 2 h.

Mulberry

1. The bags or cryotubes are cooled for 24 h at –5°C, followed by 24 h at –10°C, 24 h at –15°C and 24 h at –20°C.
2. Following 24 h at –20°C the bags or cryovials are directly transferred into a deep freezer (–135°C or –150°C) or immersed in LN.
3. For warming, the bag or cryotube is placed at 0°C to equilibrate.

Viability assessment (two options)

1. Sterilize buds with 70% ethanol for 1 min followed by 15 min in a sodium hypochlorite solution (10 % household bleach). Rinse with sterile water. Dissect shoot tips and culture on recovery medium.
2. Graft onto seedling rootstocks. Maintenance of good vascular tissue in the buds to be cryopreserved is crucial for high survival.
   a. Micrografting of cryopreserved pear buds is feasible provided the bud has good vascular tissue.
   b. For mulberry buds, graft buds with vascular tissue onto 1-year-old seedlings.

Recovery

Recover shoot on recovery medium at 25°C with a 16 h photoperiod under white fluorescent light (52 µmol m^{-2} s^{-1}).

Notes

1. There were no significant differences in survival among pear buds stored at –135°C, –150°C and –196°C. Higher survival was obtained by slow warming at 0°C than by rapid warming in water at 37°C.
2. Partial dehydration of dormant mulberry buds collected in late autumn or early spring also allowed their use for cryopreservation.

16.4.3 Controlled Rate Cooling of Silver Birch and Aspen Dormant Buds

By Leena Ryynänen, Soile Jokipii, and Hely Häggman based on Ryynänen 1996; 1999; Ryynänen et al. 2002; Jokipii et al. 2004

Dormant silver birch (*Betula pendula* Roth) and hybrid aspen (*Populus tremula* L. × *P. tremuloides* Michx.) *in vivo* buds are used. The buds can be obtained from field (Ryynänen 1996, 1999) or from greenhouse-grown plants that have been cold-stored at +2°C (Ryynänen et al., 2002, Jokipii et al., 2004). The protocol is also suitable for genetically modified material (Ryynänen et al. 2002, Jokipii et al. 2004).

Materials needed

1. Flow hood, Petri dishes, forceps, preparation knives, 70 % ethanol
2. Cryovials, 2 ml (e.g. Sarstedt, Nümbrecht, Germany)
3. Controlled rate freezer (e.g. Lauda Ultra-Kryomat Low Temperature Circulators, Königshofen, Germany)
4. 37°C water bath
5. Liquid nitrogen and storage Dewar
6. Culture media for silver birch:
   Regeneration: woody plant medium (WPM; Lloyd and McCown, 1980) containing 4.4 µM 6-benzyladenine (BA), 2.0% sucrose and 0.8% agar in 10-ml test tubes,
   Proliferation: WPM containing 4.4 µM BA, 2.0% sucrose and 1.0% agar in baby-food jars with Magenta caps
   Rooting: WPM without any growth regulators, containing 1.0% sucrose and 1.0% agar in baby-food jars with Magenta caps
7. Culture media for hybrid aspen:
   Regeneration: WPM containing 2.22 µM BA and 0.005 µM naphthalene acetic acid (NAA), 1.5% sucrose and 0.8% agar
   Proliferation: MS medium (Murashige and Skoog,1962) containing half strength of macro nutrients, all micronutrients, 2.22 µM BA and 2.85 µM indole-3-acetic acid (IAA), 1.5% sucrose and 1.0% agar
   Rooting: MS medium without any growth regulators, containing half strength of macro nutrients, all micronutrients and 1.5% sucrose
8. Indole-3-butyric acid (IBA; pH 5.5) 0.5 mM
9. peat-perlite (1:1)

Collection and storage

1. Collect materials in winter during deep dormancy.
2. Bud material from plants hardened in an unheated greenhouse (minimum 5°C) are transferred to a cold room (3°C) and kept in the dark to maintain dormancy until use.
3. Branches from dormant trees growing outdoors are sealed in plastic bags with their bases in snow or crushed ice and then stored in a cold room (–5°C) until use.

Sample preparation

1. Approximately 1 cm stem segments containing a bud are cut from the branches or plants and sealed in the cryovials.
2. Vials are placed in drawers/canes and kept at 0°C (up to 36 h).

Cooling and warming

1. Cool the material at 0.17°C min to –38°C. Hold the cryovials at –38°C for a few minutes or as long as 24 h.
2. Submerge samples in liquid nitrogen.
3. Warm in a 37°C water bath for 2–4 min, and then keep in ice for 0.5 min and subsequently at room temperature.

Viability assessment

1. Surface sterilize the samples by shaking in ethanol for 1.5 min. Do not wash with sterile water.
2. Peel the scales and most of the leaves from the bud under a stereomicroscope. Cut off the twig and all of the bark from the base of the bud. Take care that there remain no extra tissues on meristems that could prevent subsequent growth.
3. Transfer excised buds to culture tubes (1 bud/tube) containing recovery medium. Plug the tube, wrap with laboratory film and place in a 22°C growth room with a 16 h photoperiod (light intensity 85–120 $\mu E\ m^{-2}\ s^{-1}$.)
4. Decrease the light intensity by shading the tubes with gauze for 1 week.
5. Cultivate for 4 weeks: recovering buds either develop into shoots directly or via adventitious buds from nodular basal callus of the thawed bud.
6. Transfer into glass jars for multiplication and later for rooting.
7. Establish plants in the greenhouse.
8. Dip the rooted shoots in 0.5 mM IBA solution (pH 5.5) for a few seconds to enhance the root growth.

9. Pot the plantlets in peat-perlite (1:1, v/v) and cultivate first at high relative air humidity for 2 weeks, after which continue the cultivation in decreasing humidity for two more weeks. After acclimation, transfer the plants to greenhouse conditions.

Notes

1. Cultivation in test tubes is recommended because of the risk of contamination; however, the medium in the test tube easily becomes desiccated and cracked so shoots may require transfer to new medium.
2. The physiological/morphological status of a cryostored bud affects the recovery: collecting too late in the year or the presence of catkins within the vegetative buds (not possible to detect before bud burst) decreases recovery.
3. When buds in good condition are cryopreserved, there is no significant difference in the growth of unfrozen controls compared to cryopreserved buds of silver birch.
4. No genetic instability has been found when the regenerated silver birch plants are compared to the original donor trees using RAPD assays together with chromosome analysis.
5. Cox and Stushnoff (2001) cryopreserved dormant *Populus tremuloides* using a controlled rate cooling procedure with 20 cm twig sections. Bud break was used to quantify survival.

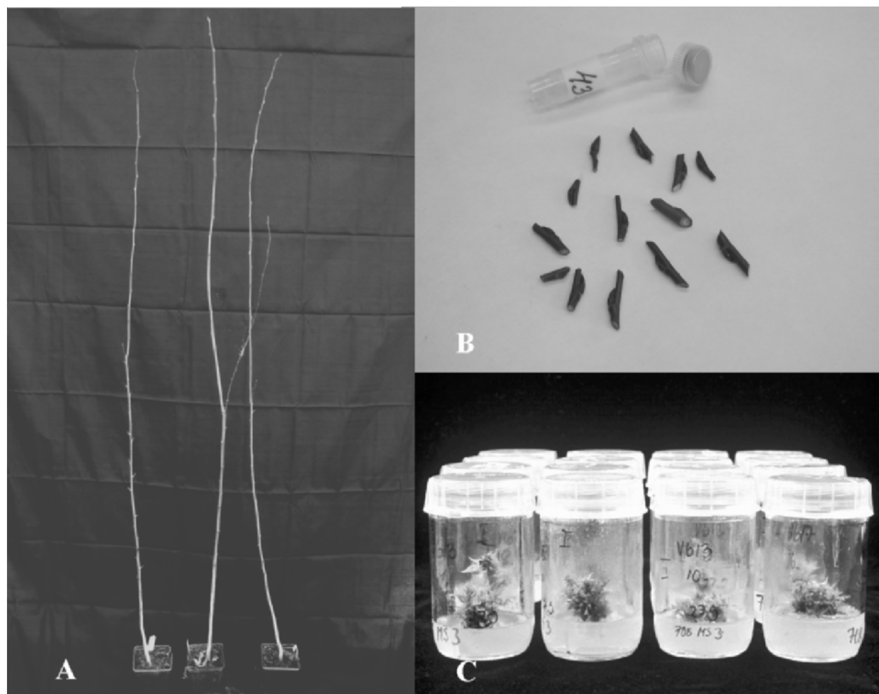

Fig. 16.1 Cryopreservation of dormant buds is an appropriate method for germplasm conservation of hybrid aspen (*Populus tremula* × *tremuloides*) A. Greenhouse-grown, cold stored saplings. B. Excised *in vivo* buds ready for cryopreservation. C. Vigorously multiplying *in vitro* shoots regenerated after cryostorage

16.4.4 Cryopreservation of Willow (Salix) *Dormant Buds*

By L Towill, G Volk, J Waddell, R Bonnart, M Widrlechner based on Towill and Widrlechner 2004

Materials needed

1. Polyolefin tubes (3M Corp.) (3M Company, 19mm-42mm diameter tubes)
2. Heat sealer
3. Liquid nitrogen Dewar
4. 2–4°C room for warming samples
5. Moist peat for regrowth
6. Controlled rate freezer (ThermoForma, CryoMed Freezer)
7. Rooting hormone
8. Sterilized medium (1:1:1:1 perlite: vermiculite: peat moss: sand)
9. Crisper containers

Collection and storage

Twigs (4–10 mm diameter) from *Salix* species were cut in January and February from field plantings, wrapped in plastic bags and stored at –3.5°C up to 5 months until processing for storage.

Sample preparation

Twigs were cut into sections approximately 4–6 cm in length (2–3 buds per section), placed within the polyolefin tubes, with 5–6 sections per tube and the tubes were heat sealed.

Cooling and warming

1. Tubes are placed in a controlled rate chest type freezer at –3.5°C and cooled at –5°C day^{-1} (–1°C h for 5 hours per day and held overnight at each intermediate temperature) to –35°C.
2. Tubes are held at –35°C for 24 h and then placed in liquid nitrogen vapor for at least 24 h.
3. Warm the tubes in air at 2–4°C for 24 h.

Regrowth

1. Buds that will be below the surface of the medium are removed with a razor blade and each section is notched slightly with a razor blade on each side of the basal end to expose the cambium layer. Scions are dipped into 20X Dip-N-Grow (500 ppm IBA, 250 ppm NAA) for 3–5 s before putting into a sterilized medium (1:1:1:1 perlite: vermiculite: peat moss: sand) that has been saturated with sterile water.
2. Scions are inserted 2–3 cm into the 3–4 cm deep medium contained within crisper containers (26 × 32 cm).
3. Crispers are placed on a heat mat within a 3°C cold room. Temperatures are stabilized to provide 13°C in the root zone and 4°C above the medium in the crisper. Low light (25 umol m^{-2} s^{-1}) is provided by a single cool-white fluorescent bulb (10 h day).
4. Lids are kept open 1–2 cm and cuttings are misted daily and medium is kept moist until rooting is complete after 6 weeks. Successfully rooted scions have roots 3–60 mm in length. Bud break is scored as any buds that have opened with visible leaves and shoots.
5. Volk (unpublished) has modified the method using direct rooting for recovery of cryopreserved shoots.

16.4.5 Vitrification of Persimmon (Diospyros) *Dormant Buds*

By T Matsumoto based on Matsumoto et al. 2001, 2004

Materials needed

1. Cryotubes
2. Sterile filter papers
3. Preculture medium: MS (Murashige and Skoog 1962) with 0.3 M sucrose and 0.7% agar in Petri dishes
4. Osmotic loading solution: MS with 2 M glycerol, 0.4 M sucrose
5. PVS2: 30% (w/v) glycerol, 15% (w/v) DMSO and 15% ethylene glycol, 0.4 M sucrose in liquid MS
6. Rinsing solution: MS with 1.2 M sucrose
7. Recovery medium: half strength MS medium with 3% sucrose and zeatin 1 mg l^{-1} and 0.7% agar in Petri dishes

Collection and storage: Twigs are collected in January after exposure to temperatures of 10–15°C.

Sample preparation

1. Excise shoot tips (about 1 × 1 mm) from surface sterilized dormant buds.
2. Preculture the shoot tips for 1 day with a 16 h photoperiod at 25°C.
3. Place 10 precultured shoot tips/cryotube, add osmotic loading solution and hold for 20 min at 25°C.

Cooling and warming

1. Remove loading solution and add PVS2 (25°C). Set timer for 20 min.
2. After 5 min, drain PVS2 and replace with fresh PVS2 (25°C).
3. After 19 min remove PVS2 and add 1 ml of fresh PVS2.
4. After 20 min plunge the cryotubes into liquid nitrogen.
5. For warming, place cryotubes in 40°C water for 1 min with stirring.

6. Drain the PVS2 solution immediately, add rinsing solution and hold for 20 min at 25°C.
7. Transfer shoot-tips onto sterile filter paper discs on top of recovery medium and culture on the recovery medium under a 16 h photoperiod (50 μ mol s^{-1} m^{-2}) at 25°C.
8. After one day, transfer the meristems onto a fresh paper disc over the same medium and culture under the same conditions.

Notes

1. During draining and adding solutions using Pasteur pipette, shoot tips in cryotubes are easily injured.
2. PVS2 solution is harmful for plant material. Be exact in the 20-min exposure prior to liquid nitrogen, and after warming remove the shoot tips from PVS2 immediately.
3. Matsumoto et al. (2004) and Ai and Luo (2005) cryopreserved nodal sections of winter twigs of persimmon using a controlled rate cooling procedure and recovered plants *in vitro*.

16.4.6 Cryopreservation of Dormant Garlic Bulbs

By D Ellis and G Volk, based on Volk et al. 2004; Ellis et al. 2006

Materials needed

1. Preconditioning medium: Gamborg (Gamborg et al. 1968) B5 basal medium with 3% sucrose, 0.5 mg l^{-1} 2-(γ,γ-dimethyl-allylamino) purine (2ip) and 0.1 mg/L α-naphthalenacetic acid (NAA) and solidified with 0.65% agar (Sigma A-7002) in Petri dishes
2. Osmotic loading solution: liquid MS medium (Murashige and Skoog 1962) with 2 M glycerol and 0.6 M sucrose (pH 5.7)
3. PVS2: 30% (w/v) glycerol, 15% (w/v) DMSO and 15% (w/v) ethylene glycol, and 0.4 M sucrose in liquid MS
4. PVS3: 50% glycerol (w/v) and 50% sucrose (w/v) in MS medium
5. Rinsing solution: MS with 1.2 M sucrose
6. Sterile aluminum foil strips (0.5 mm × 15 mm)
7. Recovery medium: Gamborg B-5 basal medium with 3% v/w sucrose, 0.5 mg l^{-1} 2ip, 0.1 mg l^{-1} NAA with 0.65% agar

Collection and storage

Bulbs are field harvested in Washington State in the United States in mid-August, dried for ~22 days at ambient temperatures (27°C days/20°C nights) under forced air and stored at 20°C for a minimum of 8 weeks. Bulbs are then shipped to the lab for cryopreservation.

Sample preparation

1. Bulbs are broken into individual cloves, the skins are removed and the basal end of the clove is pared down, using a razor blade, to 1cm cubes containing an intact shoot tip with the basal plate shaved free of debris but left intact (~0.5 mm thick).
2. The 1-cm cubes are sterilized in 70% ethanol with gentle agitation for 10 min, drained and placed in a 20% commercial bleach (6.0% sodium hypochlorite) solution with one drop of Tween 20 and gently agitated for an additional 20 min. Cubes are then rinsed three times with sterile distilled water for 15 min per rinse.
3. Shoot tips, 1–2 mm × 1–2 mm, consisting of a basal plate and a shoot tip with 1–2 leaf primordia are aseptically excised from the cubes with the aid of a dissecting scope and placed on preconditioning medium at 5°C for 24 h in the dark.

Cooling

PVS2

1. Place reconditioned shoot tips in 25°C osmotic loading solution and hold for 20 min.
2. The loading solution is removed, replaced with chilled (0°C) PVS2 and kept on ice for 15 min.
3. After exactly 15 min, 4–5 drops of PVS2, each containing a single shoot tip, are placed on sterile foil strips and plunged into a shallow vessel of LN.
4. Cryovials are placed below the surface of LN and the foil strips are inserted into the 1.2 ml cryovials.
5. The vials are lifted from the LN, the lids closed, and then replaced in the LN. The LN in the tube will evaporate during this process.

PVS3

1. Place preconditioned shoot tips in 25°C osmotic loading solution and hold for 20 min.
2. The loading solution is removed and replaced with room temperature PVS3 for 120 min.
3. Shoot tips are moved to 1.2 ml cryovials containing 0.5 ml of PVS3 and plunged into LN.

Warming

PVS2

1. Cryovials are opened in LN. Foil strips are removed and submerged into 25°C rinsing solution for 20 min with the solution refreshed once after 10 min.
2. Foil strips are removed from the solution once the shoot tips became detached (within the first minute).

PVS3

1. PVS3-treated shoot tips are rapidly warmed by plunging the cryovials into a 40°C water bath for 2 min.
2. Vials are dried and cleaned with 70 % ethanol.
3. The PVS3 is quickly removed from the vial, 1 ml of rinsing solution is added and held for 10 min at 25°C.
4. After 10 min, the solution is refreshed and the shoot tips are poured into a Petri dish of rinsing solution.

Regrowth

1. After 20 min in rinsing solution, shoot tips from both PVS2 and PVS3 are transferred to Petri dishes of recovery medium, sealed with Parafilm, and placed in the dark at 25°C.
2. After 24 h, shoot tips are moved to fresh B5 medium, resealed and kept in the dark for an additional 5 days, and then transferred to low light (25°C/16 h photoperiod) (6 µmol s^{-1} m^{-2}) for another 5 d prior to transfer to a well lit growth chamber (25°C/16 h photoperiod) (42 µmol s^{-1} m^{-2}).
3. Shoot tips remain on this medium until they develop well formed shoots at which time they are moved to Magenta GA-7 culture vessels containing 75 ml of solid B5 medium.
4. Only shoot tips with a visible and organized center of growth, subtended by leaves, are scored as viable (regrowth). Callus and greening is evident on some explants, however this is not considered as viable regrowth.

Notes

1. Preparation of the 1 cm cube containing the shoot tip from the clove was done in a separate room from the media prep or transfer room to avoid potential contamination from the bulbs.
2. Some genotypes respond better to PVS2, while others respond better to PVS3. We routinely run shoots from each genotype through both PVS2 and PVS3 to ensure cryopreservation success from every genotype because we found no correlation of PVS type to particular types of garlic.
3. Survival of recovered cryopreserved shoot tips in the greenhouse is excellent.
4. The resulting plants will have multiple shoots and form many bulbs.

Chapter 17
Cryopreservation of Pollen

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17.1 Introduction

Cryopreservation of pollen is required for carrying out investigations in both fundamental and applied aspects of pollen biology. Besides the already existing role of pollen cryobanks in breeding, there are many promising applications which have come to focus with the recent advances in allied bio-scientific areas. Crossing desirable genotypes involves multiple and staggered plantings in order to synchronize flowering. This can be avoided when cryopreserved viable pollen is available, facilitating hybrids between genera, species and genotypes. This could effectively conserve field and greenhouse space. The international transfer of germplasm in the form of dry pollen is not generally restricted (Hoekstra 1995). Moreover, this will eliminate the need to grow plant populations to produce pollen. Pollen is usually subjected to less stringent quarantine restrictions. The importance of pollen cryopreservation in horticultural species is well documented (Ganeshan and Rajasekharan 2000a; Kobayashi et al. 1978).

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

Manipulations of the physiological status of a given plant species, cryopreservation technique and recovery steps provide information that suggests some generalities in approaching the overall problem of cryopreservation of pollen. In most species the genotype effect, stage of pollen beyond complete maturity, physiological status of plant, cold treatment and the methodology followed are the major factors influencing post cryopreservation survival. In most tropical species, dry-mature pollen grains freshly dehisced from anthers are in ideal physiological condition to be processed for cryopreservation.

17.3 Protocols for Cryopreservation

The objective of a useful pollen cryopreservation protocol is to collect dry mature pollen from the desired species and conserve using methods which allow retention of its normal function; ultimately assessed by its ability to germinate *in vivo* and effect normal fertilization (Hanna and Towill 1995). Alexander and Ganeshan (1993) and Ganeshan and Rajasekharan (1995) reviewed the work on pollen storage in fruit crops and ornamental crops. Hoekstra (1995) assessed the merits and demerits of pollen as a genetic resource. Grout and Roberts (1995) detailed the methodology for pollen cryopreservation. Barnabas and Kovacs (1997) and Berthoud (1997) stressed the importance and need for pollen conservation. Ganeshan and Rajasekharan (2000b) reviewed the current status of pollen cryopreservation research and its relevance to tropical horticulture. Rajasekharan et al. (2003) developed a user-friendly database software ‘Polbase’ for digitizing accessions collected and maintained in a pollen cryobank. Generally, dry pollen, as collected, requires no pretreatment. Specific needs for candidate species will be discussed in this chapter.

17.3.1 Rewarming

The usual rewarming procedure, after a desired period of cryopreservation, is to place the sample at room temperature for 30 to 60 min, without the use of a water bath or any rewarming systems.

17.3.2 Viability Assessment

Pre-and post-cryopreservation viability assessment is usually followed for pollen through *in vitro* and *in vivo* assays. The common procedures include a staining method to differentiate aborted and non-aborted pollen (Alexander 1980), the hanging drop technique (Stanley and Linskens 1974), modified cellophane technique (Alexander and Ganeshan 1989), PEG technique (Shashikumar 2006), and in vivo staining pollen tubes in pistil (Alexander 1987), in addition to assessment of fertilizing ability in the field to determine the ability to produce normal fruit and seed set.

17.3.3 Theoretical and Practical Considerations

1. Techniques of cryogenic storage are new for many tropical fruit tree species. The success rate is very high for bi-cellular pollen material from tropical regions.
2. Training in cryogenic operations, handling procedures, safety precautions, etc. are essential. Proper handling equipment, such as cryogloves, long forceps or tongs, and face guards, is essential.
3. It is generally advised not to use glass vials for cryogenic storage, as they can explode due to seepage of liquid nitrogen (LN) into the vial during storage.
4. Commercially available storage containers with a long static LN holding time and with low static evaporation rates are desirable. The canisters designed to hold vials, pouches, etc. in liquid and vapor phase are to be used. Dewars should be topped after opening. The LN level in storage cryocans will have to be monitored and filled regularly depending on the type of Dewar used.
5. Ensure transfers or retrievals in and out of the canisters as quickly as possible, so that samples held in storage at –196°C are not allowed to warm up.

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17.4 Protocols

17.4.1 Cryopreservation of Mango Pollen (Mangifera indica)

(Rajasekharan and Ganeshan 1992, 2001–2002; Shashikumar 2006)

Pollen Collection

Anthers are collected for pollen collection and processing for storage. (There is only one anther which produces viable pollen; dehisced anthers are ash colored). Mature anthers are collected in clean Petri dishes from healthy panicles from mature trees between 10 and 11 AM. The anthers are brought into the laboratory and transferred to empty gelatin capsules.

Procedure

1. Gelatin capsules containing anthers are enclosed in laminated poly-aluminum pouches and sealed airtight.
2. Pouches are stacked in canisters of a cryobiological system (Mach SM 43 MVE, USA) and lowered gradually into LN and stored for desired durations.
3. Canisters are capped with perforated lids to prevent the vials from floating out into the Dewar during refilling of LN.

Retrieval and post-storage fertility assessment

Anthers are warmed to ambient temperature, by initially holding the canister over LN vapor phase for 15 min, then pouches with anthers are removed from the canister and held at room temperature. Field pollinations are carried out or pollen germinated.

Mango pollen germination medium composition

1. Boric acid 100 ppm
2. Sucrose 15% w/v
3. Polyethylene glycol 10% w/v

17.4.2 Cryopreservation of Papaya Pollen (Carica papaya *and* Carica cauliflora)

(Ganeshan 1986a)

Pollen collection
Pollen of *Carica papaya* L. 'Washington' and *Carica cauliflora* L are to be collected in Petri dishes from plants bearing male flowers between 9 and 10 AM. Species-wise bulked pollen samples are to be transferred to empty gelatin capsules or butter paper pouches, individually packed and in turn transferred to laminated aluminum pouches.

Viability assessment
Viability of fresh and cryopreserved pollen is assessed in terms of germinability *in vitro*. Pollen is germinated in hanging drops of medium (6% sucrose in distilled water) on depression slides at 25±2°C (Shivanna and Rangaswamy 1993). This process involves suspension of pollen grains in germination medium.

Cryopreservation
Pollen in gelatin capsules or butter paper pouches are sealed in poly aluminum pouches, loaded to canisters and cryopreserved.

Retrieval and post-storage fertility assessment
Anthers are warmed to ambient temperature by initially holding the canister over LN vapor phase for 15 min, then pouches with anthers are removed from the canister. Field pollinations are carried out or pollen is germinated on slides.

17.4.3 Cryopreservation Citrus Pollen (Citrus limon)

(Ganeshan and Alexander 1991; Ganeshan and Sulladmath 1983; Rajasekharan et al. 1995)

Pollen collection
The species handled for pollen studies include *Citrus limon, Citrus aurantifolia, Citrus sinensis* and *Poncirus trifoliata.*

Inventory
Petri dishes, butter paper, forceps, muslin cloth fixed to a 10 cm. cylindrical ring with the help of a firm rubber band, clean razor blade.

Procedure
1. Pollen collections are usually made on a bright sunny day between 8 and10 AM.
2. Staminate flowers are harvested at peak anthesis and the dehiscing anthers are gently caressed over the muslin cloth fixed to the cylindrical ring, which acts as a sieve.
3. Dehiscing anthers are carefully removed from pistillate flowers and pollen is extracted over the muslin cloth sieve.
4. Pure pollen is extracted in clean Petri plates or butter paper.

Precautions: The following points should be noted:
1. Quality of pollen collected depends on the correct stage of anthesis/ anther dehiscence.
2. Pollen should be free of anther debris.
3. Do not collect pollen from infected or insect damaged flowers.
4. Decide upon bagging of flowers depending on insect activity.
5. Do not collect pollen on a rainy day or if it had rained overnight.
6. Do not force out pollen from anthers.

Viability assessment
Pollen collections are subjected to viability indexing by germination *in vitro* by the hanging drop technique (Shivanna and Rangaswamy 1993). This process involves suspension of pollen grains in germination medium.

Germination medium

Prepared in deionised, double distilled water.
20% Sucrose
100 ppm H_3BO_3
300 ppm $Ca(NO_3)_2$ 4 H_2O
200 ppm Mg SO_4 $7H_2O$
100 ppm KNO_3
pH 7.3

Cryopreservation

1. Pollen samples are packed either in gelatin capsules or butter paper packets, sealed air tight in polyethylene aluminum laminated pouches and lowered into a canister of a cryoflask.
2. The canister is capped with a perforated lid and plunged into LN contained in the cryoflask.

Retrieval and post storage viability/fertility assessment

Pollen samples are held at ambient temperature for 10–15 min prior to a viability test or field pollination.

Pollen samples are viable if:

1. Germination *in vitro* is as good as fresh pollen.
2. Germination time taken is not more than that of fresh pollen (pollen vigor).
3. Estimated germination is in the range of 60–80% of fresh pollen.

17.4.4 *Cryopreservation of Grape Pollen* (**Vitis vinifera,** *and* **Vitis labrusca**)

(Ganeshan 1985)

Pollen collection
The method of Olmo 1942.

1. Tap the 2nd or 3rd dehiscing inflorescences onto clean Petri dishes.
2. Blow out the floral debris and retain the yellow pollen mat.
3. Consolidate the collected pollen using a clean razor blade.
4. Pack the pollen into gelatin capsules or butter paper pouches.

Viability assessment
Pollen viability is tested by its ability to germinate in an artificial medium. Fresh pollen samples are cultured *in vitro* following hanging drop technique (Shivanna and Rangaswamy 1993). This process involves suspension of pollen grains in germination medium. The germination medium consists of 20% sucrose solution in which pollen germinates profusely. Hanging drop cultures are incubated for 5 h at 25±2°C after which pollen growth is arrested by staining with a drop of Alexander's stain (1980). Pollen whose tube lengths are measuring more than the grain diameter are considered viable.

Cryopreservation
Storage at –196°C is accomplished by direct immersion in LN after precooling to –20°C.

Retrieval and post-storage fertility assessment
After warming pollen samples the vials are kept at ambient temperature for 30 min before taking to field. Fertility of pollen cryopreserved in LN is tested by controlled field pollinations on established male-sterile lines.

17.4.5 *Cryopreservation of* Gladiolus *Pollen*

(Rajasekharan et al. 1994)

Pollen collection and processing for cryostorage

1. The flowers are tied with thread at the bud stage in order to prevent contamination by stray pollen and to obtain pure samples.
2. On the day of collection, the flowers are harvested and brought to the laboratory. Petals are carefully separated and pollen grains are extracted by scraping the mature anthers which are about to dehisce, with a blunt needle, passing traversely along the lobe of the anther.
3. Bulked pollen samples are transferred to empty gelatin capsules, packed in laminated poly aluminum pouches, sealed airtight.
4. Lower gradually into canisters of a LN cryobiological system.

Precaution: Care must be taken not to scrape off the tapetal tissue, which could contaminate the pure pollen.

Viability assessment

Germinate pollen on cellophane strips soaked in pollen germination medium (Rajasekharan et al. 1994). Field pollinations may be used to confirm viability.

Pollen germination medium composition

15% Sucrose
300 mgl^{-1} Ca $(NO3)_2$ 4 H_2O
200 mg 1^{-1} Mg SO_4 $7H_2O$
100 mg 1^{-1} KNO_3
100 mg 1^{-1} Boric acid

17.4.6 *Cryopreservation of Rose Pollen*

(Rajasekharan and Ganeshan 1994)

Pollen collection and processing for storage

1. Pollen of previously bagged flowers (which are due to open the following day) is collected on a bright sunny day.
2. On the day of collection bags are removed between 10 and 11 AM and the blooming flowers are brought into the laboratory.
3. After separating the petals, pollen grains are collected by gently tapping the flower hips onto butter paper.
4. Collections from flowers of a given cultivar are bulked before assessing viability *in vitro*.

Viability assessment

Assess viability by germination of pollen in a hanging drop culture. Field pollinations can also be used to assess viability.

Germination medium

15% Sucrose
150 g l^{-1} $Ca(NO_3)_2$ $4H_2O$
200 mgl^{-1} $MgS0_4$ $7H_2O$
100 mg 1^{-1} KNO_3
100 mg l^{-1} H_3Bo_3

Cryopreservation

1. The pollen samples carefully transferred into gelatin capsules enclosed in a laminated aluminum pouch.
2. The pouches with the capsule are sealed air tight and stacked in the canisters of a cryobiological storage system, and lowered gradually into LN.

Retrieval and post-storage fertility assessment

Requirement: pollination kit—Petri dishes, butter paper, forceps, polyethylene covers (for bagging if necessary) needles, clean razor blade, painting brush, etc.

17.4.7 Cryopreservation of Asclepiadaceae Pollinia—General procedures

(Shashikumar 2006)

Pollen in asclepiadaceous members are arranged in compact sac like structure called a pollinium. A fine needle is used to extract each pollinium from freshly opened flowers. Pollen is binucleate, number and size of pollen in a pollinium will vary with species. Fresh pollinia are processed for cryopreservation. From 20 to 30 pollinia are placed in a gelatin capsule, which is then sealed in poly aluminum pouches.

Procedure for extraction and processing asclepiadaceous pollinium for cryostorage

1. Collect coronary corolla of asclepiadaceous members at 10–11 AM.
2. Dissect the coronary corolla using fine needles. Pullout corposculum of each pollinium using fine-pointed forceps.
3. Collect the pollinia on a clean butter paper.
4. Immediately transfer pollinia to gelatin capsules.
5. Place the gelatin capsules inside aluminum pouches and seal.
6. Label the pouches with inventory information.
7. Place the pouches inside the canisters and close the lid.
8. Immerse the canisters slowly into the LN containers.

Retrieval of pollinia from cryogenic containers

1. Gradually lift canisters from the cryogenic containers.
2. Retrieve the aluminum pouches and allow the pouch to warm to room temperature.

Viability assessment

1. Viability in pollinia is assessed using the hanging drop method (Shivanna and Rangaswamy 1993) with 4–5 pollinia in a drop of germination medium. Boric acid 100 ppm, sucrose 20%, pH 7.0
2. Incubate the slides in a humidity chamber (RH-90%) for 4–5 h.

Qualitative and quantitative estimation of germinated pollinia extracted from asclepiadaceous members

The pollen in Asclepiadaceae is in a compact mass called pollinia, which pose difficulties in qualitative and quantitative estimation after germination of pollen grains. In order to overcome this difficulty, follow the technique described below:

1. Transfer germinated pollinia into a 5 ml test tube containing 1–2 ml of pollen germination medium.
2. Agitate test tube using a single tube mixer (‘Rotex’ or ‘Vortex’) for about 5–10 min. (This process loosens the compact mass of germinated pollen, further rupturing the pollinial wall).
3. Transfer the mixture onto a clean slide over a drop of Alexander’s stain.
4. Lower clean cover slip on the germinated pollen mixed in a drop of stain, gently tapping on the cover slip. (This results in individual separation of germinated pollen grains, for photomicrography as well as quantitative estimation.)

Possible problems

1. Dissection of pollinia after extraction may result in loss of viability.
2. Culturing more than 2–3 pollinia may over crowd and pose problems for counting.

17.4.8 Cryopreservation of Tropical Orchids

(Anonymous 2006)

Physiological status

Orchids in general possess pollen tetrads collected into highly organized waxy pollinia with appendages. The pollen are tightly packed in the pollen sac (pollinia) generally surrounded by a viscous fluid. In species like *Vanilla,* pollen grains are unicellular and held together by a viscous fluid. Sometimes the pollinial tetrads are organized into many granular packets, prolongations of which form the caudicle as in *Pecteilis, Habenaria, Satyrium*. Especially in those cases the pollen are more tightly packed without much surrounding fluid. Even though information on the nature of pollen is scanty, it is known that both binucleate and trinucleate are present among orchids.

Pretreatment

Protocols for pollinia cryopreservation of orchids with pollen with surrounding viscous fluid packed in pollinia are described below. A short treatment in vitrification solution formulated by Sakai et al. (1990) is preferred for cryopreservation. The treatment includes exposure to an osmotic loading solution containing 2 M glycerol and 0.4 M sucrose prepared in BK medium (Brewbaker and Kwak 1963) for 15 min and PVS2 solution containing 30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose in BK medium for 5–20 min. Simple desiccation under laminar airflow is also effective in some species.

Cryopreservation

Successful cryopreservation of pollinia typically uses direct immersion of a vial (1–2 ml) containing the PVS2-treated pollinia into LN. For

experimental purposes, overnight exposure in LN is more than sufficient to assess successful vitrification and LN tolerance of treated pollinia. Dried pollinia are also cryopreserved through vitrification.

Rewarming

To avoid de-vitrification the LN-treated pollinia need to be rewarmed rapidly. The vials are removed from LN and dropped into a 40°C water bath. The rewarmed pollinia are washed in a solution of 1.2 M sucrose prepared in BK medium. Pollinia cryopreserved through simple drying does not require any further washing or rehydration, but can be directly used for germination assay or pollination.

Viability assessment

Immediate survival is determined by placing a small sample on a cavity slide, adding one to two drops of diluted fluorescein diacetate solution and observing under a fluorescent microscope after a few minutes. Germination is determined by plating samples of rewarmed and washed pollinia in two–three drops of BK medium containing 0.5–5% sucrose onto cavity slides and incubating at 24±2°C for 12–24 h.

Protocols for specific species

Cymbidium bicolor pollinia simple drying

C. bicolor is an epiphytic orchid with two pollinia that has pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen are binucleate and easily germinate in BK medium (Brewbaker and Kwak 1963) with 0.5–5% sucrose.

Prepare in advance

1. Sterilized BK medium containing 0.5% sucrose.
2. 0.02% methyl blue in lacto-phenol: lactic acid 20 ml, phenol 20 ml, glycerol 40 ml, distilled water 20 ml, 1% cotton blue 2 ml
3. Harvest freshly opened flowers
4. Assess germination capability in BK medium with 0.5% sucrose

The procedure

1. Isolate pollinia from flowers using a fine tip forceps.
2. Put the pollinia in a sterile Petri dish and place it open in a laminar airflow cabinet for 30–75 min.
3. Collect the dried pollinia in 1 ml cryovial, place on cane and submerge in LN.
4. Warm in a water bath at 40°C for 2 min.
5. For an *in vitro* germination assay, place dried and LN treated pollinia in cavity slides, add two–three drops of BK medium containing 0.5% sucrose and place in a humid chamber for 12 h. Observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and germination percentage assessed.

A 75 min desiccation under laminar airflow is required to achieve about 76% *in vitro* germination after cryopreservation (desiccation control was 87%). However, 30–60 min desiccation may also be tried to determine optimum period, considering genotype, seasonal and regional differences.

***Arundina bambusifolia* pollinia silica gel drying**

Arundina bambusifolia is a terrestrial orchid with two pollinia with unicellular pollen compactly packed and surrounded by a viscous fluid. It easily disperses in liquid media. It is more convenient to use a drying protocol rather than vitrification. The pollen easily germinates and pollen tubes grow in BK (Brewbaker and Kwak 1963) medium containing 0.5–5% sucrose.

Prepare in advance

1. Germination: sterilized BK medium containing 0.5% sucrose
2. Methyl blue 0.02% in lacto-phenol: lactic acid 20 ml, phenol 20 ml, glycerol 40 ml, distilled water 20 ml, 1% cotton blue 2 ml
3. Harvest freshly opened flowers

The procedure

1. Isolate pollinia from flowers using a fine tip forceps.
2. Put the pollinia in aluminum foil boats and place in a glass bottle packed with charged silica gel and close it air tight. Keep the bottle at 24±2°C for 60-120 min.
3. Collect the dried pollinia in 1 ml cryovial. Place on cane and submerge in LN.
4. Warm in a 40°C water bath for 2 min.
5. For *in vitro* germination assay, place dried and LN treated pollinia in cavity slides, add two–three drops of BK medium containing 0.5% sucrose and place in a humid chamber for 12 h and observe under microscope for pollen germination and tube growth. If germination is confirmed, pollinia may be stained in a drop of lacto-phenol blue, tapped for dispersing pollen and scored for assessing germination percentage.

A 60 min desiccation with charged silica gel is required to get sufficient dehydration to achieve about 80% *in vitro* germination after cryopreservation (desiccation control is 79%). However, a 60–120 min desiccation may also be tried to determine the optimum period, considering genotypic, seasonal and regional differences.

Dendrobium ovatum pollinia vitrification

Dendrobium ovatum is an epiphytic orchid with two pollinia that have pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen tetrads will easily disperse through gentle tapping. The pollen easily germinates and pollen tubes grow in BK (Brewbaker and Kwak 1963) medium with 0.5–5% sucrose.

Prepare in advance

1. Germination: sterilized BK medium containing 0.5% sucrose
2. Methyl blue (0.02% in lacto-phenol): lactic acid 20 ml, phenol 20 ml, glycerol 40 ml, distilled water 20 ml, 1% cotton blue 2 ml
3. Osmotic loading solution: BK medium containing 0.4 M sucrose and 2 M glycerol
4. PVS2: BK medium with (w/v) glycerol 30%, ethylene glycol 15%, DMSO 15% and 0.4 M sucrose
5. Rinsing solution: BK medium containing 1.2 M sucrose
6. Harvest freshly opened flowers

The procedure

1. Isolate pollinia from freshly opened flowers.
2. Put the pollinia in 1 ml loading solution in a 2 ml cryovial and kept at room temperature (24±2°C) for 15 min. Do the experiment in two vials, one for control and another for LN exposure.
3. Transfer the pollinia into 1 ml-chilled PVS2 in a 2 ml cryovial and keep for 5–10 min.
4. Place the tube for LN treatment on a cane and submerge in LN.
5. To warm, plunge into a 40°C water bath for 2 min.
6. Rinse the control samples in rinsing solution immediately after PVS2 treatment and rinse the LN-treated samples after rewarming. Remove 1 ml from the vial and add 1 ml of rinsing solution and hold for 2 min. Repeat five times without waiting.
7. Transfer pollinia to a piece of sterile filter paper to drain.
8. For *in vitro* germination: place pollinia in cavity slides and add two–three drops of germination medium. Place in a humid chamber for 12 h. Pollinia may be stained in a drop of lacto-phenol blue and tapped for dispersing pollen.
9. A 5 min PVS2 treatment results in 78% *in vitro* germination that is similar to the control (81%). Up to 10 min, PVS2 exposure may give satisfactory results, considering genotype, seasonal and regional differences.

Luisia macrantha pollinia vitrification

Luisia macrantha is an epiphytic orchid with two pollinia with pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen easily germinates and pollen tubes grow in BK (Brewbaker and Kwak 1963) medium with 0.5–5% sucrose.

Use the same protocol as for *Dendrobium ovatum*

A 10 min PVS2 treatment is required to achieve 62% *in vitro* germination comparable to the PVS2 control (67%). However, 5–15 min exposure may give satisfactory results and thus may be tried to determine optimum period, considering the genotype, seasonal and regional differences.

Rhyncostylis retusa pollinia vitrification

Rhyncostylis retusa is an epiphytic orchid with two pollinia with pollen tetrads compactly packed and surrounded by a viscous fluid. The pollen easily germinates and pollen tube grows in BK medium with 0.5–5% sucrose.

Use the same protocol as for *Dendrobium ovatum*

A 20 min PVS2 treatment is required to achieve 82% *in vitro* germination after cryopreservation almost equal to the corresponding PVS2 controls (84%). However, 10–20 min exposure may give satisfactory results considering genotype, seasonal and regional differences.

17.4.9 Cryopreservation of Solanaceous Species

(Rajasekharan et al. 1998; Rajasekharan and Ganeshan 2003)

The procedures followed for solanaceous species, especially tomato, eggplant and bell pepper, are similar due the flower-bearing habit.

Pollen collection and processing for cryopreservation

1. Pollen extraction: carefully clip anthers from healthy flowers at the time of dehiscence.
2. Place flowers in clean Petri dishes in a desiccator containing activated silica gel under ambient conditions.
3. The anthers dehisce after 30–45 min, releasing pollen.
4. Tap gently over a clean butter/waxed paper.
5. Transfer collected pollen to gelatin capsules.
6. Enclose gelatin capsules in small laminated pouches and seal.
7. Lower canisters into a LN cryoflask.

Alternative method

1. The just opened flowers are brought to the laboratory.
2. Keep flowers in an incubator with light at 25°C for 1 h.
3. Remove the style and cut the anther cone at the end using a small scissors. Hold the flower upside down and tap out the pollen.
4. Bulk the pollen sample and transfer to a gelatin capsule and seal in a laminated aluminum pouch.

Viability assessment

1. Tomato pollen is germinated by the hanging drop technique (Shivanna and Rangaswamy 1993) in BK medium with 20% sucrose.
2. Eggplant pollen is germinated *in vitro* by the improved cellophane method (Alexander and Ganeshan 1989). *(Eggplant pollen fails to germinate in hanging drops, since pollen sinks to the bottom of the drop).*

   Germination medium
   15% Sucrose
   300 g l^{-1} $Ca(NO_3)_2$ $4H_2O$
   200 mgl^{-1} $MgSO_4$ $7H_2O$
   100 mg l^{-1} KNO_3
   100 mg l^{-1} H_3BO_3
3. Incubate at 25±2°C for 4–6 h and stain (Alexander 1980).
4. Field pollination may be used to determine viability.

17.4.10 Cryopreservation of **Allium cepa** *Pollen*

(Ganeshan 1986b)

Pollen collection and processing for cryopreservation

1. Plants of *Allium cepa* are grown in insect-proof cages under field conditions. Pollen is collected from umbels in partial to full bloom.
2. Umbels are gently tapped on clean Petri dishes between 11AM and 1 PM. Pure pollen samples collected from several umbels are thoroughly mixed before filling gelatin capsules (25 mg in each capsule).
3. Capsules are individually packed in laminated aluminum pouches and sealed.
4. Cryopreservation of pollen samples was accomplished by direct immersion in LN after precooling the culture tubes at –20°C for 2 h.

Viability assessment

1. Viability of pollen was assessed in terms of germinability *in vitro* and fertility by controlled pollinations.
2. Fresh, non-frozen (control) and frozen pollen samples are germinated in 20% sucrose medium (prepared in double glass-distilled water) using a hanging drop technique (Shivanna and Rangaswamy 1993).
3. Capsules with pollen are removed from LN and brought to ambient temperature and samples drawn for viability tests *in vitro.*
4. Pollen is held for 30 min at ambient temperature before it is dusted on the medium and incubated in a humid chamber at 25°C ± 2°C for 6 h. Cultures are stained with a drop of Alexander's stain (Alexander 1980).

Fertility assessment

Controlled pollinations with cryopreserved pollen may be carried out on a male sterile line in 50–60% relative humidity conditions or under greenhouse conditions. Warm frozen samples at ambient temperature and hold for 30 min before pollinating.

Chapter 18
Cryopreservation of Recalcitrant (i.e. Desiccation-Sensitive) Seeds

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18.1 Introduction

Unlike orthodox seeds, mature seeds of some species do not survive desiccation and are often referred to as "recalcitrant" (Hong et al. 1998). Approximately 10–20% of angiosperm species produce seeds that acquire some, but not full, tolerance of desiccation during maturation (Dickie and Pritchard 2002). Incidence of recalcitrance does not distribute along phylogenetic clades, though some plant families include many species producing recalcitrant seeds (e.g., Fagaceae, Lauraceae, Sapindaceae, Meliaceae) while other families apparently lack species exhibiting this trait (e.g., Solanaceae, Asteraceae, Amaranthaceae). Life history traits of the plant, such as a long lived, perennial nature, and its habitat, such as aquatic or rainforest, are associated with seed recalcitrance, but not all plants with these characteristics produce recalcitrant seeds. The term "recalcitrant" is also used to describe seeds that are particularly difficult to germinate because they have deep dormancy or an unknown dormancy release mechanism. Though frustrating to work with, seeds with this dormancy physiology are amenable to

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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cryopreservation using straightforward procedures described for "orthodox" seeds and will not be addressed here.

Tolerance to desiccation is acquired during embryogenesis (Berjak et al. 1989; Vertucci and Farrant 1995; Pammenter and Berjak 1999, 2000). After fertilization, the water content of the seed is very high and it decreases as the seed accumulates dry matter. The duration that seeds accumulate dry matter varies among species, and this phase ends when the vascular connection between the parent plant and seed is severed at physiological maturity. Just before physiological maturity, both orthodox and recalcitrant seeds survive stress to water potentials of about –5 megapascals (MPa). (note: most vegetative tissues are tolerant to only –1.5 MPa, which is known as the "permanent wilting point."). After physiological maturity, the water content of the seed continues to decrease until it comes to equilibrium with the relative humidity of the air. Orthodox seeds survive at ambient relative humidity, usually between 10–75%, which converts to water potentials of between –350 and –40 MPa at 25°C. In contrast, the level of tolerance in recalcitrant seeds is brief and usually limited to about –12 MPa. All germinating seeds become progressively less tolerant of desiccation (Buitink et al. 2006). The functional definition of recalcitrance, as limited tolerance of desiccation, implies that all embryos are recalcitrant during histodifferentiation and early reserve deposition and again during the germination phase of their development.

Damage to cells at water potentials greater than –5 MPa (i.e., relatively mild stress) is attributed to mechanical problems resulting from cell shrinkage during dehydration (Walters et al. 2002a). Replacing vacuolar volume with food reserves during the dry matter accumulation phase of embryo development reduces the amount that cells shrink, and so increases the minimum water content at which the cells can survive (also known as the critical water content). Stresses at water potentials less than –5 MPa (i.e., relatively high stress) are attributed to appressing macromolecular surfaces as they squeeze together during intense dehydration or to free radical attack as metabolism becomes unbalanced (Walters et al. 2002a). Protection from these stresses is believed to arise from the production of molecular antioxidants and metabolically inert, highly water soluble carbohydrates and proteins. Production of these putative protectants is induced in both recalcitrant and orthodox seeds during late embryogenesis, but, hypothetically, to a greater extent in orthodox seeds.

The distinction between recalcitrant and orthodox seeds provides a useful dichotomy upon which to base cryopreservation procedures for dry and hydrated materials (Engelmann 1997). Dried germplasm does not contain water that readily freezes and irreversibly damages cells. Hence, dry orthodox seeds can be stored at any subzero temperature, and the decision to store

under freezer or cryogenic conditions is based on the required longevity of germplasm, the quantitative effects of temperature on aging kinetics, and the relative costs of various storage regimens in delivering the desired shelf life. In contrast, recalcitrant seeds must be stored under conditions that preclude lethal ice formation. This truism is common for all hydrated germplasm, so basic principles of cryoprotection and cryoexposure, described throughout this manual, apply to recalcitrant seeds and vice versa. Essentially, cryopreservation of cells involves applying stresses faster than the resulting potential damage can occur. The stresses are desiccation and ice formation, and damage results from the myriad of mechanical, structural and biochemical changes that disrupt cell structure in response to changes in water properties. The strategies used during cryopreservation steps to maintain viability and genetic integrity of stored germplasm depend on the physical, physiological and genetic attributes of different tissue types (Table 18.1).

Table 18.1 Considerations of various germplasm forms that influence strategies used for cryopreservation

	Dry embryo	Recalcitrant embryo	Vegetative tissues
Water potential (ψ_w)	$\psi_w < -100$ MPa; extremely low probability of ice	$-1 > \psi_w > -20$ MPa; ice formation likely between −10°C and −50°C	$\psi_w > -1$ MPa; ice formation at < −5°C
Cytoplasm density	Extremely dense; dry matter occupies ≥90% of cell-volume; no vacuoles	Moderately dense; dry matter occupies between 30% and 75% of cell volume; some vacuolation	Dilute; dry matter occupies <20% of cell volume; often highly vacuolated
Tissue size	Less than 0.001 mg to more than 1g and constitutes between 0.5 and 5% of seed dry mass	0.1–15 mg and constitutes between 0.5% and 5% of seed dry mass	0.1–0.5 mg dry mass for shoot tips. Cold-hardy dormant buds from woody tissues are comparable to seeds.
Stress tolerance	Extreme toleranceto desiccation (< 0.01 g H_2O/g fw; $\psi_w < -350$ MPa) and cold	Moderate and short term tolerance of desiccation (0.18 to 0.3 g H_2O/ g fw; $-10 > \psi_w > -25$ MPa); variable tolerance to chilling (0 and 15°C)	Low to moderate tolerance of desiccation (> 0.8 g H_2O/ g fw; $\psi_w > -1.5$ MPa); variable tolerance of chilling and cold (15–40°C)
Recovery medium	In vitro not required	In vitro required for excised axes	In vitro or grafting
Conservation target	Genes, genotypes or populations, depending on mating system and sampling methods		Genotype of parent plant

18.2 Physical Factors

Successful cryopreservation treatments have been achieved for recalcitrant seeds produced by species originating from temperate environments. In these cases, cryopreservation is largely an engineering problem, and the physical attributes of the tissue or propagule are dominant factors in developing successful procedures. The main physical attributes to consider for various cryopreservation strategies are the water potential, water content and size of the selected tissue to be cryoexposed. Methodologies are developed to accommodate the various constraints.

Water potential affects the range of temperatures at which water freezes and the cooling rate required to traverse that temperature range before ice nucleation occurs (Fig. 18.1). The relationship between water potential and water content is described by water sorption isotherms (Walters et al. 2001), and an example of that relationship can be derived from comparison of the values for the bottom and top axes in Fig. 18.1. At maturity, the water potential of recalcitrant seeds is about –1.5 MPa, necessitating cooling rates faster than 5,000°C/sec between –5°C and –100°C to prevent ice formation. Most recalcitrant seeds survive short-term exposure (a few hours) to water stresses as low as –12 MPa without addition of exogenous protectants. At –12 MPa, freezing transitions are severely limited and only a minute amount of water freezes even if cells are cooled and warmed from –25 to –80°C at about 0.2°C/sec (10°C/min) (Fig. 18.1). Many cryo preservation protocols for recalcitrant seeds make use of the apparent coincidence between the critical water potential that limits both desiccation damage and freezing of water (Vertucci et al. 1991). Freezing at water potentials less than about –15 MPa is so limited that viability does not depend on rapid cooling; orthodox seeds can therefore receive slow cooling treatments. Cryoprotectants alter the freezing properties of water and change the relationship between water content and required cooling rate (Volk and Walters 2006).

The water content of the tissue affects the changes in volume during drying and cooling, the heat capacity of the material and the amount of water available for freezing. Water content is a measure of the ratio of water within cells relative to other constituents. Expressed on a fresh mass basis, water content correlates with the relative volume of cells occupied by water, and therefore the extent of cell shrinkage experienced during dehydration (Figs. 18.2 and 18.3). For example, nearly mature cotyledon cells, which usually have high concentrations of dry matter and dense cytoplasm, contain about 0.35–0.40 g H_2O/g fresh mass when water potential is near –1 MPa. Adjusting the water potential of these cells to –12 MPa by dehydrating them reduces the water content to about 0.20 g H_2O/g fresh mass,

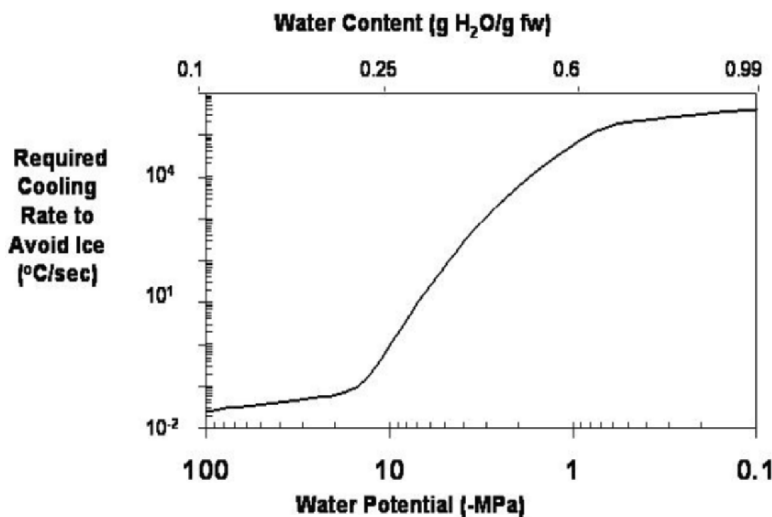

Fig. 18.1 Relationship between water potential and minimum cooling rate necessary to avoid ice formation in aqueous solutions. Water contents corresponding to mature embryonic axes of *Aesculus hippocastanum* with water potentials of less than –1.5 MPa are provided on the upper abscissa. Water contents of sucrose solutions are provided for water potentials greater than –1.5 MPa. Cooling rates are calculated from measures of water viscosity at 5°C (From Buitink and Leprince 2004; Wesley-Smith et al. 2004a, b; Walters 2004), the size of Aesculus embryonic axes (From Wesley-Smith et al. 2001a) and the crystallization rate of pure water (From Franks 1985)

causing a mere 15–20% reduction in cell volume (Fig. 18.2, solid line). In contrast, a partially mature embryonic axis may contain about 0.8 g H_2O/g fresh mass at –1 MPa and 0.2 g H_2O/g fresh mass at –12 MPa, resulting in a 60% volume change if dehydration alone is used to protect cells from ice during cryoexposure (Fig. 18.2, solid line). The severe change in volume may cause irreparable damage to cells according to the hypothesis that most cells tolerate <50% change in volume (Walters et al. 2002a). Less intense drying will avoid the mechanical stress, but will increase the cooling rate required to avoid lethal ice formation. For example, the embryonic axis will shrink to about 40% of its original volume if dried to –5 MPa (Fig. 18.2, dashed line); however the higher final moisture level will necessitate cooling at about 200°C /sec (Fig. 18.1).

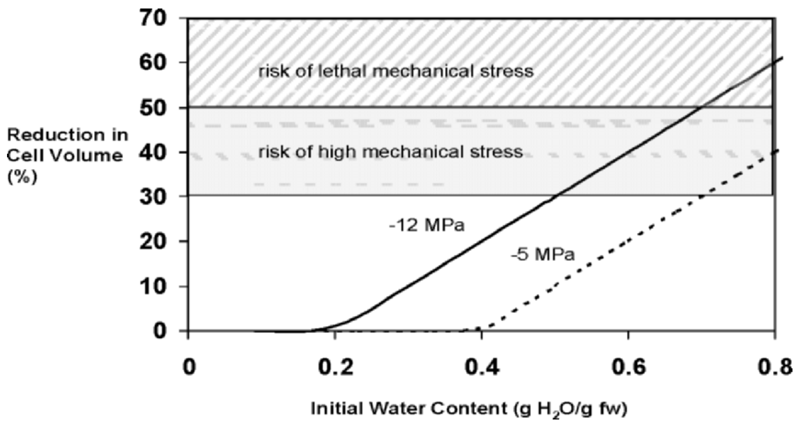

Fig. 18.2 Reduction in volume when water potential of cells is reduced to -12 MPa (solid line) or –5 MPa (dashed line). Calculations assume water contents of axes at –12 and –5 MPa are 0.2 and 0.4 g H_2O/g fw, respectively. Calculations assume constant temperature and do not account for decrease in volume associated with cooling

Water content measurements also reflect the heat capacity of the system, which is one of the parameters that drives the maximum cooling rate that can be achieved by convective cooling (Fig. 18.3). For example, heat capacities for the cotyledon and axis examples above are calculated as about 2.3 and 3.6 J/g/°C at –1 MPa, assuming heat capacities of water and dry matter of 4.2 and 1.2 J/g/°C. Given the same total mass and cooling procedure, cooling will be slower in axis compared to cotyledon tissues because there is more energy to dissipate from the water in the sample. Adjusting water potential to –12 MPa by removing water reduces the heat capacity of both cotyledons and axes to about 1.8 J/g/°C and facilitates rapid cooling in both systems. However, as discussed in the previous paragraph, reducing water content to –12 MPa also increases viscosity and reduces the rate of crystallization and, thus, the stringency required for rapid cooling. Water content, especially when expressed on a dry mass basis, also provides an accounting of the mass of water participating in freezing transitions. For example, a cotyledon contains about 0.6 and an axis 6.0 g H_2O/g dry mass at –1 MPa, implying a nearly ten-fold difference in the availability of water for freezing reactions.

Tissue size affects thermal mass and surface area to volume ratios, which, in turn affect rates of dehydration, cooling, and cryoprotectant penetration (Fig. 18.4). The large size of seeds, compared to other cryopreserved tissues and germplasm, precludes rapid drying, rapid cooling and

cryoprotectant penetration. Very small seeds (<1 mg dry mass) can be cooled faster than 1000°C/sec if water content is adjusted appropriately (Fig. 18.4). Seeds and explants containing ≤6 mg dry mass and a broad range of water contents can be cooled at rates between 100°C and 500°C/sec. As sample dry mass increases, the allowable range of water contents narrows and it becomes physically impossible to cool hydrated tissues fast enough to prevent lethal freezing injury. To circumvent the problem of too much thermal mass and to reduce the volume of material in cryostorage, cryopreservation protocols for recalcitrant seeds recommend storage of embryonic axes excised from the seed, and using *in vitro* systems following recovery (see physiological attributes). If the embryo or embryonic axis is very large (>12 mg dry mass), this approach has limited possibilities since it is unlikely that both desiccation and freezing damage can be avoided.

For successful cryopreservation, desiccation-rehydration and cooling–warming must occur faster than the damage that these stresses cause. The rate at which samples can be dried or cooled depends on the physical constraints described above and efficient moisture and heat transfer. Movement of water and heat occurs through conduction or convection depending upon the sample size and barriers to flow. High surface area to volume ratio of the sample, good contact between the sample and desiccant or cryogen, and movement increases the efficiency of moisture or heat transfer through convection. Hence, good ventilation facilitates drying, and projecting or waving samples through cryogens with low surface tension increases cooling rate (Wesley-Smith et al. 1992, 2001a, b, 2004a, b; Pammenter et al. 2002). The rate of moisture or heat transfer through either diffusive or convective mechanisms is dependent on the water potential or temperature difference, respectively, between the sample and the surrounding environment. Faster drying occurs when the surrounding relative humidity is quite low or when barriers that impede water movement are removed. Air desiccants that lower the relative humidity, thereby creating a large water gradient, include vacuum, dry gases, silica gel, or the atmosphere in equilibrium with saturated salt solutions such as LiCl or $ZnCl_2$ that reduce relative humidity to less than 20%.

Extreme desiccating forces create the risk of inadvertently over drying very small samples or generating a gradient within large samples which tends to over dry outside surfaces and under dry interior cells (Wesley-Smith et al. 2001b). Rather than creating large water gradients, solution-based cryoprotectants probably function by increasing water permeability and may therefore be effective in improving water movement throughout the sample. Analogous to water transfer, heat transfer is faster when the surrounding cryogen is extremely cold. Liquid nitrogen slush, formed by

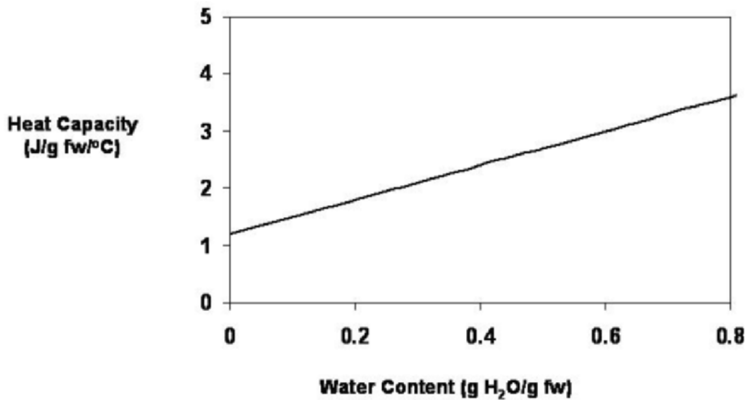

Fig. 18.3 Heat capacity as a function of the water content. Calculations assume heat capacity of pure water and dry biological material are 4.2 and 1.2 J g°C^{-1}

pulling a vacuum over liquid nitrogen, is a favored cryogen because of its extreme low temperature (–210°C) and lower surface tension. Better thermal contact between cryogen and sample is achieved using liquid nitrogen slush, which reduces the formation of an insulating vapor barrier at the sample surface known as the Leidenfrost Effect. Cooling rates in excess of a thousand degrees per second can be achieved in tiny (< 1 mg) samples forcibly propelled into sub-cooled liquid nitrogen using spring-loaded plunging devices or compressed air (Fig. 18.4) (Wesley-Smith et al. 2001a). Cooling rates of about 10°C /min can be achieved by placing standard (2 ml capacity) cryovials into liquid nitrogen vapor (Vertucci 1989). Approximate cooling rates attained using different container sizes, liquid nitrogen states and heat transfer methods are provided in Table 18.2. Hydrated samples must be stored cryogenically until they are thawed and placed in recovery. For recalcitrant embryos, the optimum storage temperature is largely undetermined, and it is unknown whether there is a benefit or possible disadvantage to storage at –80°C (mechanical, two- stage compressor), –135°C (approximate temperature of vapor above liquid nitrogen) or –196°C (temperature of liquid nitrogen). Stability of tissues depends largely on the temperature at which structural rearrangements of molecules become restricted because of extreme viscosity. This temperature is referred to as the glass transition temperature (*T*g). *T*g increases with increasing solute concentration from near –135°C for pure water to near –50°C for aqueous solutions at –12 MPa and above 0°C when cells are dried to less than –50 MPa (Buitink and Leprince 2004).

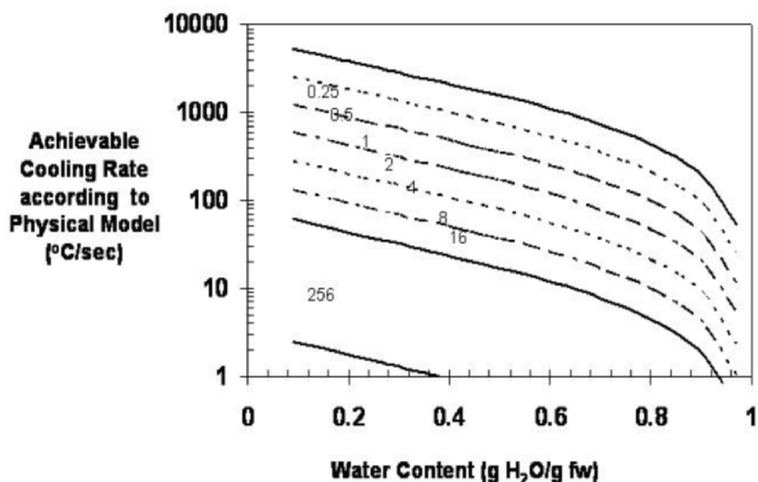

Fig. 18.4 Cooling rates of embryos exposed to liquid nitrogen predicted by a combination of conduction and forced convection heat transfer mechanisms. The model considers thermal mass based on dry mass of axis in milligrams (indicated by numerals), heat capacity calculations given in Fig. 18.3 and thermal conductivity of pure water and dry biological material as 0.59 and 0.2 mW mm°C^{-1}. The shape of the axis is assumed to be a sphere in this model and the total mass of the sample is the sum of the dry mass (indicated) and water mass (*x* axis)

Table 18.2 Approximate cooling rates achieved using different sample containers, liquid nitrogen phases and entry procedures (From Vertucci, 1989; Wesley Smith et al. 2004a)

Container	Liquid nitrogen state	Heat transfer mechanism	Approximate cooling rate
Cryovial insulated with 10 cm styrofoam	Vapor	Conduction	< 1°C/min
Cryovial of diameter (1 cm) or straw (up to 25 cm long)	Vapor	Conduction	20–50°C/min
Cryovial (2 ml)	Liquid	Conduction	100–200°C/min
Sample (100 mg)	Liquid	Conduction	500–800°C/min
Sample (1–3 mg)	Liquid	Convection	100–500°C/sec
~ 0.5 mg Sample	Slush	Convection	~1000°C/sec

Consequently, the degree to which embryos are dried before cryoexposure should profoundly affect the storage temperature at which they are

stable on a practical timescale. Hence, estimations of stability of cryogenically stored materials and optimization of storage temperature must account for the temperature dependence of molecular reorganization within the glassy phase (Walters 2004). This temperature dependence is known as glass "fragility."

18.3 Physiological Attributes

The sensitivity to desiccation stress in recalcitrant seeds, as for all tissues and germplasm, is the fundamental physiological feature dictating cryopreservation strategy. The moderately dense cytoplasm and moderate tolerance for dehydration of mature recalcitrant embryos allow manipulations to lower cell-water potentials, easing the stringency of exogenous applications of cryoprotectants and super-fast cooling. Hence, many of the procedures used to cryopreserve recalcitrant seeds focus on balancing damage by desiccation and freezing stresses, and mitigating the latter through rapid cooling treatments.

Achieving maximum tolerance to desiccation frequently involves rapid drying procedures of no more than a few hours. Rapid drying limits the time cells are exposed to intermediate water contents where metabolism becomes unbalanced and molecular mobility is sufficient to allow structural changes in macromolecules. Slower drying procedures, over the course of a few days, occasionally increase desiccation tolerance; but this is mostly observed when embryos are immature and the additional time at water potentials between –1 and –2 MPa allows completion of the maturation process (Vertucci and Farrant 1995; Walters et al. 2002a). More often, slow drying exacerbates desiccation stress, probably because protective reactions are not highly inducible in recalcitrant seeds, and depending on the developmental stage, the time at elevated water potentials allows germination to progress and/or metabolism-linked damage to accumulate (Berjak et al. 1989; Walters et al. 2001).

Shelf life of recalcitrant seeds directly influences cryopreservation success. Shelf life is promoted by postharvest moisture and temperature conditions that minimize both degradation and germination during storage. Storage conditions that take advantage of chilling tolerance and stratification requirements of seeds adapted to overwintering can slow germination, retard microflora proliferation and minimize desiccation stresses. Associated fungi, and less frequently, bacteria, pose a particular problem, especially in material of tropical provenance, proliferating during seed storage under the necessarily high relative humidity conditions (Sutherland et al.

2002). Unless eliminated, the microflora will survive cryostorage, flourishing subsequently under *in vitro* conditions, and obviating successful seedling production. Hence it is imperative to develop appropriate treatments both for the seeds and for explants. Germination is difficult to delay in recalcitrant seeds from warmer climates that generally lack dormancy mechanisms and may exhibit sensitivity to temperatures below 15°C. Hence, while seeds from temperate areas can be processed for cryopreservation several months after harvest, seeds from tropical areas usually must be processed immediately.

Low survival following nonlethal desiccation and sufficiently rapid cooling to liquid nitrogen temperatures is often reported for axes from immature seeds or for mature axes from seeds that are not adapted to over wintering. Clearly, these embryonic axes lack protective mechanisms that are found in other recalcitrant embryos. Applications of exogenous protectants may improve survival in these situations (Walters et al. 2002b). Application of cryoprotectant solutions containing glycerol, DMSO, or propanediol can bolster recovery percentages while reducing required cooling rates, but these compounds may have toxic effects depending on species and exposure times. Other methods to enhance survival include applications of ABA and tetcyclacis in the preexposure media (Beardmore and Whittle 2005), addition of calcium and magnesium to the cryoprotectant solution (Mycock 1999) and antioxidants to reduce oxidative stress during recovery.

Recovery of tissues from cryogenic storage is a critical part of the cryopreservation procedure, and depends on both thawing and rehydration procedures, as well as subsequent culture conditions. It is imperative that thawing occurs rapidly to avoid ice crystal growth, and the mode and medium used for this and for rehydration, appears to be critical to success. Use of a thawing and rehydration solution containing 1 μM Ca^{2+} and 1 mM Mg^{2+} has been shown to promote normal seedling formation (Berjak and Mycock 2004), while direct immersion facilitates far better recovery than does equilibration hydration of the partially dehydrated explants (Perán et al. 2004).

Because of size constraints in recalcitrant seeds, the embryonic axis is usually excised and germinated *in vitro.* Viability is usually accessed by swelling and greening of the axis and normal development is scored when both roots and shoots are formed. Root without shoot development is a common abnormality, especially after cryoexposure, and suggests that the radicle and epicotyl are not equally tolerant of the combined stresses of wounding during excision, sterilization, desiccation and cryogenic temperatures. Plant growth regulators are sometimes added to the growth

medium, but do not appear to be critical for normal development of mature embryonic axes. Poor growth conditions, overzealous sterilization or excessive drying may not produce symptoms in control (no low temperature treatment) axes, but may exacerbate damage in cryoexposed tissues.

18.4 Genetic Considerations

Efforts to cryopreserve recalcitrant seeds arise from our need to conserve genetic resources for future use. With this utilitarian objective, accountability measures focus not so much on preserving viability per se, but rather on preserving the allelic richness within an accession or the genetic composition of a specific individual. Recalcitrance is genetically regulated: it may be inherited (or lost) in interspecific crosses (Dussert et al. 2004), and it becomes less severe as generations become more adapted to longer maturation periods (Vertucci et al. 1994). Additionally, the degree of development prior to shedding, which is influenced by climatic conditions during development, appears to influence the desiccation response of seeds of some species (Daws et al. 2006). Incidence of recalcitrance among Angiospermae suggests that species producing these types of seeds may possess the appropriate tolerance genes, but fail to express them during late embryogenesis or express genes with competing effects. Consequently, genebanking recalcitrant seeds may actually impose severe and inadvertent selection pressures on genes that regulate phenology, embryo development and germination. Methods to guard against this type of genetic erosion include assessments of shifts in allelic frequencies and linkage disequilibrium between neutral and expressed loci in samples before and after cryoexposure, especially when mortality is high (Richards et al. 2004). Storing alternative germplasm, such as pollen, may forestall the loss of genetic integrity in accessions stored as recalcitrant embryos, and provide genetic resources to bolster diversity in *in situ* reserves as well as increase efficiency during regeneration and aid breeders making controlled crosses.

Many of the cultivated crops that produce recalcitrant seeds are out crossing species and desired genotypes are captured and maintained through vegetative cuttings or somatic embryos. This approach ensures genetic uniformity during mass regeneration of elite genotypes; however, it does not replace the need to preserve recalcitrant seeds, despite the inherent difficulties. Methods to preserve recalcitrant seeds are important for conservation and breeding programs because genetic diversity is most efficiently captured through seeds of a regenerating population (Richards et al. 2007), and zygotic embryos are the only way to retrieve progeny from

conventional crosses. Collections of zygotic embryos are more prone to genetic erosion arising from random changes in small samples or nonrandom changes from lethal damage during preservation or sampling bias during harvest, such as selection of mature fruits.

18.5 Practical Aspects of Recalcitrant Seed Storage

18.5.1 Plant Information and Seed Handling

In advance of storage, a number of practical items must be considered. First gather information on the basic phenology of the plant. When does flowering occur? When does fruit mature? What were the growing conditions between flowering and fruiting (early frost, irrigation, major pests?). If plants are in a remote location, can maturation date be predicted reliably to facilitate collection plans?

18.5.1.1 Plant Information

Next gather information on the basic anatomy of seed and embryonic axis. What are the seed mass, water content and water potential at maturity? Where is the embryonic axis relative to the storage reserves? What are embryonic axis mass, water content and water potential at maturity? Is the seed polyembryonic and are embryos zygotic and/or originating from maternal tissue? What is the texture of fruit and storage reserves? Will you need a scalpel, knife, hammer, vice, or slicer to dissect tissues. Will you need a dissecting microscope to examine tissues?

18.5.1.2 Seed Processing

Seeds will require decontamination before further processing, for which the appropriate treatment needs to be ascertained. Sodium hypochlorite is commonly used, but may damage seeds and/or excised axes. Other sterilants, such as calcium hypochlorite, mercuric chloride and alcohol, are options if sodium hypochlorite adversely affects tissues (usually observed as discoloration).

18.5.1.3 Seed Physiology

Gather information on basic growth characteristics of seed and excised embryo. What are dormancy breaking requirements and good germination conditions? Will seeds survive under refrigerated (~5 to 10°C) conditions?

Can embryonic axis be germinated *in vitro*? Do dormancy breaking requirements still apply? Does addition of plant growth regulators promote normal development such as both root and shoot growth?

18.5.1.4 Seed Source

Gather information on selected source population for seeds. If seeds are collected from the wild, does the site represent the appropriate genetic diversity? Is it a hybridization zone among species? If seeds are collected from within a plantation or orchard, is open pollination a problem? Would controlled crosses capture the conservation target better? Identify storage behavior of seeds from population or species (see next section).

18.5.1.5 Acquisition and Restrictions

Arrange for import permit if seeds are to be shipped from a different country. Import permit should specify port of entry (if known), species, size of shipment, and whether fleshy fruit is allowable. Know quarantine restrictions in your area and risks of inadvertent introduction of pests. Establish shipping protocol with supplier. Try to ship whole fruits that have been surface sterilized. If a phytosanitary certificate is needed (e.g., international shipments), make sure shipper has proper protocols and contacts so that the process can be expedited. Otherwise, arrange for expedited inspections in home country. Package fruits/seeds in moistened peat moss sealed in plastic bags. Use insulated containers for packaging. Include a copy of the import permit, phytosanitary certificate and a letter identifying the package contents and contact information from the originating and destination laboratories for inspection agents. Use expedited mail services.

18.5.2 How to Identify Storage Behavior

Initially it is important to check the Compendium of Information on Seed Storage Behaviour (Hong et al. 1996), Seed Information Database (SID) http://www.kew.org/data/sid, local botanical databases, Web of science, Scopus or other scientific databases for existing information on species. Use the Latin binomial and “seed” as keywords. Internet “Google” search Latin binomial.

Evaluate developmental characteristics of seed that may be germane to cryopreservation success. For example, cryopreservation is difficult if seeds are immature (green color, soft food reserves) or germinating (protruding radicle). Cryopreservation is more difficult in embryonic axes containing >2.0 g H_2O/g dw.

Evaluate the desiccation tolerance of the embryonic axis. If seed mass is less than 5–10 mg, use whole seeds; otherwise consider working with excised axes. If seed or axis contains less than 0.15 g H_2O/g dw upon harvest, then confirm viability using a standard germination test. If seeds or axes are wetter, expose to air or desiccant for different times and measure water content and viability. Viability of embryonic axes (not whole seeds) can be approximated in about 2 h using electrolyte leakage measurements. Definitive assessments of survival require a germination assay which may take weeks or months. Increases in leakage or loss of germination potential when seed tissues are dried to water contents $\leq$0.10 g H_2O/g dw are indicative of nonorthodox behavior. Most recalcitrant axes are damaged when water contents decrease below about 0.25 g H_2O/g dw.

Place a subsample of seeds in the refrigerator and monitor viability after a week and then monthly. This will tell you how rapidly you must process seeds. Protocols 18.6.1, 18.6.2 and 18.6.3 outline information on drying, cryopreserving, and recovering recalcitrant seeds.

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18.6 Protocols for Working with Recalcitrant Seeds

18.6.1 How to Dry Seeds and Measure Water Status

An isotherm relating water potential to water content for embryonic axes and whole seeds (Walters et al. 2001) provides useful information on maturity status and critical water contents. Rapid drying is preferred. If seeds have not completed their embryogenic program, a few days within fruits before initiating drying may induce greater desiccation tolerance. Our preferred drying procedures flush or circulate dry gas onto excised embryos for a predetermined time or until a predetermined mass of water has been removed. The device used by the Durban, South Africa lab is illustrated in Pammenter et al. 2002. Desired time or water loss is estimated from the initial water content, previously determined drying time courses and the target water content. Target water content considers water contents that limit desiccation damage (above the critical water content), the cooling rate required to limit ice formation (Fig. 18.1), and available cooling protocols that provide the necessary cooling rate (Table 18.2).

Drying time should not exceed a day or two at most, and time spent at intermediate water potentials ($-10 \leq \Psi w \leq -3$ MPa) should be minimized. Samples that are dried to the target water content should be processed immediately into liquid nitrogen. Samples recovering from liquid nitrogen should be rehydrated immediately. Generally recalcitrant seeds are too large to dry at the rate required to avoid desiccation damage or to achieve the desired cooling rate commensurate with dry mass and water content of seed (Fig. 18.4).

1. It is generally necessary to excise the embryonic axis at the start. Although increasing explant size and hence thermal mass, inclusion of some cotyledonary tissue may be desirable to protect epicotyl from damage (see Goveia et al. 2004). We prefer to delay surface sterilization until the recovery procedure.
2. To measure water content of milligram-sized samples (embryonic axes), place sample in pre weighed foil packets. These packets can be made by wrapping small strips of aluminum foil around the tip of a pencil. Use sub samples receiving the same treatment as the bulk of tissue being processed. Measure fresh mass. Measure dry mass after heating 24–40 h at 95°C. It is important to specify whether water content is expressed on a fresh or dry mass basis.

18.6.2 How to Cool Seed Tissues to Liquid Nitrogen Temperatures

1. Determine necessary and achievable cooling rates based on tissue size and water properties (Figs. 18.1; 18.4).
2. Naked embryos are more prone to loss or damage, so if mass allows, prepackage embryos in foil packets. Foil packets can be made by wrapping small strips of aluminum foil around the tip of a pencil or by cutting foil in about 1 mm^2 rectangles and placing axes in a single layer (not touching), folding the foil rectangle in half and folding over edges to seal. Otherwise, place naked embryos on aluminum foil strip or stick onto pins used for cryosectioning (Leica, Austria) using a dot of glycerol.
3. Select a cryovial "system" that holds liquid nitrogen and samples, but allows liquid nitrogen to drain. The modified cryovial should be easily transferable from the cooling station to long term storage in a cryovat, and then into warm water. Our cryovial system is modified from a 5 ml polypropylene tube with a snap-on cap. The base of the tube is cut off and replaced by fine mesh. The top of the cap is also cut off and the fine mesh is held in place by sliding the cap walls over the base of the tube. Another cap is used to cover the cryovial before transfer to the cryovat.
4. Place a Styrofoam cup filled with liquid nitrogen and containing an open cryovial in a vacuum-sealing desiccator. Pull a vacuum on the desiccator using a >1.5 kW (2 hp) vacuum pump and allow liquid nitrogen slush to form. Turn vacuum off, vent desiccator slowly and rapidly introduce samples individually to slush using forceps, and subsequently place foil strips/packets into the cryovial.
5. Alternatively, propel embryonic axes mounted on pins into slush using a compressed air injector or similar forcible entry mechanism. Process desired number of axes, then seal cryovial and transfer to cryovat for storage. Regenerate slush after approximately 1–2 min of use, as it is essential to maintain nitrogen in a slushy state (about –210°C). An alternative cooling procedure is to place embryonic axes in a Styrofoam cup and rapidly pour liquid nitrogen slush into it.

18.6.3 How to Recover Seed Tissues from Liquid Nitrogen

1. Directly immerse foil packets into water or a solution containing Ca^{2+} and Mg^{2+} warmed to about 40°C for 1 min, or place naked embryos in liquid medium at about 25–40°C for 1 min.
2. After about 1 min transfer axes to medium at room temperature.
3. Surface sterilize by swirling tissues for 10 min in a 10% commercial sodium hypochlorite solution (or calcium hypochlorite, mercuric chloride, ethanol). If bacteria are a problem, then additional treatments (bactericides; antibiotics) will be required.
4. The germination medium usually contains standard basal nutrients such as Linsmaier and Skoog (1965), full or half strength Murashige and Skoog (1962) or woody plant medium (Lloyd and McCown 1980) with 3% sucrose and 0.8–1% agar. Activated charcoal is occasionally included to scavenge phenolic compounds.
5. Wrap Petri plates with foil for 2–3 days to give an initial dark period.

Swelling and greening of viable tissues should be apparent after 2 weeks. Germination may require 2 months to score. It is essential to differentiate between swelling and greening on the one hand, and plant production, on the other. Cryopreservation cannot be considered successful unless a seedling/plant that will survive *ex vitro*, can be established.

Chapter 19

Cryopreservation of Orthodox (Desiccation Tolerant) Seeds

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19.1 Introduction

Although there are several methods of *ex situ* plant conservation, seed banking is the most efficient for many species, particularly for ease of application and the amount of diversity conserved (Linington and Pritchard 2001). Indeed, seed storage is the main form of *ex situ* plant genetic resources (PGR) conservation globally, representing about 90% of all collections, the vast majority of which are crops, including cultivars (FAO 1996). More than half the world's PGR accessions are held in medium-term or long-term storage conditions. For long-term storage, the international standards are drying at 10–25°C and 10–15% RH to 3–7% moisture content, followed by storage at about –18°C (FAO/IPGRI 1994). Whilst less than expected seed longevity at about –20°C is known for "intermediate" or Type II seeds (see Pritchard 2004), 'orthodox' Type I seeds can also age quicker at seed bank temperatures than predicted by the seed viability equations (for explanation see Pritchard and Dickie 2003). This was revealed by an elegant experiment in which orthodox *Hordeum vulgare* ssp *distichium* cv. Proctor seed, ageing at warm temperatures, was interrupted by transfer to –20°C,

[1] Financial support from the Darwin Initiative (Project 14-056) is gratefully acknowledged.

B.M. Reed (ed.), *Plant Cryopreservation: A Practical Guide.*
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which indicated longevity parameters associated with storage at –6°C (Roberts and Ellis 1977). This observation, combined with comparisons between actual performance and extrapolation of longevity to sub-zero temperatures, suggests that the benefits of all sub-zero storage temperatures may be less than previously thought (Dickie et al. 1990; Pritchard 1995; Pritchard and Dickie 2003; Walters et al. 2004). Although the modelling of seed longevity at sub-zero temperatures is a challenge, such cooling generally enhances dry seed longevity (Dickie et al. 1990; Pritchard and Seaton 1993; Walters et al. 2004). Consequently, cryopreservation may be of particular importance for the long-term (10-100s years) storage of otherwise inherently short-lived orthodox seeds (Pritchard 1995; Pritchard and Seaton 1993; Pritchard et al. 1999b; Walters et al. 2004; Pritchard 2007).

The extra biological insurance of cryopreserving seeds was recommended-ded about 30 years ago (Stanwood and Bass 1978). Since then, numerous studies have reported dry seed tolerance of liquid nitrogen exposure of hundreds of species (see citations in Pritchard 1995, 2007). Cryopreservation studies on genetic resources in general have increased significantly in recent times (Pritchard 2002), including on dry seeds (Pritchard 2007). Most of the studies in about the last 10 years have been on species of socio-economic importance, particularly woody (about 40), horticultural (about 9) and agriculture (about 13) (for review see Pritchard 2007). Native, endangered or endemic species have also been studied. Moreover, greater collective efforts to cryopreserve unique PGR are evident recently. Such initiatives include: technology transfer of methods for vegetatively propagated crops (Reed et al. 2004), the establishment of a regional cryo centre of excellence in Africa (Darwin Initiative 2005; Wood et al. 2005), and functional regional cryo networks (EU 2006; Pritchard 2006). However, interest in the low temperature extremophily of seeds through experimentation with liquefied gases extends back to the nineteenth century, with a golden era between the 1830s and 1930s (e.g. Thiselton-Dyer [1899]; see "The latent life of seeds").

19.2 The Latent Life of Dry Seeds

As early as 1834, Edwards and Colin (1934) subjected dry seeds of barley, broad beans, rye and wheat to a temperature which "froze mercury" after which "their power of germination was unchanged." Similarly, Wartman (1860) explored the effects of different temperatures on dry seeds of nine species (*Avena sativa*, *Clarkia elegans*, *Eschscholtzia californica*, *Hordeum vulgare*, *Lepidium sativum*, *Linaria bipartite*, *Nemophila insignis*, *Portulaca oleracea*, *Triticum sativum*); in all cases, germination was "unimpaired"

after 20–30 min exposure to –110°C. Also De Candolle and Pictet (1879), De Candolle (1865) and Dewar and McKendrick (1892) noted no loss of seed vitality after exposure to liquefied gases. Brown and Escombe (1897–1898) treated seeds of 12 species in liquid air for 110 h, with no ill effects. Selby (1901) followed a similar approach by exposing seeds of 12 species (*Chenopodium album*, *Cucumis sativus*, *Helianthus annuus*, *Linum usitatissimum*, *Lupinus luteus*, *Mimosa pudica*, *Onobrychis sativa*, *Pinus sylvestris*, *Ricinus communis*, *Secale cereale*, *Triticum sativum* and *Zea mays*) to liquid air; concluding that "it is not apparent that any marked unfavourable effect on germinable seeds may be traced to the immersion." The effects of even lower temperature (1.35 K; –272°C) were assessed by Lipman (1936); with vetch, wheat, barley, tobacco, flax, buckwheat, spinach, milo, maize and *Melilotus* seeds showing no ill effects when germinated and grown in a greenhouse.

The effect of storage time at –196°C was assessed for a range of species by Lipman and Lewis (1934). Neither sugarcane, spinach, cucumber, sugar beet, buckwheat, barley, purple vetch, oat, onion, mustard and *Melilotus* seeds stored for 30 days, nor pea, corn, squash, alfalfa and sunflower seeds stored for 60 days, showed any detrimental effects with respect to laboratory/greenhouse germination or vigour. Similarly, sweet clover seeds tolerated 176 days storage in liquid air, yielding 74% germination, compared to 64% for the unfrozen control (Busse 1930). As germination of these stored seeds was not checked until 6 months after thawing, at which point the controls had only about 50% germination, Busse (1930) felt that "storing in liquid air….may be beneficial in retarding normal aging of …seeds," a sentiment that has subsequently been shown to be true. Wartman (1860) also compared seed responses (short-term exposure) at –110°C with –57°C, observing no systematic differences.

Finally, two-step freezing ("gradual transition" from vapour to immersion) was pioneered by Selby (1901), in a comparison with rapid cooling ("sudden transition" by immersion) to liquid air (–190°C). Selby (1901) also considered storage time (6–48 hours), but a full factorial design was not used, making specific insights difficult other than most of the treatments yielded similar germination to the controls.

In summary, the basis of modern seed cryopreservation was established between 170 and 70 years ago with basic experiments on the short-term (hours) tolerance of mainly crop seeds to various cryogenic temperatures, including the first consideration of two-step freezing. Only at the end of this period did attention turn to the importance of seed moisture content on survival of cryogenic temperatures.

19.3 Moisture Content

In 1934 Busse and Burnham noted that “the percentage of abnormal plants decreases with increasing moisture content up to 10 or 15 per cent” when treated with liquid air. The authors had dried oilseeds of flax for 10 h at 51°C and then manipulated the uptake of additional moisture above the dried level in an atmosphere of high humidity. The impact on seedling quality was observed as a decrease in seedlings with a double growing point and double or fasciated hypocotyls compared with the driest cryopreserved seeds. The abnormalities produced by low temperature were not inherited, however, but due to “temporary changes in the developmental processes.” Similarly, cotton seeds treated with liquid air subsequently germinated but had “cotyledonary leaves filled with cracks” and the driest flax seeds also had slightly reduced germination (–12%) after cryopreservation. Busse and Burnham (1934) opined that such injury was “due to the simple mechanical stresses and strains set up in the seeds owing to different coefficients of expansion and different rates of cooling of the different parts.” Interestingly, both rapid warming (transfer from liquid air immediately onto a piece of metal at room temperature) and slower warming (allowing liquid air to evaporate) had similar effects on the number of abnormalities in flax seed (Busse and Burnham 1934). Rapid temperature changes cause similar stresses and strains in dry soybean, with cracking of dry beans (Sakai and Noshiro 1975). Use of such stresses to positive effect is also known; a few minutes exposure to liquid air or liquid nitrogen is known to reduce hardseededness (impermeability to water), for example in seeds of sweet clover and alfalfa (Busse 1930) and trifolium (Pritchard et al. 1988). Cooling at about 10°C min^{-1} tends to overcome these stresses (Vertucci 1989a).

Moisture contents at ≤8% tend not to affect seed survival following cryopreservation. At less than 13% moisture content (fresh weight basis) no damage to germination was observed in 42 species of commonly cultivated plants (Stanwood and Bass 1978). Stushnoff and Juntilla (1978) noted that seeds with a moisture content of 5–13% were not injured by ultracooling but seeds at 13–16% moisture content were damaged. Moreover, as moisture content increases, the rate of warming (and cooling) can extend the moisture content range for survival (Sakai and Noshiro 1975). In Italian rye grass seed, rewarming rapidly (25°C s^{-1}; 1500°C min^{-1}) rather than slowly (0.5°C s^{-1}; 30°C min^{-1}) following immersion in liquid nitrogen increased the critical moisture content for survival from 24 to 30% moisture content (dry basis; 19–23% fresh weight basis). Similarly, this “critical moisture content” increased from about 16–18% to 19–21% dry basis (i.e. from about 15– 17%

fresh weight [FW] basis) in unhulled rice and winter wheat seeds following comparable treatment. Moreover, it was shown for wheat seeds at 16–18% moisture, that 30 min during warming at an intermediate sub-zero temperature (about –30°C), decreased germination by 38–50%, probably due to ice formation and growth (Sakai and Noshiro 1975).

Subsequent studies aimed to identify this upper moisture content limit for seed cryopreservation using rapid cooling and warming; including those of Stanwood (1985), who coined the term high moisture freezing limit (HMFL). HMFL in oilseeds varies, being about 9% moisture content for sesame (about 50% oil) and about 14% in soybean (about 18% oil) (Pritchard 1995). Overall, HMFL (MC, % FW) is inversely related to oil content (% dry weight [DW]); thus, HMFL = 23.1 to 0.21 oil (Pritchard 1995). Validation of this relationship was confirmed with oily seeds (37–52% DW) of four citrus species, combined with data for 12 other species, including seven *Coffea* sp. (Hor et al. 2005). Using differential scanning calorimetry, it was shown that the seed unfrozen water content, WCu, (% FW) was also related to seed (oil) lipid content (LC); % DW), thus: WCu = 23.4 – 0.28 LC (Hor et al. 2005).

The HMFL and WCu approximate to precooling relative humidities of 74–77% RH in *Citrus* (Hor et al. 2005) and about 85% in other species (see Pritchard 1995 and references therein). Thus drying below the HMFL or the WCu prior to cooling is the best way to ensure successful cryopreservation. Moisture content optima for oilseed cryopreservation are equivalent to about 65–75% RH (Pritchard 1995). It should be noted however that pre-cooling desiccation below this RH may have little negative impact on seed survival, whereas higher moisture levels significantly increase the risk of ice formation.

19.4 Repeated Cooling and Storage Temperature

As noted above, liquid nitrogen exposure can reduce the physical impermeability of hard seeds (irrespective of oil content), the effectiveness increasing with number of cooling–warming cycles (Pritchard et al. 1988). Conceptually, there may be a point at which the number of temperature cycles becomes too stressful, thereby reducing germinability. Gonzalez-Benito et al. (1998) assessed this risk in seeds of *Centarurea hyssopifolia* and *Limonium dichotomum*, finding that they were unaffected by weekly removal from liquid nitrogen compared to constant immersion for 21 weeks.

The optimal temperature in the sub-zero range for the long-term storage of dry seeds remains to be elucidated. This is particularly true of seeds that

can be dried only to intermediate moisture contents (about 8–10%). For these Type II seeds—so called because they can tolerate drying into zone II of the sorption isotherm—it has been hypothesised that storage at –18°C risks devitrification of the aqueous glass (Pritchard 2004). Consequently, it has been suggested that all dried seeds are stored at least 70°C (or K) below the glass transition temperature (Tg); in other words, at ≤ Tg–70. For orthodox seeds, Tg–70 is around 10°C, at which temperature longevity can be around 200 years (Daws et al. 2007); however, further cooling, particularly cryopreservation, has substantial benefits for seed longevity. For example, the reduction in germinability of relatively low initial quality *Dactylorhiza fuchsii* seeds during sub-zero storage was about three times slower in liquid nitrogen compared to –20°C over a 6-year period, although viability loss was not completely stopped (Pritchard and Seaton 1993). Moreover, cryogenic storage clearly prolonged shelf life of lettuce seeds, with half-lives projected as ca. 500 for seeds stored in the vapor and ca. 3400 years in liquid phases of liquid nitrogen (Walters et al. 2004), far in excess of the performance predicted at conventional storage temperature (Pritchard 2007).

19.5 Recovery

For all dried seeds there is the risk of damage during rehydration in the early stages of the germination test. This is best avoided by humidification of seeds above water. Damage is most often observed when dried seeds are placed in water, rather than on moistened (not soaking wet) filter paper or on water agar. Germination should progress under optimum conditions empirically derived for the species under investigation.

19.6 The Future

The kinetics of seed viability loss at sub-zero temperatures have been predicted from the warmer temperature responses of a range of species (Dickie et al. 1990; Pritchard 1995) and determined for one species, lettuce (Walters et al. 2004). Clearly, much remains to be elucidated about seed storage performance at low temperatures. Of particular interest is the increasing number of observations of sub-optimal performance of seeds under conventional seed bank conditions (drying and storage temperature). Examples are prevalent in tropical species (Ellis et al. 1990, 1991; Pritchard and Dickie 2003; Pritchard 2004), which form the vast majority of global plant diversity. In papaya, drying *per se* (Wood et al. 2000) and short-term

"freezing" in the dry state (Pritchard et al. 1999b) compromises germinability.

Associations have been made with the conformational state of seed lipids, particularly crystallisation at –18°C, in *Cattleya aurantiaca* (Pritchard and Seaton 1993) and some *Cuphea* species (Crane et al. 2003; Crane et al. 2006) and reduced storage performance. Such sensitivity in dry oilseeds may reflect a predilection for oil-body coalescence and massive cellular disruption during rehydration (Leprince et al. 1998; Volk et al. 2006). Slower seed imbibition over water, imbibition at high temperature, or preheating of dry seeds before imbibition (Crane et al. 2003) appear to reduce such problems. However, in some orchid species exhibiting seed cold-shock, germinability appeared independent of imbibition temperature (Pritchard et al. 1999a). Other observations that the range of sub-zero temperatures inducing cold shock in dry seeds varies in some orchids species, i.e. is not limited to –18°C (Pritchard et al. 1999b), emphasises the need for future studies in this area.

Cryogenic storage prolongs dry seed longevity compared to conventional freezer storage. Such cryopreservation of 'dry' seeds requires careful attention at three procedural steps: (1) seed dehydration to water contents below the high moisture freezing limit (~ 70–80% relative humidity [RH]); (2) relatively slow cooling and rewarming of the dry samples (~10°C –20°C min^{-1}); and (3) gentle imbibition in the germination test for viability assessment. As critical steps in the process, step (1) is important for all seeds, whilst steps (2) and (3) can be particularly important for oilseeds.

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19.7 Protocols

19.7.1 General Seed Cryopreservation

Based on Sakai and Noshiro 1975

High survival of air-dried crop (and other) seeds after immersion in liquid nitrogen may be easily obtained. Physical damage to seeds post-cryopreservation (see Pritchard et al. 1988) can be overcome usually by using slower cooling rates (Vertucci 1989).

Checklist

1. Protective clothing (gloves, apron and face shield)
2. Wide neck LN dewar (1–2 l capacity) for cooling
3. LN storage vessel and inventory system (i.e. containers and racks)
4. Long handle forceps for placing containers in and extracting from liquid nitrogen
5. Seed storage vessels/packets or polypropylene screw-cap vials. Alternatively, packets can be shaped from aluminium foil.
6. Water bath at 35–40°C

The procedure

1. Dehydrate to the correct range of moisture content: 8–15% dry weight [DW] (8–13% fresh weight [FW]) using a desiccant, such as silica gel, low relative humidity saturated salt solutions (see Protocol 19.7.3), an air conditioned room, or drying room operating at ca. 15% RH. Drying under ambient conditions where the RH is < 70% will also enable such moisture contents to be achieved.
2. Enclose seeds in aluminium foil or plastic vessel with a screw cap.
3. From room temperature, immerse the foil/vessel enclosing seeds in liquid nitrogen. The plastic vessel will enable slower cooling (and warming) as heat transfer is slower compared with foil.
4. After storage, transfer the foil/vessel to air at 0°C or room temperature (slow warming). More rapid warming can be achieved by placing the container in a 40°C water bath; this is generally not harmful to the seeds and may be important if the seeds are close to the high moisture freezing limit (see Protocol 19.7.2).

Possible problems

Ultra-dry seeds may crack when rapidly cooled/ warmed. To reduce this risk, use rates of about 10°C min^{-1}, achieved by using plastic cryotubes and vapour phase cooling, combined with warming in air at room temperature (rather than using a hot water bath).

19.7.2 Cryopreservation of Australian Wild Citrus Seed

By Kim Hamilton based on Hamilton et al. 2005

Cryopreservation of seeds after controlled drying to low moisture contents has been demonstrated as an alternative option for long-term *ex situ* storage of some cultivated *Citrus* species, for example *Citrus aurantifolia*, *C. aurantium*, *C. limon* and *C. sinensis* (Lambardi et al. 2004; Hor et al. 2005). A simple desiccation-based cryopreservation protocol for seeds has also been demonstrated in *C. australasica* (Finger lime), a southeastern Australian wild *Citrus* species grown as a bushfood. *C. australasica* seeds desiccated down to 5% moisture content had a germination level of 82% and normal morphology after short-term storage in liquid nitrogen (Hamilton et al. 2005).

Procedure

1. Extract seeds from mature fruits.
2. Dry seeds by incubation at 15% relative humidity (15°C) for 7 days (~5% moisture content). Alternatively, seeds can be dried over activated silica gel for 5–10 days (~3% moisture content).
3. Place dried seeds in cryotubes on canes and directly immerse in liquid nitrogen for storage.
4. Rapidly rewarm seeds by plunging cryotubes into water at 40°C for 1–2 min.
5. Surface sterilize seeds by rinsing in 2% sodium hypochlorite for 30 s, followed by two rinses in water. Seeds need to be cleaned to reduce fungal infection during germination testing, most likely caused by presence of seed coat mucilage. This may be a feature of seeds from other fruits.
6. Place seeds on moist filter papers (or 1% agar) in Petri dishes and incubate at 30°C with an 8 h day length. Seeds must be kept moist throughout germination testing.
7. Record germination after at least a 2 month incubation period.

19.7.3 Manipulation of Seed Moisture Content

Based on Sun 2002

An understanding of seed water (moisture) content (i.e. how much water) and an understanding of water relations terms is essential for any studies on seed longevity (Roberts and Ellis 1989), including cryopreservation experiments. Water (moisture) content on a wet (fresh) weight basis is widely used in the literature and can be easily converted to dry weight basis. Because water content is a function of seed chemical composition, particularly oil (lipid) content, it is important to consider that seeds of different species may vary in dependency of water content on relative humidity (sorption isotherm). Fortunately, the relative humidity of an environment used to pretreat seeds can be converted to water potential (i.e. how available the water is), as long as the temperature conditions are known, allowing wider comparisons to be made between seed responses and the wider literature on plant water relations.

Prepare in advance
Saturated salt solutions

Checklist

1. Saturated salt solutions in jars that can be sealed hermetically and with raft to keep seeds above the solution.
2. Forceps and scalpel (in case seed parts require dissection)
3. Electronic balance (accuracy depending on sample mass)
4. Oven (for sample drying)
5. Equilibrium relative humidity meter

Procedure

1. Place seed at 25°C over saturated salt solutions at prescribed humidity level. Higher relative humidities may hydrate seeds above the high moisture freezing limit or unfrozen water content (see Protocol 19.7.4).

   a. KOH (8%)
   b. K acetate (23%)
   c. K_2CO_3 (43%)
   d. NH_4NO_3 (63%)
   e. NaCl (75%)
   f. NH_4Cl (78%)

2. Regularly weigh seed sample over many weeks until equilibrated.
3. Determine sample dry weight by drying 1 day in oven at 103°C and calculate water content (WC).
   WC (% weight basis [w.b.]) = (fresh weight – dry weight)/fresh weight × 100.
4. To convert moisture content to dry weight basis:

   WC (% w.b.) × 100 / [100–WC (% w.b.)].

5. Determine equilibrium relative humidity of seeds in closed sample container of a humidity meter over about 30 min (record temperature of sample).
6. Co-plot water content with RH of environment (i.e. construct a water sorption isotherm).
7. Convert RH of environment to water potential:

   $$\Psi_W = (RT/V_W) \ln (\% \text{ RH}/100)$$

   Where R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T the absolute temperature and V_W the partial molar volume of water (18 cm^3 mol^{-1}).

Possible problems
Seeds in lower RH treatments may take a considerable time to equilibrate.

19.7.4 Determination of the Unfrozen Water Content

Based on Hor et al. 2005

Pre-drying orthodox seeds below the high moisture freezing limit (HMFL) or unfrozen water content (Vertucci 1989; Hor et al. 2005) is a pre-requisite for successful cryopreservation. Theses moisture limits to cryopreservation can be determined using differential scanning calorimetry.

Checklist

1. Forceps and scalpel (in case seed parts require dissection)
2. Saturated salt solutions in jars that can be sealed hermetically and with raft to keep seeds above the solution
3. High accuracy balance (for sample weight determinations)
4. Oven (for sample drying)
5. Differential scanning calorimeter (DSC), with proprietary software

The procedure

1. Equilibrate seed for 4 week at 25°C over saturated salt solutions providing a RH spectrum between 8% and about 93%.

   a. KOH (8%)
   b. K acetate (23%)
   c. K_2CO_3 (43%)
   d. NH_4NO_3 (63%)
   e. NaCl (75%)
   f. NH_4Cl (78%)
   g. $(NH_4)_2SO_4$ (80 %)
   h. KCl (85%)
   i. $BaCl_2$ (90%)
   j. KNO_3 (93%)

2. To achieve even higher moisture levels, partially rehydrate in a 100% RH atmosphere, i.e. over water in a closed container.
3. For thermal analysis, seal seed samples (about 15 mg) in aluminium pans.
4. In the DSC: Cool pans to about –120°C at maximum cooling rate of machine (often about 200°C min^{-1}).
5. Heat pans at about 10°C min^{-1} from –120°C to 20°C; record thermograms.

6. Puncture pans and determine sample dry weight by drying about 1 day in oven at 103°C.
7. Analyse heating thermograms for the determination of the peak and onset temperatures and the enthalpy of endothermic transitions.
8. Determine transition enthalpy (joules per gram DW) from the area above the baseline.
9. Estimate the unfrozen water content (WC_u) by plotting enthalpies against seed sample water content (g H_20 g^{-1} DW).
10. In oily seeds, two linear relationships will exist: for equilibrium moisture contents below and above about 80% RH.
11. Solve the two linear relationships to estimate the WCu. For non-oily seeds, WCu is the *x*-intercept for the linear regression.

Possible problems

Long-term (many weeks) equilibration of seed samples at high humidity may result in fungal contamination of the sample. Use slightly lower equilibration temperatures or shorter times.

19.7.5 Seed Rehydration and Heating Based on Lipid Melting Characteristics

Based on Crane et al. 2003

Oilseeds in particular appear to be sensitive to the rehydration phase of the germination test, possibly as a result of physical state of the lipids on ingress of water. Induction of such sensitivity appears in some species to be a function of seed thermal, especially sub-zero, history. Although in its infancy as a practice, thermal properties of seed lipids may be used as a marker for sensitivity to storage at –18°C [e.g. *Cattleya aurantiaca* (Pritchard and Seaton 1993)] and possibly exposure to other sub-zero temperatures. For example, following –18°C treatment *Cuphea* seeds with lipid peak melting at ≥27°C require heating to >45°C prior to the germination test to ensure high germination (Crane et al. 2003).

Checklist

1. Container of water for humidification of seeds
2. Oven (for sample drying)
3. Differential scanning calorimeter (DSC), with proprietary software.

The procedure

1. Dry seed to about 5% moisture content (see Protocol 19.7.3 or 19.7.4) or over silica gel.
2. Place seeds at sub-zero temperatures for at least 18 h.
3. To limit imbibitional damage, pre-hydrate samples over water for at least 18 h or treat dry seed sample at 45°C for at least 1 h before germination test (without pre-hydration).
4. Germinate under normal growth conditions for the seeds of species under investigation.
5. To determine thermal behaviour of seeds, seal about 4 mg of sample into aluminium pans.
6. Cool pans from 20°C to–100°C at 10°C min^{-1}, hold for 1 min and rewarm to 50°C at the same rate.
7. Record crystallisation events on cooling and peak temperature at the apex of the lipid transition during warming, using the highest transition peak temperature when several peaks are observed.

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