

Refinement and standardization of storage procedures for clonal crops

Global Public Goods Phase 2:

Part III. Multi-crop guidelines for developing *in vitro* conservation best practices for clonal crops

Erica E Benson, Keith Harding, Daniel Debouck, Dominique Dumet,
Roosevelt Escobar, Graciela Mafla, Bart Panis, Ana Panta, David Tay,
Ines Van den houwe and Nicolas Roux



The CGIAR System-wide Genetic Resources Programme (SGRP) joins the genetic resources activities of the CGIAR centres in a partnership whose goal is to maximise collaboration, particularly in five thematic areas: policy, public awareness and representation, information, knowledge and technology, and capacity building. These thematic areas relate to issues or fields of work that are critical to the success of genetic resources activities.

SGRP contributes to the global effort to conserve agricultural, forestry and aquatic genetic resources, and promotes their use in ways that are consistent with the Convention on Biological Diversity (CBD). The Inter-Centre Working Group on Genetic Resources (ICWG-GR), which includes representatives from the centres, FAO and the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA), is the Steering Committee. Bioversity International is the Convening Centre for SGRP and hosts its coordinating Secretariat. See www.sgrp.cgiar.org.

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This document has been produced in compliance with, and fulfilment of GPG2 Project Milestone 1.2.1 “Review *in vitro* protocols applied to clonal crops” and sub-activities, 1.2.1.5 “Guidelines prepared for adapting *in vitro* procedures to additional crops. Develop a multi-crop guideline on state of the art of the development and application of *in vitro* techniques for the medium and long term storage of clonally propagated crops and 1.2.1.6 Revise the guidelines through expert consultation.” This document also supports Milestone 1.2.2, to “Develop and implement a programme of technology transfer and capacity building to refine and standardize *in vitro* conservation for clonal crops.” As part of Activity 1.2 (Year 3) the technical information collated on *in vitro* techniques enables the production of a training manual for the use of MTS and LTS by other partners, including National Agricultural Research Stations.

Interdependent actions: This document provides information for the interdependent Milestone Activity 2.1 and its sub-activities concerning the compilation of Best Practices for clonal crops and Sub Activity 1.1 Development and implementation of risk-management procedures for individual genebanks and for collections in common. And, specifically Milestone 1.1.1 existing centre risk assessment guidelines collated. Guidelines analysed and generic risks to collections identified.



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Erica E Benson¹, Keith Harding¹, Daniel Debouck², Dominique Dumet³,
Roosevelt Escobar², Graciela Mafla², Bart Panis⁴, Ana Panta⁵,
David Tay⁵, Ines Van den houwe⁶ and Nicolas Roux⁷

¹Damar Research Scientists, Damar, Drum Road, Cuparmuir, Cupar, Fife, KY15 5RJ, Scotland, UK.
e.benson-damar@tiscali.co.uk; k.harding-damar@tiscali.co.uk

²CIAT, Km 17 Via Cali-Palmira, AA 6713, Cali, Colombia. r.escobar@cgiar.org; d.debouk@cgiar.org;
g.mafla@cgiar.org

³IITA, Nigeria, c/o Lambourn & Co, Carolyn House, 26 Dingwall Rd, Croydon, CR9 3EE, UK.
d.dumet@cgiar.org

⁴Laboratory of Tropical Crop Improvement, Katholieke Universiteit of Leuven, Kardinaal
Mercierlaan 92, 3001 Heverlee, Belgium. Bart.Panis@biw.kuleuven.be

⁵CIP, Apartado 5969, Lima 100, Peru. a.panta@cgiar.org; d.tay@cgiar.org

⁶Bioversity International-Belgium, Katholieke Universiteit of Leuven, Kardinaal Mercierlaan 92,
3001 Heverlee, Belgium. ines.vandenhouwe@biw.kuleuven.be

⁷Bioversity International, Commodities for Livelihoods Programme, Parc Scientifique Agropolis II,
34397 Montpellier Cedex 5, France. n.roux@cgiar.org

Reviewers

David Galsworthy
Central Science Laboratory
Sand Hutton
York
YO41 1LZ
United Kingdom

Florent Engelmann
Département environnement et
ressources (DER)
Institut de Recherche pour le
Développement (IRD)
911 avenue Agropolis, BP 64501
34394 Montpellier cedex 5
France

David Ellis
National Center for Genetic Resources
Preservation (NCGRP)
USDA
1111 South Mason Street
Fort Collins, Colorado
CO 80521-4500
USA

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Abbreviations and acronyms

AFLP	Amplified fragment length polymorphism
BP	Best practice
BSA	Bovine serum albumin
CABRI	(European) Common Access to Biological Resources and Information
CBD	Convention on Biological Diversity
CCP	Critical control point
CCTF	Clonal crop task force
CGIAR	Consultative Group on International Agricultural Research
CIAT	Centro Internacional de Agricultura Tropical
CIP	International Potato Centre
CRF	Controlled rate freezing
DMSO	Dimethyl sulphoxide
FAO	Food and Agriculture Organization of the United Nations
GMO	Genetically modified organism
GLP	Good laboratory practice
GPG2	Global Public Goods Phase 2
HEPA	High efficiency particle-removal air System
HSE	Health and Safety Executive (of the UK)
IARC(S)	International Agricultural Research Centre(s)
IBPGR	International Board for Plant Genetic Resources
IITA	International Institute of Tropical Agriculture
INIBAP	International Network for the Improvement of Banana and Plantain
IPGRI	International Plant Genetic Resources Institute
IPR	Intellectual property rights
ISBER	International Society for Biological and Environmental Repositories
ISO	International Standard Organization
ITC	(INIBAP) International Transit Centre
ITPGRFA	International Treaty of Plant Genetic Resources for Food and Agriculture
IVAG	<i>In vitro</i> active genebank
IVBG	<i>In vitro</i> base genebank
IVGB	<i>In vitro</i> genebank
KPIs	Key performance indicators
LN	Liquid nitrogen
LTS	Long-term storage
MC	Moisture content
MSDS	Material safety data sheet
MTA	Material transfer agreement
MTS	Medium-term storage
OECD	Organization for Economic Co-operation and Development
PVS	Plant vitrification solution
PVS2	Plant vitrification solution number 2
PVS3	Plant vitrification solution number 3

RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
SCV	Somaclonal variation
SGRP	System-wide Genetic Resources Programme
SINGER	System-wide Information Network for Genetic Resources
SMTA	Standard material transfer agreement
SOPs	Standard operating procedures
T _g	Glass transition temperature
UKNCC	UK National Culture Collections
UN	The United Nations
USDA	United States Department of Agriculture

Foreword

I feel honoured to be invited to introduce the publication “Refinement and standardization of storage procedures for clonal crops – Collective Action for the Rehabilitation of Global Public Goods Phase 2”. I am grateful to the authors of this publication, in particular to Nicolas Roux, coordinator of centres’ *in vitro* conservation specialist community.

The impact of the International Agricultural Research Centres’ work towards sustainable development largely depends on the centres’ genebanks, which hold the world’s most complete collections of plant diversity for food and agriculture. Four centres (Bioversity International, CIAT, CIP, and IITA) maintain over 28,000 *ex situ* accessions of bananas, plantains, cassava, potatoes, sweet potatoes, Andean roots and tubers and yams. From this total, 85% are also held as *in vitro* collections under slow growth conditions, and 10% of these have been placed under cryopreservation. The conservation of clonal material poses additional and unique challenges, especially when *in vitro* conservation methods are implemented.

Although the feasibility of using *in vitro* culture methods for plant genetic resources conservation was advocated in the mid to late 1970’s (e.g. by the late G Henshaw and his group), it was only in the 1980’s that the International Board for Plant Genetic Resources (IBPGR) established a working group of specialists, with the coordination of T Williams and L Withers, to look at critical aspects of *in vitro* plant conservation. As a follow-up, the IBPGR-CIAT project was implemented in 1987-89 to assess the technical and logistical aspects of establishing and running an *in vitro* active genebank using cassava as a model. In order to realize the potential of *in vitro* conservation at the CGIAR system and global levels, one lesson learned indicated that generic conservation quality standards should be developed. Early contributions towards these objectives included the IBPGR status report on *in vitro* conservation techniques by S Ashmore in 1997, and the technical guidelines for the management of field and *in vitro* collections by B Reed et al. in 2004.

A milestone of the centres’ long history of working together on genetic resources issues was the creation of the System-wide Genetic Resources Programme (SGRP) in 1994. Based on two external reviews, commissioned by the SGRP in 1995 and 1998, an investment plan was developed with World Bank funding; the plan comprised a two-phase programme. The programme’s first project “Global Public Goods Rehabilitation Project”, Phase 1 (GPG1) in 2003-06, raised the standards, and upgraded the operations of CGIAR genebanks. Centres holding clonal collections *in vitro* made substantial impact on accessions backlog processing, advanced the preparation of safety backups, and improved the health status of collections.

The second project, “Collective Action for the Rehabilitation of Global Public Goods”, Phase 2 (GPG2) aimed at enhancing the security and stewardship of the genetic resources held in trust in CGIAR genebanks. This project is the central topic of this publication which presents the outcomes, lessons learned, and points out key

challenges involved in furthering the GPG2 activity “Refinement and standardization of storage procedures for clonal crops”, sub-activity “Review of *in vitro* protocols applied to clonal crops”.

The GPG2 Project (2007-09) successfully promoted collective actions for the conservation of clonal genetic resources, specifically to increase their security, to use best practices across genebank processes needing validation, third party accreditation and risk management. In this context, the evolving role for germplasm curators was envisioned to satisfy stakeholders’ demands in meeting high standards in storage procedures (including *in vitro* slow growth and cryopreservation), to provide access to taxonomic and trait-related information, to develop modern genebank inventory systems for storage and delivery of accession data, and to develop high throughput screening techniques for new traits (such as abiotic stress, micronutrient and health-related phytochemical content).

In pursuing the collective actions for implementing system-wide priorities, attention should be placed on the use of best practices for raising the quality standards in the management for clonal collections, and on seeking qualification by International Standard Organization accreditation, e.g. the recent certification of CIPs’ genebank with ISO 17025. Maintenance of third party certifications will require continuous, rigorous controls, processes and validations within and between centres.

To successfully move ahead in implementing the GPG2 objectives, key challenges requiring collective attention still need to be tackled. These include:

- a) establishing practical risk-amelioration strategies for *in vitro* genebanks, especially in disaster-prone areas;
- b) developing simple, low cost, conservation protocols to expand the *in vitro* genebanks in developing countries;
- c) linking fundamental and applied research in *in vitro* conservation, for expanding the range of cryoresponse in the germplasm, for increasing the subculture interval of slow growth, and improving the efficiency of disease-indexing techniques at *in vitro* level, and
- d) developing high throughput screening techniques for relevant new traits, such as abiotic stress, micronutrient and health-related phytochemical content.

William Roca
Consultant
Genetic Resources and Biotechnology
Lima, Peru
(w.roca@cgiar.org)

Preface

These multi-crop, *in vitro* conservation guidelines have been produced as part of the CGIAR's System-wide Genetic Resources Programme (SGRP) project "Collective Action for the Rehabilitation of Global Public Goods Phase 2" (GPG2). The project's development goal is "*Crop genetic resources and associated biodiversity are put to use in developing countries to fight poverty, enhance food security and health and protect the environment*". The GPG2 Project will facilitate CGIAR's contribution to securing global plant genetic resources by upgrading genebank operations through the collective development of best practices. These are consolidated by adopting system-wide performance indicators, risk management procedures, protocol validation and quality assurance measures. The GPG2 collaborative activity 1.2: "*Refinement and standardization of storage procedures for clonal crops*" is mandated, through the CGIAR's *Clonal Crop Task Force*, to produce multi-crop guidelines for the medium and long-term storage of germplasm. The overarching aims of this activity are the collation of information to support the validation of best practices and the production of multi-crop best practice guidelines. Three outputs have been compiled to aid this process:

- **Part I.** Project landscape and general status of clonal crop *in vitro* conservation technologies;
- **Part II.** Status of *in vitro* conservation technologies for Andean root and tuber crops, cassava, *Musa*, potato, sweetpotato and yam;
- **Part III.** Multi-crop guidelines for developing *in vitro* conservation best practices for clonal crops.

This document comprises **Part III**, which has been constructed using information collated from: (1) a review of state-of-the-art slow growth and cryopreservation in the wider community of practice (**Part I**) and (2) a survey and workshop, which assessed CGIAR's *in vitro* genebanks' activities for their mandate clonal crops (**Part II**). Commonly held best practices and standards are core to high quality bioresources stewardship, these guidelines are therefore based on best practices from both plant (Genebank Standards 1994; Engels and Visser 2003; Reed et al. 2004) and other bioresource communities (OECD 2001, 2007; Coecke et al. 2005; ISBER 2005, 2008; NCI 2007; Benson 2008; Betsou 2008). The Office of Economic Co-operation and Development guidelines for biorepositories (OECD 2007) have been used as the main exemplar for their construction.

Summary

The Clonal Crop Task Force (CCTF) representing the CGIAR's *in vitro* genebanks, Bioversity, CIAT, CIP and IITA, hold in trust the genetic resources of *Musa*, cassava, potato, sweetpotato, yam and Andean root and tuber crops (ARTCs). These genebanks are cooperating in the GPG2 Activity 1.2: "*Refinement and standardization of storage procedures for clonal crops*". A survey of their conservation activities was conducted, followed by clarification of survey returns at a workshop, hosted by CIP in Lima, Peru in 2007. In addition, experiences gained from conserving potato, yam and cassava have been scrutinized with a view to using the lessons learned to help conserve yam, sweet potato and ARTCs. This has been undertaken by evaluating the survey returns and reviewing: (1) crop-specific technical guidelines of the CGIAR's *in vitro* genebanks and (2) the status of the wider *in vitro* conservation community of practice. The development of best practices and quality systems for safeguarding biological resources is advancing rapidly in many bioresources communities and this document is informed by their wider experiences. This collective knowledge provides the foundation for developing these multi-crop best practice guidelines. They are compiled in two parts. **Section I** comprises general operational guidelines for *in vitro* plant genebanks. **Section II** provides generic, multi-crop technical guidelines for the medium-term (slow growth) and long-term (cryopreservation) storage of crop germplasm held in *In vitro* active genebanks (IVAGs) and *In vitro* base genebanks (IVBGs) respectively. The format is structured to harmonize with GPG2's Knowledge base, best practices, risk management templates and performance indicators. The guidelines place emphasis on conserving clonal plant germplasm in infrastructures that are aspiring to develop quality management systems.

1. Introduction

Conserving clonal crops presents unique challenges not encountered in other types of genetic resources management, for which seed storage is often the preferred conservation method. This approach is not feasible for germplasm from clonal crops which are vegetatively propagated and/or do not produce seeds. Special genetic combinations must also be preserved through clonal means, as heterozygosity does not maintain their desired characteristics. Clonal plant genetic resources can be preserved in field genebanks and as dormant vegetative (e.g. tubers, buds) propagules conserved in cold stores. However, these approaches are limited by efficiency, security and the costs of long-term maintenance. *In vitro* conservation is a complementary strategy that has the advantage of safe germplasm transfer under contained (i.e. *in vitro*) phytosanitary control. Medium-term storage (MTS) extends subculture intervals and reduces processing costs by arresting growth. Long-term storage (LTS) is achieved by cryopreservation and theoretically it secures viable germplasm longevity for centuries.

The realization of best practices and external accreditation requires considerable time and resources and it is more practical to aspire to their development whilst capacity building progresses. Within this context, the generic guidelines in Section I may be best utilized as a road map to facilitate stepwise progress that are in line with the goals of high standard stewardship. In working towards best practices, some cautionary points need consideration (Genebank Standards 1994; OECD 2007). These include fixing best practices in a point in time, and the inability of some institutions to attain and maintain them. Therefore, developing best practices and quality systems should be considered evolutionary processes that keep in step with future technological advancements and the needs and capabilities of end users. Where constraints occur, institutions may need to moderate the development of their best practices to make them amenable to local circumstances. It is hoped this approach will support best practices development in the wider global plant conservation community.

1.1 Aims, scope and structure

The aim of these guidelines is to enable the continued development of quality services in the CGIAR's clonal crop IVGBs. They have been constructed in accordance with the fundamental principles and responsibilities common to all biorepositories, which are:

- **Purity:** freedom from contaminating organisms
- **Authenticity:** correct identity
- **Stability:** fit-for-purpose and trueness-to-type.

These guidelines are modelled on existing plant conservation guides (Genebank Standards 1994; Engels and Visser 2003; Reed et al. 2004). However, they have been extended to incorporate generic best practices from other bioresources communities (i.e. sectors dependent upon the safe conservation of biological resources) that have

already developed quality systems (Stacey and Doyle 1998, Stacey et al. 1999; OECD 2001, 2007; Coecke et al. 2005; ISBER 2005, 2008; Stacey 2004; Stacey and Day 2007). The scope of this document constitutes generic principles applicable to multiple crops and it is constructed within the remit of the GPG2 Project's Knowledge base and Risk management activities. The technical scope of these guidelines specifically concerns *in vitro* storage, albeit for continuity associated practices are also considered. More detailed coverage of phytosanitary regulations, registration and authentication are addressed by other activities of the GPG2 Project. These guidelines comprise two parts: **Section I** offers general operational guidelines for *in vitro* genebanks; **Section II** provides generic, multi-crop technical guidelines for medium-term (slow growth) and long-term (cryopreservation) storage. Crop-specific protocols and technical guidelines of CGIAR's clonal genebanks have already been compiled (IPGRI-CIAT 1994; Panis and Thinh 2001; Panis et al. 2005; Panta et al. 2007; IITA 2007a, b; Mafla et al. 2007; Panis 2009) or, are in progress.

1.2 Quality systems for clonal crop *in vitro* genebanks

The *In vitro* genebank (IVGB) as defined by Genebank Standards (1994) comprises two components: (1) the *In vitro* base genebank (IVBG) and (2) the *In vitro* active genebank (IVAG). The attributes of the IVBG are germplasm is preserved for the long-term and not normally distributed directly to users. Ideally, a base collection represents a comprehensive genepool of the crop or species and because of the longevity of viable germplasm held in cryobanks at liquid nitrogen (LN) temperatures (to -196°C) they are usually designated IVBGs. The IVAG holds accessions that are immediately available for multiplication and distribution and the cyclic flow of material is their key feature. The IVAG operates by successive subculturing and tissue cultures are maintained in conditions that retard their growth. This increases the efficient use of resources and staff time, and reduces the likely hood of contamination and operator errors. Developing best practices for protecting clonal food crops is an ongoing remit for CGIAR's genebanks responsible for conserving potato, sweetpotato, *Musa*, yam, cassava and ARTCs. Their activities include the collective development of protocols and best practices and their validation as well as risk management for the safe conservation of germplasm held in trust. High standards of stewardship require compliance with overarching regulations regarding germplasm acquisitions, material transfers, ownership and phytosanitary legislation. These are drivers for developing quality management systems and they are underpinned by common best practices that may be modified to meet local conditions and needs.

SECTION I. GENERAL BEST PRACTICE GUIDELINES FOR CLONAL CROP *IN VITRO* GENEBANKS

These general guidelines underpin technical best practices and standards and they are core to high quality genetic resources stewardship. Cross-cutting operations are common to all biorepositories and genebanks and they provide the operational infrastructure for the multi-crop technical guidelines presented in **Section II**.

1. Organizational and quality frameworks

In vitro crop plant genebanks should operate in compliance with institutional national and international regulations and provide sustainable infrastructures and/or alternative measures to ensure the security of their germplasm holdings and their safe transfer to other repositories, researchers, end users and beneficiaries. Information sources on national and international regulations are provided in the 'Useful websites' section of this document. If possible, the genebank's overarching organization should aspire towards operating within a formalized quality framework. A glossary of terms related to quality systems and best practices is provided in the 'Technology terminology' section of this document.

1.1 Management and organizational requirements

Management responsibilities and operations include delegation and overseeing of administrative, technical, regulatory, safety, quality and training activities, as appropriate to qualified and new staff, visitors, students, end users and beneficiaries. The IVGB Director is required to formulate an organizational chart that clearly identifies and delineates the different functional relationships of the IVGB, and how they interact with respect to responsibilities and processes. The Director, together with appropriate staff oversees personnel supervision and training, approves and maintains job descriptions and ensures the capacities and capabilities of staff to undertake genebank operations. Clear and explicit procedures should be in place for documenting and archiving staff lists, duties and protocols, including inventories of biological holdings, associated passport and regulatory information. Senior Management has responsibility for implementing, as appropriate, accreditation standards and support efficient bilateral communications across all levels to ensure system effectiveness. This is usually undertaken by leadership team meetings, group meetings and team and sectional meetings.

1.1.1 Technical management

A *Technical Manager* is responsible for overseeing the management of practical and technical aspects of individual work areas in the IVGB and works in liaison with the *Quality Manager*. Duties of the Technical Manager are the implementation,

maintenance and improvement of the technical management system. Responsibilities may include, providing resources to support quality analyses within genebanks that are aspiring to external accreditation, Technical Managers are assisted by locally identified *Accreditation Coordinators*, who have the responsibility of the day-to-day maintenance of quality, and accreditation procedures (see *section 7*). Technical management includes the supervision and training of personnel and trainees by staff that are fully trained and authorized to undertake protocols and procedures and to assess test results (see *sections 1.3 and 1.4*). As necessary, Technical Managers should have the support, authority and resources to satisfy external third party accreditation standards. They should be empowered to identify departures from their management system or testing procedures, and put in place corrective and preventative actions to address them. Staff should be appointed to deputize for absent Technical Managers.

1.1.2 Quality management

A Quality Manager should normally be appointed to ensure that the IVGB complies with appropriate quality standards (see *section 7*) and provide guidance and information to staff concerning the maintenance and improvement of standards. A Deputy Quality Manager should be appointed for absentee coverage. In the case of organizations aspiring to, or maintaining third party external accreditation, an overall responsibility of the Quality Manager is to ensure that the requirements of the external body are met (e.g. for ISO certification). The Quality Manager heads the *Quality Systems Team* and together they are responsible for implementing and maintaining the Quality Management System. This includes actions at the technical level and in the administration of quality control procedures and internal audits (see *section 7*). The Quality Manager and Quality Systems Team liaises with the external accreditation body, carries out performance and proficiency testing, responds to follow-ups and complaints and corrects non-conforming work (see *section 1.2.1.1*). In addition, the Quality Manager and Quality Team ensure technical managers (see *section 1.1.1*) and their personnel are informed of changes to the accreditation standard and the policies and procedures of the accreditation body, including updates to its publications. Further responsibilities are document control, the production and administration of quality handbooks and technical guides and archiving copies of amended manuals (see *section 1.3*). The Quality Manager and Quality Team are tasked to ensure that IVGB personnel are aware of the importance and relevance of their activities, and how they contribute to the overall management system.

1.2 *In vitro* genebank quality management systems

The purpose of a quality system is to ensure IVGBs are operating to the highest standards in compliance with their regulatory, safety and service obligations. An overarching Quality Assurance Programme (see *section 7*) or *Quality Management System* (termed 'QMS') ensures that biorepositories operate in full accordance with their best practices. The Quality Manager (see *section 1.1*) represents the IVGB on quality matters with partner institutes, external contractors and suppliers, auditors,

regulatory bodies and end users, in compliance with risk management and regulatory obligations. Processes in a QMS should provide the opportunity to improve the system and support actions to prevent and correct non-conforming work (see *section 1.2.1.1*) or deviations and departures from technical procedures and protocols.

1.2.1 Quality manuals

Policies and procedures of a QMS should be defined in a *Quality manual*, which provides the overarching objective of the quality policy statement. The Quality manual describes the roles of personnel and their responsibilities and the genebank's facilities and infrastructures. The range and scope of operations should be defined within the context of the genebank's quality policy. The manual should include the protocols, Standard Operating Procedures (SOPs) and methods that need to be in place to ensure that quality policy objectives are satisfied. Personnel should understand and be able to comply with the Quality manual, which should be assessed annually in a management review. Any changes made to the Quality manual should be undertaken in a controlled manner (see *section 1.3.1*). The review and update process should involve regular meetings with all staff concerned and take into account statutory and regulatory requirements. The Quality manual should be maintained as an electronic document and published as a workbench hard copy that is available to all staff.

1.2.1.1 Quality manuals and non-conforming work

Personnel should conform to the procedures in the quality manual and its associated documentation and report to the Quality Manager and Quality Team any issue or factor that influences their ability and capacity to comply. In cases where staff have not conformed to documented procedures and to work accredited by an external third party, the Technical and Quality Managers and Quality Team are to be informed immediately and the significance of the non-conforming work assessed. Such an occurrence is recorded as non-conforming work and the Quality Manager and Quality Team should take corrective action in a timely manner. This includes analysis allowing the root cause to be identified and rectified; corrective actions are monitored for their efficacy. Should non-conforming work cast doubt on work and procedures carried out, the Quality Manager should ensure that an audit is carried out to confirm the efficacy of corrective actions. In association with the Quality Manager, the Technical Manager should be responsible for authorizing the continuation of work. Preventative actions should be put in place to avert the risk of future non-conforming work and to improve the quality of the system and technical processes.

1.3 Documentation and records

Documents provide, publish and disseminate factual and material information. *Records* are registered information, or pieces of recorded evidence that are permanent, traceable points of reference. Records are systematically archived and accessed for regulatory and institutional inspection, and as such, they have an important role in quality management systems.

1.3.1 Controlled and uncontrolled documents

Controlled documents are verified for accuracy by internal and/or external quality audits. *Uncontrolled documents* have not been through this process and they may be designated as drafts under revision, or non-verified, un-validated documents. A full list of controlled information required for germplasm processing, starting from registration and progressing to storage and safe transfer should be available in IVGBs. Alterations to best practices and core documents should be indelible, explained, dated, and signed by the operator, supervisor and endorsed by the Quality Manager. Copies of quality manuals, best practices, and technical handbooks should be clearly marked as '*controlled*' and designated with a version number, issue date and authorization name. If controlled documents are usually maintained electronically, printed copies should be marked as uncontrolled; controlled documents are available at point of use.

1.3.1.1 Document verification and access

Procedures for verification of general/passport documentation and accompanying germplasm registration should be in place, including confirmation of safety status for quarantine and phytosanitary regulations, special and regulatory documents and other Intellectual Property (IP) restrictions such as (Standard) Material Transfer Agreements ([S]MTAs). Lists of technical documents, safety and operational manuals provided by equipment manufacturers' and chemical suppliers' Material Safety Data Sheets (MSDS) should be recorded and their access points made known to the personnel using them. Staff, visitors and students should adhere to internal documentation. Departures from SOPs and best practices should be agreed upon and confirmed with written justification by the Quality Manager.

1.3.1.2 Documentation errors

There are two basic types of errors: (1) where work has not been carried out in accordance with documented procedures and (2) where documentation of a procedure is incorrect, for both cases corrective action is required. Where mistakes and deviations detected in documents are due to inaccuracies in language translation, misinterpretation or misdirection, the error should be identified, rectified and, if required personnel re-training implemented. A Quality Manager should ensure that documents are in order, updated, amended and changes properly documented with the agreement of personnel, supervisors and Technical Managers.

1.3.2 Records

The records chain extends from germplasm acquisition, registration to storage and dispatch, inclusive of supporting practices such as phytosanitary control. The Quality Manager should be responsible for ensuring records are in place for records retention, archiving, security, confidentiality and destruction. Records should be accessible for inspection by authorized personnel as required for quality, regulatory, health and safety audits and inspections.

1.4 Regulatory issues

In addition to institutional, national and international regulations, these include observance of the Convention on Biological Diversity, the Standard material transfer agreement (SMTA), regarding plant genetic resources exchange, the International Plant Protection Convention and regulations concerning biosafety, phytosanitary control and quarantine. Genebank and biorepository personnel should be familiar with and have access to the regulatory guidelines and laws that are relevant to their work (for examples see Stacey and Doyle 1998; Stacey 2004).

1.5 Training and personnel competencies

Proper training underpins quality systems and it is essential for implementing best practices; in some countries, it can be mandatory for safety regulations. Procedures and personnel should be in place for coordinating, supervising and delivering training, this is particularly important as IVGBs are becoming increasingly multidisciplinary and depend on different types and levels of expertise. Personnel should only be allocated to, and held responsible for work for which they have been appropriately trained. Each staff member should be trained according to documented job descriptions and their development needs reviewed regularly and where appropriate training should be linked to specific objectives and measures of competence. Trained staff should also undertake the training and assessment process.

1.5.1 Training programmes

Staff should be fully informed of and trained in the quality systems and best practices used in their IVGB and are aware of the principles of quality issues and their role and importance in delivering quality services. It is the responsibility of all staff to familiarize themselves with best practice guidelines, documented protocols and the policies and procedures appropriate for their work. It is management's responsibility to ensure staff, visitors and students are provided with appropriate quality documents. A four stage training procedure is recommended:

1. Read documentation.
2. Witness of operation by trained staff.
3. Carrying out of an operation under supervision.
4. Undergo periodic assessment and produce objective evidence of competence.

Periodic re-evaluations, updates and audits of personnel can also be included in training programmes; mentoring is advisable to ensure personnel understand and are able to comply with their Quality System obligations. Staff training should be a formalized procedure, ideally reviewed on a regular basis in line with personal career development. Personnel should be supported by training when new procedures, protocols, instruments and equipment are introduced, until they demonstrate confidence and competency. Authorization to use specialist and high-risk equipment should be provided after appropriate training in their safe use by a suitably

experienced and qualified user or instructor. This may include staff training by the instrument/product manufacturer at the time of installation. Training can also be provided by formal courses, workshops and through academic qualifications.

1.6 Technology transfer

Formalized procedures for technology transfers should be coordinated with current best practices and quality systems. These can entail: (1) documented monitoring of technology transfer; (2) evidence based consensus as to whether the technology will, or will not be adopted; (3) impact assessment of the technology on current and projected operations; (4) risk and safety assessments; (5) cost/benefit and efficiency analyses and (6) staff training in new technologies. Distinctions between research development, technology transfer, internal verification, validation and best practices should be made clear to personnel. Technology transfers may be implemented via workshops, research collaboration, technical visits, Web-based posting of methods, SOPs and training attachments; these usually involve non-validated skills training. Critical point assessment of technology transfers help to identify problems and they are recommended before implementing a validation exercise and, on its completion. In this context, a critical point is a factor or issue that can affect the successful outcome of a procedure or protocol.

1.7 Validation

A formally validated *in vitro* conservation protocol should be sufficiently robust to translate into a best practice that can be used by different genebanks, over a reasonable period, by different staff. It can involve:

1. *Reproducibility testing*: comparing results for a protocol replicated several times, ideally with several genotypes if possible.
2. *Reliability testing*: comparing several methods which have the same application.
3. *Blind testing*: to prevent outcomes being influenced by laboratory/genebank bias.

1.7.1 The validation process

Validation is the process by which a protocol or method is ensured to be fit for purpose. Optimally, external validation is calibrated and corroborated by validators with different levels of expertise across different institutions. This approach tests the quality of supporting information, guidance and experimental designs and helps to ensure a robust best practice is the outcome.

1.7.1.1 Validating storage protocols

Storage protocol validation typically involves reaching consensus on a common experimental design which is applied within the same time frame across several institutes, using an identical test culture from a sole source. The first stage requires detailed consensus planning as to how the validation will be undertaken. Lead in time

is necessary to dispatch test cultures to different validators, on their receipt, the cultures should be checked for morphogenetic competency and health. Test cultures should be cultivated using the same growth media across participating laboratories and cross-checks are advised so that cultures continue to grow within expected performance criteria. It is preferable that test cultures have a proven amenability to the protocol being used. This should have been developed, tested and verified by at least one participating partner who usually takes the lead.

1.7.1.2 Quality assured validation

Identical handbooks and clear, unambiguous, complete technical instructions should be provided to all validators and scrutinized for accuracy, understanding and language translation. Precise details of solution preparation, calculations and technical detail should be checked, preferably by more than one person (see *section 2.4.6*). Adjunct training, workshops and phone/video-conferences are recommended, especially if validation is concomitant with technology transfer to personnel unfamiliar with the procedures.

2. Infrastructures and technical operations

Genebank infrastructures should include any special design elements necessary for the security and safety of germplasm holdings and the personnel charged with their stewardship. The facility should adhere to obligations as required by law and/or other regulatory bodies and the operating environment and equipment should conform to relevant national and international standards and safety regulations.

2.1 Institutional facilities

These comprise basic facilities (lighting, heating and air conditioning) and essential services (water, power, emergency), communications, computers, Information Technology (IT) support and any special requirement for bioresources conservation and management.

2.1.1 Environmental services

Environmental control of ambient temperature and humidity in laboratories, operational areas and computer systems is a quality assurance factor. Critical environmental limits should be identified to ensure the appropriate control of temperature, humidity and light in growth rooms. Adequate air conditioning is advisable to prevent excessive loads on computers, refrigerators, freezers, incubators and cold rooms, for which regular maintenance is important to extend equipment lifespan and offset breakdowns. It is important to replace equipment prior to its failure, therefore upgrading older equipment and keeping the older equipment as a back-up is critical. Operating conditions for optimal use and lifetime of mechanical refrigerators, freezers and temperature-controlled equipment should be in accordance with manufacturers' recommendations. Regular defrosting and cleaning should be undertaken in accordance with manufacturers' recommendations. Service ventilation

systems should conform to health and safety requirements for the use of solvents, irritants, LN and other harmful substances. Lighting should be adequate for safe working, particularly where samples are stored; care is required for protecting light sensitive materials and germplasm. *Appendix II* provides examples of equipment calibration and maintenance for routine technical and safety operations within IVGBs.

2.1.2 Work place design

Laboratories and work areas should be conducive to high standards of cleanliness, optimally doors and windows ought to be close-fitting, and wall and floor surfaces sealed with even and smooth curved wall-to-floor junctions. Bench surfaces should be made of fabrics that provide smooth, contained surfaces to facilitate cleaning. Physical infrastructures should be designed to aid secure germplasm movement through a self-contained, process chain. For example: (1) receipt/registration and storage of incoming germplasm accessions; (2) phytosanitary control; (3) preparation, regeneration and processing; (4) *in vitro* and cryogenic storage; (5) duplication/backups; (6) germplasm recovery; (7) germplasm transfers/dispatch and (8) decontamination, cleaning and waste processing.

2.1.2.1 Logistics

Operations and workspace design should be planned so that germplasm and materials do not become contaminated, lost or misplaced. Logistically this may be achieved by the physical delineation of clean and dirty areas and offsetting the timing of 'dirty', clean-up and sterile manipulations by having an area designated for the quarantine of new cultures. Using the laboratory on the "no way back" principle ensures that samples progress one-way through increasing levels of cleanliness and security.

2.2 Computer and IT support

In addition to usual computer usage, computers, electronic data management and storage inventories are integrated with modern analytical equipment and digital barcoding systems. Therefore, robust and efficient IT support should be provided, inclusive of a safe, anti-surge power supply and provisions should be made for the regular back-up of data and IT support. For specialist equipment (e.g. LN auto-supply tanks) contingencies should be made in the event of computer and power failure.

2.3 General laboratory services

Full and proper instructions explaining how general laboratory equipment is safely used should be written down and manufacturers' handbooks annexed as required. Equipment usage, calibration, testing and maintenance should confirm fitness for purpose and servicing should be undertaken proficiently and regularly documented by a Laboratory or Facility Manager. Qualified personnel and/or external maintenance engineers, (i.e. endorsed by equipment providers) should carry out servicing and the calibration of general equipment (see *Appendix II*). These activities

should be in the care of the Quality and Technical Managers and records made available for internal/external auditors.

2.3.1 Water quality

Fit-for-purpose high quality water should be continuously available and stored as required. Water processing systems should be regularly cleaned and serviced and the microbial, chemical and physical quality of water routinely checked.

2.4 Basic *in vitro* genebank operations

These include facilities and operations dedicated to the routine culture and *in vitro* maintenance of crop germplasm. Actions involve the regular servicing, maintenance, cleaning and calibration of basic laboratory equipment: e.g. autoclaves, pH meters, light meters, balances, stirrers, water baths, automatic pipettes, microscopes, centrifuges, laminar airflows (e.g. flow checks and filter changes) should be scheduled by rota for competent staff and verified by a check-list (see *Appendix II*).

2.4.1 Laboratory cleanliness

Staff should be trained in aseptic technique and culture facility hygiene should be observed by regular cleaning. Stringent measures are necessary to ensure asepsis control and the avoidance of procedures that might potentially introduce pathogens, nuisance microflora, adventitious organisms, pests and mites into culture areas. Ideally, different washing-up areas are delineated into clean and dirty zones equipped with sufficient sinks and space for automatic and manual washing; provisions should be made for special washing treatments and rinses. An area holding dedicated disposal containers is designated for safe recycling and/or disposal of organic and inorganic chemicals, solvents, spent media, plant wastes, broken glass, sharps and paper. Strict containment should be maintained throughout all culture operations, and as required, in positive pressure, self-contained, limited access clean rooms. Plant culture rooms, growth chambers and cabinets should be under regular, checklist surveillance and made secure from contamination risks by routine, regular cleaning.

2.4.2 Sterilization

Staff should be trained and competent in the safe use of autoclaves and filter-sterilizing units and they should understand why and how the different types of sterilization procedures are applied. Autoclaves and sterilizing equipment should be routinely serviced and checked for function and safety. In some countries it is a statutory health and safety obligation for external (third party) checks to be undertaken on autoclaves. This procedure may also include the certification of the audit and the withdrawal of autoclaves from use if they do not comply with safety standards.

2.4.3 Tissue culture use

Procedures should be in place explaining how different types of cultures are acquired, used and by whom. Process and supply chains should be monitored for their intended purpose in: (1) slow growth and cryogenic storage; (2) research; (3) phytosanitary and

molecular diagnostics; (4) for training and technology transfer and (5) black box duplication. Good practices should be instigated for culling and disposing of cultures when they are no longer needed. For example: (1) deemed unsuitable for intended purpose; (2) withdrawal from storage collections; (3) redundancy on completion of research projects and training programmes. Evidence of removal and safe disposal should be clearly documented.

2.4.4 Laboratory procedures

Procedures should contain full instructions, including basic preparatory steps that explain how reagents, solutions and culture media are made, these should provide details of formulae, dispensing weights and volumes. Calculations and formulae should be meticulously verified by more than one operator and ideally witnessed for accuracy before being incorporated into controlled, best practices, technical guidelines and laboratory handbooks. Such documents may need to be available for internal and external quality auditors (see *sections 1.2 and 7*).

2.4.5 Media and reagents

The accurate preparation, labelling and proper storage of culture media and analytical reagents are key issues in quality control. The IVGB should have well defined standards, documents and procedures explaining how to prepare and store chemicals, reagents and materials used in tissue culture, storage and associated analytical and molecular procedures. Reagents and chemicals should be sourced from a reliable supplier and their grade and purity (including water quality) should be appropriate to application. Inventories of chemicals, reagents and their suppliers, including details of shelf life, expiry dates, minimum safe storage temperatures and special requirements (e.g. light sensitivity) should be compiled and collated by the Laboratory Manager or Technical Manager, with the aid of supplier MSDS and manufacturers' product information. Creating and updating chemical and reagent inventories are components of risk management best practice (see *section 4*).

2.4.6 Explicit technical details

Explicit technical guidance (e.g. in SOPs) should be provided explaining how to prepare solutions using different types of formulation e.g. percentage, Molar, parts per million (ppm) and with clarification explaining how solutions are made up on a % weight/volume (w/v) or % volume/volume (v/v) basis. This process should take into account volumetric dispensing adjustments for specific gravity and the accurate gravimetric dispensing of viscous liquids (e.g. for cryoprotective additives). Preparation of analytical reagents and culture media should include details that describe how to precision-measure weights and volumes, prepare buffers, make pH adjustment and use solvents, acids and bases for solubilising additives. Clear instructions should be provided regarding the appropriate choice of sterilizing procedure (e.g. filter or autoclave) and the type of storage (e.g. refrigerated, dark).

2.4.7 Media and reagent stock control

Media batches and working solutions should be made using standard documents and templates. Culture media, analytical reagents and solutions can be formulated from constituent chemicals, pre-prepared packs or stock solutions and these should be double-checked for shelf-life expiry, diluent concentration and numerical accuracy. Final solutions should be dispensed into clearly labelled, storage vessels, dated for expiry and with the name/operator coded. Stock-controls and storage inventories should be carefully documented; ideally solutions/media should be labelled in a bar-coded inventory system. Traceability of records should be assured for the entire process, from basic reagents and stock solutions, to culture media preparation, sterilization and batch storage. For example, a binder of every sheet used to make media should be kept so that it is possible to refer back to records if issues arise and each item should be checked off as it is added during media/solution preparation. Exact comments as to how calculations are used to make up solutions and stocks should be included (see *section 2.4.6*).

2.5 General *in vitro* facilities

Physical infrastructures, housing and general storage facilities should be safe, secure and environmentally controlled. Documented procedures for surveillance, alarms, regular inspections and maintenance (see *Appendix II*) should be in place and vigilance checks undertaken for contamination/pest infestations. Mechanical freezers, refrigerators, incubators and walk-in environmental cold rooms holding critically important materials should ideally operate with dual or back-up compressors. Calibrated temperature and light regimes should comply with storage requirements and range settings should take into account normal (acceptable) operating variations and max-min differentials. When thermal probes are used for calibration and surveillance they should be positioned at different locations to identify hot/cold spots and condensation points and actions should be taken to reduce their effects. A temperature profile should be constructed for environmentally controlled facilities prior to using and after servicing in order to identify areas that are not conforming to requirements. Optimally, continuous monitoring should be undertaken using an environmental monitor-data logger, noting that capacity loading in static mechanical refrigerators and freezers can change thermal characteristics compared to empty chambers. Provisions should be made for containment when growth rooms/cold storage facilities require defrosting, servicing, maintenance or for breakdown or emergency contingencies (see *section 2.6.1*).

2.6 *In vitro* genebank equipment

Manipulations should be undertaken in regularly serviced, routinely checked laminar airflow cabinets with specification grade-clean filters. Environmental support systems, air conditioning units and positive pressure rooms should be checked for optimal performance.

2.6.1 Servicing and breakdowns

Specialist analytical, instrumentation, diagnostic and molecular biology equipment should be regularly calibrated and routinely checked by appropriately trained staff (see *Appendix II*). Ideally, spare and replacement parts should be held in store to ensure continuity of operations. Qualified maintenance engineers should service culture rooms, incubators and growth chambers regularly. Service records should be maintained and equipment failure incidents and correction procedures recorded methodically. The personnel that operate equipment should be kept informed about its status by means of a user log to verify functionality and personnel should be notified of outcomes of emergency interventions before planning to use equipment. In some countries, certain materials and equipment (e.g. pressurized cryogenic gases, radiation, and autoclaves) are regulated and they require mandatory safety inspections (see *Appendix II*). Procedures should be in place to ensure these comply with manufacturers' health and safety specifications, the Quality and Technical Managers and Safety Officer should cooperate in undertaking these measures.

2.6.1.1 Replacement of essential equipment

A policy should be in place for the repair and replacement of equipment, working on the principles of preventative maintenance, availability of replacement parts, depreciation, and using optimal environmental conditions in the area in which the equipment is used and or/stored. Documented long-term replacement plans should ideally be developed that account for expected, safe lifetimes of essential equipment, based on manufacturers' recommendations and usage and replacement needs.

2.7 Suppliers and contracted services

Suppliers of chemicals, equipment and materials should be reputable, reliable and providers of proven quality products, ideally contracted services should be quality assured by accredited certification. Copies of purchase orders, maintenance and servicing contracts should be held on file and documented as part of the quality audit. Suppliers and external contracted services should provide support in full compliance with the IVGB's safety regulations, best practices and quality assurance systems.

3. Inventories, documentation and knowledge management

Well-documented procedures are core to quality assured management, inclusive of both hard copy and electronic information. Templates should be used for record management and documentation should be securely stored and backed up as authenticated and validated information and data (see *section 1.2*). Standard terminology and formats should be used for germplasm registration data, general data management and exchange of best practice protocols and procedures. Where possible, common standards should be in place for data transmission and sharing across federated networks at the local, domain, regional and global levels. This should take into account existing data processing and inventory systems and the need to review regularly IT services in line with new developments. Regular updating and archiving of information is part of a robust knowledge and information management system.

3.1 Inventory procedures

Depositors and registrars should ensure IVGB inventory systems and procedures comply with best practices and that quality assured information is associated with the biological material entering the genebank for registration. This should be: (a) verified and (b) accompanied by all the required regulatory and legal documents.

3.1.1 Data entry, labelling and barcoding

Manual, hard copy labelling should be avoided, when necessary it should be accurate, legible and witnessed as authentic to ensure correct transfers of label information, this is especially important when several operators are involved in the process over extended periods. Electronic barcoding systems are not infallible and measures should be taken to ensure the accurate input of electronic data, such as duplicate readings of barcodes prior to data being uploaded.

3.1.2 Data quality control

Control measures should ensure identification and correction of errors accumulated in the electronic inventory over time. Optimally, each record should comprise a minimum data set, a recommended data set and a full data set with language-specific spell checks for every field built-in to avoid cumulative errors. A standard approach should be applied for scientific symbols, abbreviations, and alternatives to symbols that cannot be entered into electronic inventories (e.g. ° for temperature, Greek letters, subscripts/superscripts). Unfamiliar data entries should be cross-checked for ontology and scientific names by using authorized lists to prevent errors and the miss-typing of complex or unfamiliar names and nomenclature. A procedure to detect manual, hand written and electronic data entries for transcription errors is critical to quality information management. Data quality and consistency of data handling procedures should be documented as a best practice and appropriately described e.g. as archived, currently existing and newly entered data (see *section 1.2*).

3.2 Sample traceability

The genebank inventory should be sufficiently robust to allow trouble-free sample location and traceability of germplasm accessions held in store, together with their accompanying passport, regulatory and crop-specific information. It should be possible to track germplasm with precision and efficiency as it passes through different IVGB processes and phytosanitary treatments.

3.3 Database content and access

Inventory and informatics systems should be constructed in a standard format enabling linkages across appropriate facilities within the local IVGB and as required in federated genetic resources networks. Ideally, databases should contain all the relevant information for germplasm requisitions and depositors and inform on germplasm holdings status, including identification of duplicated/redundant accessions and/or inventories. Data should remain active so long as the germplasm remains viable, loss or purposeful removal of an accession should be recorded and documented (date, person responsible and reason) and the entry removed, indicating that it is no longer available; this information may also be archived or placed in reserve for future reference.

3.4 Safeguarding data

Informatics and IT staff should cooperate with IVGB personnel to ensure regular backups and off-site duplicate data storage for high-risk institutions. Back-up files and electronic storage devices should be stored in secure, locked and fireproof cabinets. Data archives, including procedures and manuals should be reviewed for redundancy, obsolescence, omissions and information loss. Documented standards and quality systems should be used to ensure data systems operate to best practices. Information should only be accessed by authentic users by means of ID and passwords and when necessary confidential data or information should be protected using encryption and restricted IP addresses.

3.5 Accessibility and publication

As appropriate, the IVBG makes data available externally regarding their biological holdings, in accordance with organizational principles and regulations. When required, users of restricted or confidential data should be authenticated and passwords provided to allow access to different domains, validity of identifiers and passwords should be checked. Publication of data and information, including on websites should comply with international copyright laws, regulations and be appropriately accompanied by authorship credits, acknowledgements, disclaimers and copyright permissions. Updating websites regularly includes auditing the material on a yearly basis depending on the type of material [protocols (less frequent) or data on accessions (more frequent)].

4. Safety, risk management and biosecurity

All IVGB personnel should be aware of their own responsibility towards genebank safety, the importance of audited risk management and its regulation. In some countries it is a statutory requirement for institutions to have robust risk management and safety procedures that are subjected to internal and external audits. Regulations governing safety are under the jurisdiction of diverse institutional, regional, national and international regulatory controls. Management, administration and the IVGB Safety Officer should be cognizant of all potential threats and hazards to personnel, germplasm holdings and operations. These can be broadly categorized into chemical electrical, biological, physical, radiological and fire. Non-adherence to regulations is also a risk. Protective facilities (airflow benches, containment cabinets) should be used for hazardous substances and organisms and cleaned appropriately after use to avoid subsequent contamination. Equipment and operations should be formally assessed and audited for risk and risk mitigation. Personal protective clothing and equipment (PPC and PPE), safety alarms and healthcare measures (e.g. vaccinations, oxygen monitors) should be supplied to personnel as appropriate.

4.1 Risk management infrastructure

Usually the Director or other designated senior member of staff holds primary responsibility for safety and risk management in an institution; however, staff are also responsible for the safety of operations in which they are involved. It is usual to appoint a *Safety Committee* which assigns a *Safety Officer* to administer and oversee risk management and safety programmes. The Safety Officer, in cooperation with the Safety Committee establishes risk management plans, runs safety training, monitors and maintains adherence to procedures, coordinates and delivers regular and spot-check monitoring (including checking safety equipment (first-aid kits, bioshowers, eye wash, fire extinguishers, etc.)). This committee also acts on, evaluates and reports incidents and injuries and regularly reviews, updates and recommends changes to risk management procedures. Ideally, updates should be undertaken no less than annually as well as in response to new information and incidents. Safety Officers are usually the main point of contact with external health and safety bodies and as such they should work closely with supervisors to ensure regulatory compliancy. The Safety Officer should be responsible for the safekeeping, updating and archiving of risk management documentation and literature, ensuring its accessibility to personnel. Data and information associated with safety, regulations and phytosanitary control should be retained for traceability, auditing and proof of compliancy with regulatory bodies and legal authorities.

4.1.2 Safety training

All personnel and visitors participating in practical operations should, as appropriate, undertake training in safety, risk assessment and mitigation. Including instruction on how to conduct their own risk management exercises using metrics and risk

assessment forms. Individuals undertaking specific tasks in their job description and related operations should be trained in identifying and mitigating against risks and the hazards involved. Correct completion of personnel safety assessments should be signed off by supervisors and the Safety Officer, who should retain a copy for endorsement by the Safety Committee. Staff should retain copies of risk assessments so that information is nearby for reference purposes. Regular training updates in risk management should be instigated for personnel.

4.2 External security

Genebanks should be made physically secure from break-ins and malicious intruders and ideally have an access code and 24 h security system in place for monitoring (e.g. security cameras) and limiting admittance to appropriate staff and visitors and protecting against intrusion from unauthorized persons. Doors, access points, locks/keys should be regulated and as required alarmed. An access policy should be in place for visitors, this might include sign-in sheets, logbooks and badges, and only authorized persons should be assigned to the genebanks and permitted rights of entry. Freezers, cryobanks, growth rooms and storage areas should be secured, if appropriate, in locked or restricted areas, taking into account the need to clearly mark and maintain emergency exits.

4.3 Physical security

The stringency of physical security within an IVGB will depend upon its associated activities and requirement for phytosanitary containment. Higher levels of security may be necessary for institutes undertaking genetic modification projects, for example, when using recombinant DNA technologies the level of biosecurity risk management may be expected to be higher. For these cases containment may be governed by statutory, national and international obligations for the management of genetically modified organisms (see *section 4.2*). Stringent measures may also be required for the containment of notifiable pathogens, in which case delineation into different areas of containment may be a mandatory obligation. Containment can range from *Level 1*, general security (negligible to low risk); *Level 2*, restricted areas (moderate risk) and *Level 3*, high security (high risk). The first demarks the institute from the surrounding area and it is the first point of entry and has external access control. Restricted areas in Level 2 have an additional area of security and through which only authorized personnel pass, optimally this is enclosed on all sides from general areas and without shared boundaries with public access. Level 3 areas are encased within a restricted area with additional pass security for only authorized personnel. Levels 2 and 3 may require intruder alarms and key and/or coded access. Emergency exits and incident response plans (see *section 4.13*) must not be compromised by security and containment systems.

4.4 Regulatory risks

The IVGB should ensure its processes are compliant with regulations, agreements and international undertakings in the safe storage, movement, transfer and phytosanitary control of plant germplasm. Measures, checks, and cross-checks should be documented and in place to obviate risks of non-compliance in the regulatory chain. This should include timely, responsive updating of regulatory obligations, in line with new policies, agendas and laws. The use of the database to tag accessions is critical, this involves using a database to tag restrictions per accession which are presented as pop-ups which have to be approved when ordering and filling out orders.

4.5 Phytosanitary risks

Detailed treatment of phytosanitary aspects of genebank risk management are specifically covered by GPG2 Project Activity 2.1. "Consolidate, refine, disseminate and promote best management practices for collections, including germplasm health in storage". Therefore, in these guidelines phytosanitary risks will be confined to general issues. Compliance with phytosanitary, quarantine, disease diagnostics and indexing procedures and regulations is required, including plant health restrictions for collection in one country and transfer to another. Introduction of pests and diseases in the host country to the IVGB and *vice versa* should be obviated by accompanying incoming material with phytosanitary certification from the country of origin. When appropriate, in coming materials should be regenerated in containment facilities, quarantined and subject to phytosanitary testing. These include special treatments such as thermotherapy, cryotherapy, meristem culture, and health diagnostics (e.g. use of microbial media to test for contaminants). Risk assessment and mitigation plans should be created for the *in vitro* detection and testing of pests, pathogens, nuisance microorganisms using temperature treatments, meristem excision, culture and regeneration and labelling. Incoming germplasm should be delineated from clean *in vitro* materials held in the IVAG and IVBG (including cryotanks) until material is fully checked, decontaminated and declared disease, pest and pathogen free. New *in vitro* samples should be checked for microbial flora, systemic infections, endophytes, phytoplasmas, viruses and viroids.

4.6 Transgene and pathogen biosecurity

For these guidelines, contingencies relate to sources of potential harm arising from pathogens, genetically modified organisms (GMOs) and biotoxins held in IVGBs for phytopathological research, diagnostics, omics studies and genetic improvement. In accord with the Cartagena Protocol on Biosafety (see useful websites) the nature of transgenes, their potential for proliferation and spread outside a contained environment should be documented; if required, information should be cited as to how they can be detected. The IVGB should keep a detailed inventory of all their biological materials and identify personnel responsible for their safe storage, use and dispatch. A risk assessment should be conducted for the biosecurity required for

pathogens and toxins used for research and *in vitro* diagnostic tests. This should include information on infective dose (smallest quantity able to cause infection); pathogenicity (disease causing ability) and transmissibility (ease by which a pathogen spreads by vector and host).

4.7 Material safety and germplasm acquisition

Genebanks should be cognizant of risks associated with failure to collect and preserve vulnerable genetic resources under threat *in situ*. Genetic materials should not be collected, acquired and transported by the IVGB without prior knowledge and authorization of donor countries and their national control/contact points. Germplasm acquisition should comply with national/international procedures for obtaining permissions/permits under FAO's various codes of conduct and the Convention on Biodiversity (CBD). Access to and use of germplasm should be verified with donors and undertaken in compliance with a Germplasm Acquisition Agreement between donors and IVGBs. And, in accord with the Standard International Material Transfer Agreement, the CBD's benefit-sharing provisions and The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA). Best practices should underpin collecting missions and representative samples/information should be transferred using efficient methods and robust documentation. The IVGB should maintain a comprehensive inventory of all bio-materials entering the facility.

4.8 Safe germplasm shipment and transfers

Processing of germplasm requests should be in accordance with appropriate risk management procedures. Biomaterials should be dispatched using safe transit measures and sent to facilities that ensure their continued safety on receipt (see *section 6*). Acquisition request and transfer documentation should be available for inspection by appropriate national and international authorities/quality auditors, including documented evidence explaining where, which and when external institutes have made material requests and if they have been accepted or declined, and for what reasons. Transfer and tracking of shipments should be timely and controlled with contingencies in place should a shipment go missing, be delayed or diverted, e.g. held up in transit and/or at border and customs control points. Materials should be appropriately labelled with full details, cautionary notices and regulatory documentation to ensure that dispatches are risk-managed effectively (see *section 6*).

4.8.1 Safe internal transfers

High-risk materials should not be left unattended or, outside a high security area. A code of practice should be instigated for personnel with custody control of sensitive high-risk biomaterials, this should be applied during internal transfers and across physical infrastructures with different security and phytosanitary control levels.

4.8.2 Safe external transfers

Adherence to regulatory guidelines is required in the shipment of materials and germplasm in accord with the International Air Transport Association (IATA) transport of Dangerous Good Regulations and regulations for rail, road and sea transfers. Procedures should ensure that all shipment requirements are met and that staff undertaking dispatches are fully trained and competent in procedures for transferring dangerous and potentially hazardous goods.

4.9 Safe disposal

Safe waste disposal measures should be in place for biological and non-biological materials, including correct procedures for microbial, toxic and dangerous wastes, solvents and acids. Contaminated plant materials should be treated (e.g. autoclaved) and discarded safely in accordance with phytosanitary regulations (see *section 2.4.1*).

4.10 Duplication and black boxes

Duplication of germplasm collections held in MTS and LTS in other repositories and institutes should be undertaken with formal, reciprocated risk assessments, inclusive of transit and black box collections held by partner institute. Risk management provisions should be made in the event black box security fails, this may arise in the emergency recovery of germplasm held in a black box due to systems failure or a *force majeure*, (e.g. earthquakes, floods, conflict) or the need to contain potentially hazardous black box materials.

4.11 Risk management in the *in vitro* active genebank

Loss of *in vitro* samples due to processing backlogs should be prevented by optimizing the extension of cultures held in slow growth. Visible inspections should be undertaken regularly to: (1) avoid treatments that cause deleterious stress responses, (2) control spoilage due to microbial contamination, especially from covert systemic organisms and (3) offset risks of somaclonal variation and deleterious phenotypic and epigenetic changes.

Cooling/air conditioning units in culture rooms should be serviced regularly to ensure critical temperatures and lighting for slow growth are maintained. Surveillance probes, data loggers and remote sensing devices should be used for risk mitigation related to potential environmental control problems (e.g. power surges, equipment failure, intermittent faults). Ideally, they should be connected to audio and visual alarms should failure occur or a door be left open accidentally. Optimally, a High Efficiency Particle-removal Air System (HEPA) should be installed to enhance filter efficiency and reduce the risks of contamination. If possible, growth rooms used for MTS should be backed up with a dual compressor and power use should be controlled to avoid system overload. Ideally, contingency power supplies should be independent of the rest of the power circuitry in the IVGB and it is prudent to alternate the testing and use of back-up supplies and their compressors with the main supply system as

this will ensure both are operating optimally. Personnel working in confined refrigerated spaces and cold rooms should be protected (e.g. emergency alarms, inside opening) from the risk of being accidentally locked in a low temperature facility.

4.12 Risk management in the *in vitro* base genebank (Cryobank)

Risk assessment of basic tissue culture procedures are common to MTS and LTS however special consideration is required for the use of LN and cryogenic equipment (see *section 4.1*).

4.12.1 Personnel safety

Cryogenic equipment and LN present special risk management issues as compared to the usual hazards to which personnel are exposed in general laboratory operations. Therefore, special awareness training is required for staff working in cryobanks, specifically staff should be knowledgeable about the hazardous properties of LN and trained in the handling of cryogenic gases and made aware of the four main hazards of LN:

- Frostbite and cold injury.
- Asphyxiation.
- Mechanical injury incurred on explosion of a pressurized vessel containing LN or non-cryogen resistant instrument or device.
- Pathological and toxicological injury due to uncontrolled release of a dangerous substance from a pressurized cryovial or LN storage vessel.

Liquid nitrogen, its vapour and any container or implement exposed to it can cause severe frostbite and cold burns to skin and eyes. Special cryogenic safety equipment and surveillance measures are required when using LN and these can be statutory obligations in certain countries. LN-resistant personal protection includes clothing, gloves, aprons and face shields/screens, goggles, glasses and resilient, closed toe footwear. LN spills can crack ceramic and vinyl tiles, posing safety threats and contamination hazards. Only LN-resistant vessels and instruments guaranteed by the manufacturer should be exposed to LN and their safety advice adhered to concerning differential resistances of their products to LN liquid and vapour phases. Cryogenic equipment and cryovials have different tolerances to liquid and vapour states of LN and special care is needed when using pressurized vessels that contain, supply and transfer LN. Even small passive Dewars can become explosive if ice plugs inadvertently pressurize them and LN leakage into cryovials is potentially dangerous as the vapour expands rapidly on rewarming and can potentially explode the cryovial.

Transport of LN with personnel in attendance in enclosed spaces (vehicles, lifts) is prohibited where adequate ventilation cannot be ensured and particularly if a Dewar or pressurized cryotank ruptures during transit. Special LN storage and delivery vessels are often heavy and care in their safe manipulation/transport in accordance with manual handling regulations. It is important to avoid personal injury and catastrophic breakages if a heavy Dewar is dropped and explodes. Routine safety inspection and certification of pressurized LN systems, including pressure gauges and

cryotanks is a statutory regulation in certain countries. Asphyxiation due to oxygen deficiency in atmospheres flooded with nitrogen gas is potentially lethal making adequate ventilation essential. Personnel and laboratory oxygen sensing monitors that sound an alarm at critically low levels of oxygen should be supplied, dependent upon the handling environment. The provision of these devices and their routine servicing/checking is a statutory requirement in certain countries (see *Appendix II*). Stringent rescue procedures should be in place and adhered to in areas where LN is dispensed, used and stored.

4.12.2 Sample safety

In cases of sample loss, it is necessary to take into account: contamination, mislabelling and misidentification in the cryobank and rescue procedures should be in place for samples lost in cryotanks. Maintaining LN levels and/or its vapour phase atmosphere is critical to the maintenance of storage stability; low-level alarms should be fitted as required and frequent operator surveillance and cryogen top-up procedures instigated (see *Appendix II*). Excursions in and out of cryotanks for sample input and retrieval causes minor fluctuations in temperature that might potentially damage samples, particularly if they are vitrified and stored at temperatures close to their glass transition temperatures (T_{gs}). Over long storage times, multiple rewarming and cooling cycles become significant and the inclusion of 'sentinel cryovials' is good practice to test the stability of samples in cryotanks. It is recommended that cryobanks duplicate their cryopreserved genetic resources in a different cryotank and preferably in a different location to that of the primary genebank.

4.12.2.1 Cryovials

Cryovials are manufactured to withstand cryogenic temperatures and they have to be sufficiently robust to prevent penetration of LN, which should it leak into the cryovial could pose a containment threat and a potential hazard to the operator. Cryovials penetrated by LN become pressurized when vapour is formed, creating the potential for explosions on rewarming at rapid rates. Securing cryovials in plastic cryosleeves before submerging them in LN and their retention in the same sleeves on rewarming can help to contain potential explosions. For germplasm that has not undergone phytosanitary treatment, the contents of an exploded cryovial can form pervasive and penetrating aerosols, potentially capable of transmitting microorganisms by adventitious contamination. Most manufacturers of cryogenic equipment do not advise storage of cryovials in liquid phase LN and they only recommend their use in the vapour phase. Suppliers make cryovials with special safety features (internal threads, silicone 'O' rings) but reiterate that they should be used with care and chosen appropriately for totally submerged LN storage. Some manufacturers (Benson 2008) have developed polyethylene membrane sealants for the safe storing of cryovials in liquid phase LN.

4.12.3 Cryotransit

Dry shippers are manufactured for the safe movement of cryopreserved materials and manufacturers designate their dry shippers as International Air Transport Association (IATA) approved. These vessels are engineered to be durable, lightweight and fabricated with adsorbents that hold LN, allowing spill-free safe shipment in the vapour phase. Transport in the vapour-phase may compromise glass stability in some samples that require their Tgs to be maintained at liquid phase-LN temperature. Cleaning of dry shippers is recommended between uses.

4.13 Emergencies and incident response plans

As appropriate, the genebank building should be constructed to withstand environmental catastrophes such as floods and typhoons and if necessary built to earthquake standards; in some cases conflict risks (explosions, artillery fire) may need to be considered. Standard checks of genebanks should be undertaken during extreme weather, together with regular fire alarm and sprinkler checks and safety drills. A check list of key personnel and “on call” contacts should be provided in the eventuality an essential service fails and contingencies for cover need to be made. Genebanks should be able to maintain uninterruptible essential services for power, water, light and LN supply and in the event of failure contingencies need to be activated for backup, power supplies (battery, generators). Ideally, the institute Director or Security/Safety Officer(s) should have a direct link to local providers to prioritize service restoration. Measures should check emergency services/supplies and personnel training, drills and regular practices may be instigated to ensure the familiarity and compliance of staff with emergency and crisis procedures. Critical storage facilities should be under alarmed surveillance and visual inspections undertaken regularly. In case of power failure, portable emergency, lighting should be made available for making safe any ongoing operations, back-up facilities need to be regularly checked for utility and battery status. Incident response plans should be devised in anticipation of potential breaches of security, catastrophes, fires or accidents and staff should be trained in incident procedures and know their emergency roles. It is precautionary to have alternative back-up, local facilities available for germplasm re-location if a primary storage facility fails.

5. Guidelines for germplasm registration, verification and processing

These guidelines pertain to the registration, verification and authentication of new plant materials and their accompanying documentation on genebank entry.

5.1 Recording and registration

The IVGB should have a checklist comprising a common, generic template for documentation, which is used to register biological materials. This might normally comprise information from the donor (and/or collector), legal documents, SMTAs germplasm access agreements, phytosanitary certification, quarantine requirements, passport data, a germplasm list and its sources; a plant import permit is also a local requirement. To avoid duplication, it is best practice to check if material is already in the genebank, if this is the case, a new lot of the same accession number is assigned, identifying the material as the same accession but from a different source. If the germplasm is not in the genebank, it is assigned the next available accession number, and is registered with information about the donor and any other relevant information is entered into the database, thus making a permanent, complete and accurate record of the acquisition. For accuracy purposes all information should be verified with the donor and/or collector

5.2 Explant source and starting material

A generic procedure should be entered into a standard template describing: (1) germplasm source [GPS coordinates, *in situ* provenance/habitat, field genebank] (2) collection time, date and season; (3) donor, mother plant-explant, local name(s) and other secondary IDs such as herbarium sheets and images for future reference; (4) *in vitro*/genebank or *ex vitro* origin; (5) how the material is collected, cleaned, pretreated with fumigants, pesticides and antifungal agents; (6) physiological status; (7) sterilization solutions and procedures used for culture initiation and (8) axenic status. Starting material should be described as one of the following: (1) *ex vitro*, axillary or apical shoots or meristems, buds, dormant/active, zygotic seeds, (orthodox, intermediate, recalcitrant); (2) embryos, embryonic axes, or seedling shoots and (3) *in vitro*, plants, apical, axillary or adventitious shoots or meristems; somatic embryos, pre-embryogenic masses, totipotent embryogenic callus and cell suspension cultures. Special treatments and precautions should be noted e.g. that prevent and reduce phenolic and oxidative problems (blackening and browning) on initiation.

5.3 DNA extraction and storage

Some germplasm from each accession sample may need to be sequestered as either leaf material for DNA extraction or extracted DNA. Lyophilization, low and ultra low (cryostorage) temperature storage are preferred long-term conservation options for providing stabilized '*type*' DNA-materials used for authentication and genotypic

analysis. DNA banks should be subject to best practice guidelines for documentation, storage, regulatory documentation, safety duplication, tracking, inventories and recording as for other types of genetic resources.

5.4 Phytosanitary assessments and procedures

Conservation activities should interface with the repository's phytosanitary control laboratory and confer with specialist crop health personnel on risk assessments/best practices for *in vitro* plant phytosanitary diagnostics, control, quarantine, disease indexing and eradication. No pathogen or pest-infected materials should be placed in the IVGB which should be strictly contained from points of infection. Special *in vitro* treatments for thermo-therapy, virus eradication, meristem culture and diagnostic tests for bacteriological indexing and contamination should be documented as best practices for: (1) crop-specific pests and pathogens and (2) generic procedures for detection, control and eradication of endophytic, covert and adventitious microbial agents and pests (e.g. mites).

5.5 Verification and authentication

Verification and authentication of culture identity and genotype should be confirmed for new accessions using appropriate phenotypic, genotypic, molecular diagnostic and DNA fingerprinting tests. This procedure may also confirm duplicate accession and sample status.

5.6 Monitoring new acquisitions

Newly acquired *in vitro* materials or freshly initiated germplasm accessions should be carefully monitored for contamination, necrosis, viability and physiological vigour. Frequent monitoring should be undertaken during culture establishment and following transfer from another IVGB, this helps to preclude covert contamination, confirm disease-free status and confirms that cultures are responding effectively to their new conditions.

6. Distribution of materials from *in vitro* genebanks

Common policies and procedures should be in place for distribution and access to the genetic resources held by IVGBs, taking into account crop/accession-specific issues. These include: (1) deployment practices; (2) policy exemptions, (3) National and International laws and regulations, including permits/certificates in countries commonly holding the crop; (4) crop-specific phytosanitary regulations; (5) feedback to users regarding expected time frame to dispatch and critical factors influencing delivery; (6) customer assurance information related to client requests; (7) feedback request forms recording the most relevant information required by the donor from the customer/client; (8) quantity and status of the germplasm dispatched; (9) any special handling requirements for germplasm deployment and receipt and (10) risk assessment and mitigation protocols to secure safety of the germplasm supply chain

from dispatch to receipt (see *section 4*). Distribution of materials from IVGBs should be undertaken in accordance the phytosanitary guidelines and the safe handling of germplasm (see in this document, '*Useful Websites: Plant Protection*').

6.1 Distribution records

Templates of checklist procedures are used to ensure requirements are met to dispatch germplasm safely and securely in compliance with regulations, examples include:

1. *Check accession request for availability/stock status*
2. *Action steps required if stock is low and requires replenishment*
3. *Anticipate any special phytosanitary or biosecurity requirements*
4. *Check requirements for material transfer agreements*
5. *Register request*
6. *Check passport data*
7. *Prepare list of accessions available*
8. *Generate labels*
9. *Label accession containers*
10. *Check inventory files and locate germplasm in the IVAG*
11. *Remove requested samples from the IVAG*
12. *Verify accuracy of labels/confirm authentication of sample labels*
13. *Perform any special preparations, pretreatment and growing on procedures*
14. *Undertake phytosanitary inspections required before dispatch*
15. *Prepare information list to accompany materials for dispatch*
 - a. *Passport data*
 - b. *Phytosanitary certificate*
 - c. *Characterization data to confirm accessions*
 - d. *Cover letter*
 - e. *Material Transfer Agreement information*
 - f. *Any other special requirements.*

6.2 Shipping logs

Shipping logs should be maintained for germplasm dissemination and receipt, ideally these are computerized, interfaced with accession inventories and if required connected to other genebank activities to allow for sample tracking. Each shipment should be given a unique number/code, possible log examples may include:

1. *Shipment reference number*
2. *Consignee responsible for handling the shipment and designation*
3. *Names and addresses of sending and receiving organizations*
4. *Recipient source and type of organization*
5. *Purpose of germplasm request and use*
6. *Phytosanitary certificate and reference/code numbers*
7. *Export permit reference/code number*

8. *Dates shipped and received*
9. *Courier company/name and ID code for their tracking of the shipment*
10. *Germplasm description*
11. *Reference confirmed for Standard Material Transfer Agreement*
12. *Number of samples sent and received*
13. *Requirements for stabilizing shipment (temperature, LN, dry ice)*
14. *Signature of receiving person*
15. *Deviations from the shipping log and the actual shipment*
16. *Details of any problems with the shipment.*

6.3 Preparing samples for distribution

Consignment specifications should be determined by the nature of the germplasm/materials, the transit route and destination of the shipment once it is deployed. The consignee should determine which regulatory and legal requirements need to be met, the physical conditions required for stabilization and the level of containment required for secure dispatch.

6.3.1 Regulatory issues

The consignee should be trained in the regulatory, risk and safety procedures required to support safe and secure shipment of plant genetic resources. Biological samples to be transported should be classified with respect to potential dangers/hazards and high-risk consignments should be identified and risk mitigation procedures put in place. Any media, cryoprotectants and cryogenic substances used to support cultures during transit should be classified in terms of their risk, in compliance with IATA and national shipping regulations.

6.3.2 Temperature

Temperature tolerance limits (high/low) should be identified for germplasm and for chilled/cryopreserved samples, critical temperatures (e.g. T_g for vitrified samples) should be specified and actions taken to ensure thermal stability during transit. Dry shippers contain LN absorbed in a porous matrix in compliance with IATA safety regulations. The stability of LN should be test-calibrated for new dry shippers regarding the amount of LN required to fully charge and maintain the temperature of the dry shipper over required transit times and conditions. It is best practice to ensure refrigerated materials are subjected to cold chain management to ensure that their cryogenics are in sufficient supply to maintain temperature during a transit period. It is prudent to allow at least 24 h for a safety margin for delays in arrival.

6.3.3 Quality control of shipments

For precious shipments that are heat sensitive or have critical temperature requirements for stability such as T_g stabilization it may be prudent to attach a temperature-probe/logger to ensure temperature limits have not been exceeded. For these cases, test shipments may be simulated before dispatch of the actual materials.

6.3.4 Packaging

Robust, secure and self-contained culture packages and vessels should be selected for optimum dispatch security and spaces between them should be filled with packing to prevent movement during transit. Shipment packs should be clearly labelled and indelible. The consignee is responsible for safe dispatch and should ensure appropriate containment and check all packaging regulations are met.

6.4 Special shipments

Special permits may be unique to certain countries and regions with respect to phytosanitary regulations, therefore good shipping practices should offset risks of delay, loss or confiscation where additional documentation may be required. For consignments dispatched outside the usual domains of operation or in regions of border dispute and conflict, confirmation of additional requirements should be sought. For example, in accord with the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Contingencies should be made for possible delays in completing additional paper work, particularly for temperature sensitive materials and consignments needing cryogen replenishment. Where delays at customs are anticipated, it is wise to build in an additional few days of coolant supply. Special shipments should include an institutional letter documenting the content and handling requirements attached to the import and phytosanitary permits.

6.5 Tracking in transit

Both the consignee and recipient should track the germplasm/materials during their transit and confirm dispatch and receipt. The recipient should confirm and send an electronic shipping manifest before dispatch, confirming that proper personnel, facilities and regulatory support (e.g. plant health inspectors) are available on arrival of the materials. The shipper should provide a 24-h emergency contact for packages transporting at risk and sensitive goods and transit during holidays/weekends should be avoided to ensure a safe supply chain.

6.5.1 Processing on receipt

The time between sample arrival and processing of germplasm should be taken into account, particularly for chilled and cryogenic shipment and the collection, processing and storage times should be assessed by the recipient to ensure successful recovery of the germplasm; holidays and weekend absences should be factored into receipts. Cooling/warming cycles should be avoided for cryopreserved germplasm and this should be dispatched to the cryobank for immediate receipt. Consideration should be given to containment, sterility and the physical stability of the consignment on its arrival. On receipt, germplasm should be processed as described in *Section I* of this document, *section 5. Guidelines for germplasm registration, verification and processing.*

7. *In vitro* genebank quality assurance and management systems

A quality assurance programme (or system) supports the integrated management of best practices and quality issues in the IVGB in compliance with expected performance indicators. These may be designated for the different levels of genebank operations:

1. *Registration*
2. *Regulation*
3. *Documentation*
4. *Data recording, collection and management*
5. *Accession inventory systems and process tracking*
6. *Verification (authentication)*
7. *Source material and processing*
8. *Basic tissue culture*
9. *Conservation strategy*
 - a. *Medium-term storage: Slow growth*
 - b. *Long-term storage: Cryopreservation*
10. *Biosafety and security*
 - a. *Containment, contamination, transgenes, stability, security*
11. *Phytosanitary procedures and healthcare*
12. *Storage monitoring*
13. *Regeneration, rejuvenation and recycling*
14. *Germplasm testing*
15. *Germplasm distribution*
16. *Training*
17. *Risk management*
18. *Technology transfer*
19. *Validation*
20. *Quality Control (QC).*

7.1 Quality control and performance indicators

Quality control (QC) is a system devised to check that activities are operating to a level of performance against defined standards or performance indicators. Internal quality control checks should be in place for documentation and monitoring IVGB operations within the overarching quality management infrastructure.

7.1.1 Key performance indicators

Optimally, Key performance indicators (KPIs) and metrics are devised to enable and verify that IVGBs are meeting their conservation and service obligations and goals. At the institutional level this can be assessed by external review procedures of

independent panels and timely commissioned impact assessment studies, including evaluating the influence of the IVGB on networked and global conservation communities, international policies, regulations and agendas.

At the customer level, KPIs assess and correlate: (1) germplasm requisition processing; (2) feedback from client satisfaction surveys and (3) impacts on recipients, collaborators and beneficiaries. KPIs also monitor the diversity of genebank holdings, their usage by end users/recipients, the purposes for which they are used and their impacts on beneficiaries as verified by genebank documentation. Research KPIs monitor the use of specific types of germplasm, which is quantified as accession requests related for example to omics research. Or, crop specific information can be reported in formal scientific outputs as the acknowledged use of germplasm that has been provided by an IVGB. Performance of a genebank as a knowledge/information source can be verified using dissemination and accessibility information based on web-user statistics. Operational KPIs can comprise quality assessments of the material distributed that is based on recipient/client satisfaction. This can be documented as customer feedback surveys that are linked to genebank quality management systems.

7.1.1.1 Genebank diversity and accessibility performance indicators

These may be devised to assess the diversity in different types of holdings and ideally they are linked to passport data which supplies their genetic, geographic and taxonomic distributions. Status information should be available regarding the part of the crop's total diversity held in the IVGB and the proportion of which is readily available, held in trust or constrained. For example, in terms of accessibility, e.g. low viability or stock and phytosanitary restricted or storage/regeneration recalcitrant.

7.1.1.2 Technical and security performance indicators

These include phytosanitary diagnostics and viability/stability tests that are used to assess the practices that ensure disease-free germplasm is held in genebanks with acceptable levels of viability. Indicators also include regeneration and functional tests following germplasm removal from slow growth and cryopreservation. These metrics ideally include an automatic alert that warns if the actual performance falls below a critical level. For example, indicating a regeneration backlog due to poorly performing accessions, or a containment breach putting germplasm at risk of pandemic infection. It is important that technical KPIs are interfaced with risk management and safety and security operations and that they include black box collections outside the 'home' genebank.

7.1.1.3 Key performance indicators for efficiency

These are devised to ensure that the operational, technical and management best practices are cost effective, efficient and that they positively contribute to the long-term sustainability of the IVGB and the security of its holdings within any budgetary constraints. It is prudent to note that efficient and cost-cutting measures should not jeopardize any risk management and mitigation issues and that they should support and retain regulatory and legal compliance.

7.2 Quality audits

To maintain QM systems once they have been established, it is important that annual, internal audits are carried out to ensure policies and procedures are undertaken in accordance with the quality system and its best practices. The programme should cover all activities including documentation, supply chains, accessions, databases, germplasm acquisition, distribution, training records, equipment, facilities use and maintenance, orders supplies, services and complaints. Quality audit procedures should be undertaken in cooperation with Management and the Safety Committee (see *section 4*) to ensure continued compliance with regulatory obligations. The production of new and updated technical guidelines and handbooks should be processed via internal and ideally independent peer review.

7.2.1 Third party assessment and accreditation

Third party assessment and accreditation may be an option and IVGBs may wish to aspire to (external) third party accreditation status, for which ISO standards and certification are one possibility (Betsou et al. 2008). Audits should be undertaken to acquire and maintain accreditation status and these will necessarily need to be supported by the rigorous upholding of internal quality standards. Translation of IVGB standards, best practices and operating guidelines into ISO-compatible formats offer useful opportunities to assist the process of accreditation, as is currently the case in the wider bioresources community (Betsou et al. 2008; ISBER 2008). However, when considering the adoption of third part accreditation, it should be undertaken in a management framework that accounts for the additional training and workload that the process of external accreditation will generate. Specifically, an accreditation process should not detract from ongoing genebank activities (e.g. characterization, regeneration, evaluation, collecting missions, database management) but rather, in the longer term it should add to the overall efficiency and quality of genebank activities.

7.3 Developing best practices for clonal crop *in vitro* conservation

To ensure parity and the quality control of procedures, processes, data and inventories; crop-specific best practice formats should be created by consensus and technically verified in house; ideally, the protocols should then be validated across federated genebanks. The standardized formatting of methodologies as best practices supports quality management systems for which exemplars include:

1. **Title of best practice (BP):** to prevent confusion this should be a unique name.
2. **Number:** for ease of identification, a unique code, including revision version to ensure the most recent update is used.
3. **Date:** date the procedure was first created/most recent update and by whom.
4. **Department/personnel:** to whom the BP applies.
5. **Safety and protection:** details of risk management and mitigation, special safety measures and personal protective clothing needed to undertake the BP.
6. **Equipment:** list of equipment required to undertake the BP.

7. **Consumables, reagents, and media:** list of supplies needed to undertake the BP.
8. **Special treatments:** a list of any special treatments and any processes involved.
9. **Critical points:** details of specific technical issues affecting successful outcomes.
10. **Method:** step-by-step technical guidance explaining how to undertake the procedure or protocol. This should contain sufficient detail to allow the BP to be repeated and reproduced by trained, personnel without ambiguity. It is useful to include technical explanatory notes, media formulations, and explicit guidance for each step; any special conditions and critical factors may also be included.

The BP format can also be adapted to create specific SOPs. The development of best practices for clonal crops and genebanks is facilitated by reaching consensus on generic technical guidelines from which future best practices can be developed for specific crops and if needed on a genotype basis. To enable this process *Section II* of these guidelines presents generic technical guidelines common to clonal crops. Best practice guidelines may also be used to help formulate SOPs within IVGBs and across federated genebanks. *Sections I and II* are supported by *Appendix I* which comprises bibliographies of technical and quality assurance information, literature concerning CGIAR's mandated clonal crops, research and technical publications.

SECTION II. MULTI-CROP TECHNICAL GUIDELINES FOR *IN VITRO* CONSERVATION IN CLONAL GENE BANKS

These guidelines should be used in conjunction with Section I “*General best practice guidelines for clonal crop in vitro genebanks*”. The objective of **Section II** is to provide technical information to support the development of multi-crop best practices for clonal crop *in vitro* genebanks within the context of the GPG2 project and CGIAR’s clonal crop genebanks. The technical guidelines comprise basic and generic techniques and procedures for the tissue culture (including associated manipulations), slow growth and shoot meristem cryopreservation of clonally propagated crop plants, as well as specific storage protocols.

The technical guidelines presented in Section II are generated from the results of the GPG2 Project CCTF survey and the wider published literature (see Benson et al. 2011a, b). In sequence, these guidelines *first* present the technical details of the basic equipment, materials and methods that collectively comprise the generic procedures (e.g. culture initiation, subculture, pretreatments, encapsulation and cryoprotection regimes) that are most commonly used in CGIAR’s clonal crop genebanks (see *sections 1-8; 10 to 10.5*). *Secondly*, particular protocols that are used for the medium-term (slow growth) and long-term (cryopreservation) conservation of CGIAR’s clonal crop germplasm are described in more specific step-by-step detail (*sections 9 and 10.6*). Critical point factors connected with each component step of a basic procedure or storage protocol are highlighted. Section II concludes with general guidelines (see *sections 10.7 to 10.11*) that concern associated practices and troubleshooting issues.

The technical requirements for executing the manipulations, procedures and protocols presented in Section II are based on the minimal requirements for propagating tissue cultures in a basic *in vitro* laboratory (FAO 1985), specialist facilities/equipment are noted as required.

1. Generic technical guidelines for culture initiation

Shoot cultures are initiated from suitable explants (e.g. shoots, tuber spouts, cuttings) comprising either *ex vitro* materials (e.g. from field genebanks, shade or glasshouses) or propagated materials from axenic *in vitro* cultures. The following basic protocols are based on the premise that donor plants and explants have already been processed via quarantine, phytosanitary testing, disease eradication and virus indexing procedures.

1.1 Equipment and materials

- Surface sterilizing solutions: e.g. 70% (v/v) alcohol, 5-10% (v/v) sodium hypochlorite [NaClO], prepared from commercial bleach [ca. 10-30% NaClO], optional wetting agent [Tween 20].
- Sterile distilled water dispensed into 20-50 ml bottles for washing tissues.

- Optional antifungal, antibiotic and antimicrobial agents (e.g. Plant Preservation Mixture PPM®).
- Sterilized dissection instruments, filter papers, Petri dishes.
- Binocular microscope with a sterile operating surface (i.e. sterile filter papers, open sterile Petri dish).
- Culture medium (crop-specific) dispensed into suitable culture vessels.

1.2 Procedure

1.2.1 Non-sterile manipulations

1. Record all accession details.
2. Select a sufficient number of explants (e.g. nodes, bud cuttings, apical or axillary shoots) for creating a new accession and take into account the potential need to:
 - a. Test non-axenic/non-indexed cultures for phytosanitary purposes.
 - b. Replenish losses caused by contamination during initiation.
 - c. Replenish losses due to latent infections by systemic endophytic flora.
 - d. Replenish losses due to lack of or poor growth.
3. Check *ex vitro* donor materials for pests, thrips, and mites.
 - a. Wash in mild detergent, rinse several times in laboratory tap water to remove any contaminating pests.
4. Cut each explant to a suitable size for sterilization (e.g. nodal bud + 1cm of stem either side, one apical bud with sub-basal tissue and non-expanded leaves).
5. Take care to prevent dehydration of the cuttings.
6. Use minimal cuts to reduce wound-induced oxidation (browning/blackening).

1.2.2 Sterile manipulations

1. These should be undertaken in a laminar airflow bench using aseptic technique.
2. Soak the explants in 70% (v/v) alcohol, with the duration optimized for 1-5 min.
3. Optimize surface sterilization by testing explant exposure to 5-10% (v/v) sodium hypochlorite solution (with wetting agent as required) for 10-15 min (or longer as required).
4. If necessary, apply a topical or systemic fungicide treatment, concomitantly or sequentially with the sodium hypochlorite solution.
5. Use percentage survival and percentage contamination as performance indicators to optimize sterilant concentrations and exposure times.
6. Using sterile forceps remove explant materials from the sterilant/fungicide and wash 3-5 times in sterile distilled water, ensuring that all the surfactant is removed.
 - a. For persistent infections, the fungicide may be retained during the first stages of culture initiation and re-applied after the washing treatment.

7. Place the explant on a sterile surface, (filter paper/open Petri dish) and use a sterile scalpel to remove wounded tissue from: (a) the cut edges of a nodal cutting and/or (b) the basal tissue and expanded leaves of shoot tips.
 - a. Wounded tissue will usually be bleached white or oxidized black/brown.
8. Optionally, use a binocular microscope to aid the dissection of shoot meristems by locating the apical dome, and with the aid of sterile hypodermic needles, or scalpels cut away any expanded leaf primordia and tissue subjacent to the apical region.
9. If necessary, blot the explant dry on a sterile filter paper to remove excess water and transfer to the appropriate crop-specific culture initiation medium.
10. Take care that the orientation of the explant is appropriate for shoot growth.
11. Leaving the 'planting end' of a nodal cutting a little longer is a good point of reference for positioning the explant correctly in the culture medium.
12. It is a precautionary measure to place one explant/culture vessel to limit cross-contamination losses.
13. Seal culture vessels with Parafilm^R, (or equivalent) or cling film may be used as a cost effective alternative.
14. Label clearly, using a standardized format applied either manually or by a data logger-barcode, the label should be positioned so that it does not obscure visual inspection of the culture.
15. Transfer the culture to a quarantined environmental control room set at the appropriate crop-specific temperature/light regime.
16. Inspect isolates daily for the first two weeks and remove any contaminated cultures; check twice weekly thereafter to remove contaminated cultures and isolate growing axillary buds from the original stem. Contaminated isolates should be autoclaved prior to opening the tissue culture vessel.

1.3 Critical points

- *These guidelines are generic, crop-specific best practices include technical details of any special and optimal treatments required for successful culture initiation.*
 - *Examples include: photoperiod, light intensity, temperature, media additives (charcoal, plant growth regulators, glucose as an alternative metabolite, antioxidants) and crop/genotype specific media.*
- *Vigilance limits the risks of contamination occurring during the initiation of cultures from field-derived materials, woody perennials and soil-sampled root and tuber explants.*
- *Culture initiation should be optimized by taking into account seasonal, physiological and developmental factors as these can be critical to successful outcomes.*
- *When using antimicrobial agents/antibiotics it is important to limit the risks of microbial resistance occurring.*
- *Quality control measures should be in place to help identify potential points and sources of infection and cross-contamination e.g. from personnel, media and lack of containment.*

- *It is good practice to test new cultures for systemic infection 4-6 months after initiation using bacteriological indexing procedures.*
- *Some wrapping products (e.g. cling film) may produce volatiles such as ethylene that are inhibitory to plant growth.*
- *Should an adventitious or systemic contamination incident arise it is prudent to develop isolation and containment procedures to prevent pandemic losses of germplasm in the IVGB.*

2. Generic technical guidelines for *in vitro* multiplication

Best practices are required for optimizing growth media and culture regimes for actively growing cultures as these provide material for slow growth and cryopreservation; they are also used in post-storage recovery. Procedures comprise the micropropagation of established tissue cultures for the IVAG and IVBG and culture dispatch and regeneration. In addition, cultures are used for quality control, stability assessments and research. All procedures are performed using aseptic technique applied under sterile conditions in a laminar airflow bench.

2.1 Equipment and materials

- Cultures to be micropropagated.
- New culture vessels containing fresh medium.
- Sterile instruments, filter papers.

2.2 Procedure

1. Select established shoot cultures at an optimal time in their subculture cycle.
2. Check for contamination and physiological status.
3. Discard any infected materials and unhealthy cultures.
4. Using aseptic technique, remove a shoot from the culture vessel.
5. Transfer tissue to a sterile surface e.g. filter paper, Petri dish.
6. Take micro-cuttings of shoot cultures as required for apical and/or nodal sections (e.g. for cryopreservation or slow growth initiation).
7. For axillary nodes, cut at either side of the stem, ensuring each section contains a node and a stem segment, in most cases trim tissues to have half-expanded leaves.
8. Apices should be undamaged and of an optimal size to support shoot growth.
9. Transfer nodal cuttings/shoot apices, correctly orientated, to new medium.
10. It is precautionary to place one explant/culture vessel to limit cross-contamination.
11. Seal culture vessels with Parafilm[®] (or equivalent) or cling film may be used as a cost effective alternative.
12. Label clearly using a standardized format either manually or a data logger-barcode.
13. Transfer to a culture room regulated at the appropriate crop-specific, temperature/light regime.

2.3 Critical points

- *Micropropagated cultures should be checked regularly for systemic infection, adventitious and endophytic contamination, health and vigour following each subculture cycle.*
- *Species and genotypes especially susceptible to hyperhydricity, somaclonal variation (SCV), loss of totipotency, habituation, in vitro ageing and culture recalcitrance should be monitored more carefully and procedures (e.g. culture optimization, regeneration) should be developed to prevent or reduce any specific physiological, culture problem.*
- *Unambiguous, technically complete protocols should be written for each type of crop-specific tissue culture, including full details of medium preparation, subculturing practices, type of culture vessel, closure and culture room parameters (light regimes [photoperiod, quality and intensity], temperature and humidity).*
- *Strategies for the rejuvenation and recycling of materials held in active tissue culture should be devised to take into account loss of vigour, totipotency and a decline in culture performance over long periods of maintenance in vitro.*
- *Rejuvenation and regeneration cycles should be especially undertaken for ageing cultures.*
- *The risks of adventitious microbial contaminants and instability (e.g. SCV) should be assessed on a regular basis particularly in older cultures.*
- *Young, vigorous and totipotent cultures should be preferentially selected for slow growth and cryopreservation.*

3. Generic technical guidelines for detecting microbial contaminants

These procedures are used to test initiated explants for systemic and latent infections that arise from endophytic or covert bacteria and microbial flora or spores that are resilient to standard surface sterilizing procedures.

3.1 Equipment and materials

- Tubes containing nutrient enriched (e.g. containing, peptone, yeast extract, glucose) broad spectrum, liquid bacteriological test medium made in full or ½ strength plant culture medium.
- Petri dishes containing broad spectrum, semi-solid bacteriological test medium.

3.2 Procedures

3.2.1 Screening for systemic contaminants during surface sterilization/initiation

1. Explants are surface sterilized as described above and transferred to liquid-form bacteriological test medium. Contaminated explants will be revealed by the cloudy or flocculated growth of microorganisms.
2. If cultures are contaminated, discard and repeat the surface sterilization procedure on a new batch of explants.

3. If cultures are not contaminated, proceed to culture initiation and proliferation; continue stringent surveillance for the first three months after initiation; longer may be required for persistent, endophytic and covert infections.

3.2.2 Screening of suspect contaminants for quality control

1. Remove the suspect plants and shoots from standard culture.
2. Streak the base of the plants on Petri dishes containing broad spectrum, semi-solid bacteriological test medium and incubate the culture for 2-14 days at 25°C. Alternatively, a stem or node explant can be placed directly onto the bacteriological test medium.
 - Noting that it can sometimes take several weeks or longer for covert contaminants to be revealed.
3. Plates should be checked with a hand lens or microscope.
4. Transfer individual plants/shoots to standard culture medium and retain for the results outcome.
 - Plants/shoots that test positive for contaminated streak-plates should be discarded and their accessions can be reinitiated if necessary.
 - Plants/shoots that test negative can be reinstated.

3.3 Critical points

- *Bacterial indexing should be applied as appropriate:*
 - *on culture initiation,*
 - *before material is placed in the IVAG and IVBG,*
 - *as routine quality control checks (e.g. every 3-5 MTS cycles),*
 - *when covert and endophytic infections are suspected,*
 - *before the dispatch of cultures,*
 - *before cultures are used for diagnostic tests and research purposes.*

4. Keeping technical records

Newly initiated cultures should be processed in batches and their progress through the system monitored, ideally using electronic inventories and barcodes. Only successful introductions to the genebank should be entered, although in some cases information related to problematic germplasm and unsuccessful initiation may be useful for troubleshooting and optimizing new procedures. These types of entry should be considered for separate archiving as appropriate.

4.1 Exemplars of recording data

- Batch No.
- Accession No.
- Date introduced *in vitro*
- Number of explants introduced

- Contamination status
- Necrosis status
- Vigour status
- Dispatched for 1st multiplication cycle
- Contaminated in 1st multiplication cycle
- Necrotic in 1st multiplication cycle
- Vigour loss in 1st multiplication cycle
- Number of plants, seedlings, proliferating shoots sent to genebank
- Operator responsible for batch.

Once an accession is newly introduced into the bank, its accession number is entered into the database and progress through the system may be recorded, for example as follows:

- Accession No.
- Date introduced *in vitro*
 - where there is a replacement accession, previous entries are discarded.
- Explant type:
 - meristem/nodal cutting/plantlet/ proliferating shoots/tuber sprouts.
- Phytosanitary status:
 - tested virus-free yes/no,
 - bacteriologically indexed yes/no.
- Sequential genebank updates (dd/mm/yy) as to status of:
 - progressive subculture cycles,
 - Inventory status:
 - No. tubes going in and out of the bank with a reason given e.g.
 - eliminated due to contamination, vigour loss, necrosis death;
 - dispatched for utility purpose (research, genebanking, duplication, multiplication/distribution, diagnostic testing, phytosanitary testing, stability, authentication, quality control).
 - Contamination during multiplication cycles.
 - Loss of vigour during multiplication cycles.
 - Necrosis during multiplication cycles.
 - Viability/death.

These common formats are applicable to cultures and germplasm maintained in active growth, MTS and LTS.

4.2 Critical points

- *A comprehensive record as to the progress and outcomes of newly initiated germplasm in relation to accession status in MTS and LTS should be maintained.*
- *Ideally, electronic barcoding systems should be used for tracking.*
- *Robust recording and inventory systems are needed to ensure the production of accurate, safe and secure records for cultures that are held in IVAGs and IVBGs for extended periods.*
- *For in vitro collections that have not been systematically documented or characterized, procedures should be in place to upgrade records, documentation and inventories.*
 - *These may need to be authenticated by a field regeneration cycle in which plants are grown alongside taxonomic reference accessions for verification using crop descriptors.*
- *The issues of variable quality and accuracy of archived and contemporary inventories should be addressed and there should be procedures in place for systematically upgrading (e.g. by barcoding and electronic inventories) archives that are connected to crop specific knowledge bases. These should be hosted by well-maintained websites.*

5. Generic technical guidelines for germplasm monitoring

A standard monitoring template should be devised for cultures maintained in, and recovered from active *in vitro* growth, MTS and LTS and robust performance indicators should be developed to assess consistently and accurately culture growth. These procedures should be verified by different internal operators and ideally they should be validated across federated/collaborating genebanks that hold crops in common and use the same storage methods. Assessment scales and metrics need to be sufficiently robust to extend over significant time scales and changes in personnel. Main monitoring criteria are: (1) plant health, (2) extension of subculture interval (for MTS), (3) viability after cryostorage (4) contamination frequency and (5) capacity to recover and perform fit-for-purpose after storage stress. The periodic grow-out of cultures is advised to ensure that they can still be acclimated to glasshouse conditions and are “true-to-type”.

6. *In vitro* culture and storage performance indicators

Monitoring descriptors and performance indicators could utilize computer-based image analyses. Where observational descriptive scales are used (e.g. for viability, vigour and health status) they should be qualified with visual exemplars and quantified, without ambiguity, on point scales (e.g. 1-5) or as a percentage of the overall population/explant. Exemplars include (see Reed et al. 1998, 2003):

6.1 Point scale

- 0 = **dead**, all the plantlet is brown or bleached, no visible indication of growth
- 1 = **very poor**, doubtful viability, brown, necrotic shoots, with some green
- 2 = **poor**, mainly brown/bleached, most shoot tips are necrotic, some shoot tips remain green, leaves/stems mainly brown, explant base necrotic

- 3 = **acceptable** some browning/beaching, some shoot tips necrotic, shoot tips and upper leaves green, etiolation present, base green
- 4 = **good**, elongated shoots, shoot tips generally healthy, green leaves, stem, limited etiolation and necrosis
- 5 = **excellent**, green leaves and shoots, no etiolation, browning or bleaching

6.2 Percentage scale for monthly assessments of stem necrosis

- 100% **Lost-dead**
- 30-70% **Bad**
- 10-30% **Medium**
- 0-10% **Good**

Performance indicators for MTS can also be devised as leaf senescence indexes (ratio of green: dead leaves) or micropropagation potential (number of green apical shoots, nodes and stems available for subsequent culture and rooting capability). Performance indicators for post-cryopreservation recovery are: (1) viability; (2) shoot regrowth/multiplication/regeneration, (3) plant regeneration; (4) field-based assessments of development and trueness-to-type using crop descriptors and (5) cytological, biochemical, genetic and epigenetic stability assessments.

6.3 Critical points

- *Abnormal, adventitious and dedifferentiated development should be noted as negative performance indicators because:*
 - *they may indicate compromised genetic stability, however they can be used to assist the optimization of recovery protocols (e.g. choice of plant growth regulator).*
- *Surveillance of cultures at all stages of processing and storage is critical, regular reviews are recommended on a daily or 1-4 monthly basis, dependent upon the crop, and subculture cycle extension during MTS.*
- *Clear guidance should be provided for making decisions as to discard, rescue and transfer cultures falling outside the normally expected limits of germplasm loss.*
 - *Ideally these should be based on a systematic register of visual and documented performance indicators (e.g. using surveys extended over more than one slow growth cycle) and performed for all accessions held in the IVGB.*
- *On recovery from MTS and LTS phenotypic and genotypic stability assessments should be applied as a quality control measure as appropriate.*
 - *These may be optimally confirmed by performance testing in field trials; more than one complete regeneration cycle is recommended for clonal crops/genotypes that are more susceptible to SCV and off-types (Scowcroft 1984).*

7. Quality control checks for routine monitoring

A standard format should be developed for the quality control of newly initiated and actively growing cultures and germplasm held in and recovered from slow growth and cryostorage. Quality control usually comprises four basic checks: (1) contamination/phytosanitary health; (2) physiological stress and vigour; (3) viability and (4) stability. Where semi-quantitative descriptors and observational metrics (e.g. good or bad scales/numerical codes [1-5 or, 0-100%] or a necrosis index (e.g. colour degree of greening, blackening, browning, necrosis, hyperhydricity) for culture health status are applied, an image key should be constructed as this provides a comparator between different operators. Optimally, observational tools should be aided by computer-based imaging systems (e.g. digital photographs). Vigilant and regular contamination checks should be undertaken with particular care to detect covert, endophytic infections and prevent pandemics arising as a result of delayed microbial proliferation.

8. Verification (authentication)

Identity verification should be undertaken as a routine quality control measure across the IVGB and in the responsive testing of cultures and regenerants that display uncharacteristic growth habits that are not true-to-type for standard descriptors. It is prudent to authenticate materials at strategic points for example: (1) for more frequently requested accessions (even though all accessions are equally important); (2) less frequently requested accessions, which should be targeted to avoid them falling through 'surveillance cracks'; (3) germplasm required for breeding purposes and (4) landraces requested for distribution. When differences between accessions/clones are observed molecular, genotypic and phenotypic diagnostic tests should be applied to ascertain if the differences are due to mislabelling, misidentification or instability. Ideally, mislabelled clones should be identified by comparing *in vitro* materials with field-grown clones using descriptors, databases and donor supplied information. Genotype verification is best undertaken by a genetic quality laboratory/unit which has the capacity to identify duplicates, redundant accessions, mislabelling and identification errors. It is also important to confirm the stability of plants that display aberrant growth habits before their germplasm is placed in storage and following recovery.

9. Generic technical guidelines for medium-term storage: slow growth

These guidelines pertain to the tissue culture and associated practices that are used to extend routine subculturing intervals of *in vitro* germplasm stored in slow growth in the IVAG, noting that detailed technical guidelines for MTS should be produced on a crop-specific basis and that microtuber *in vitro* storage is an alternative method for some crops. Inventories should be in place to document the type (e.g. wild species, cultivar, landrace, breeding lines) and nature of the explant (*in vitro* shoots, seedlings,

cultures, tubers) that are used to establish MTS collections. The number of replicates per accession should be recommended using evidence based knowledge of recovery, accession requests, regeneration frequency and the potential for germplasm loss. Where possible well-defined performance metrics should be used to assess recovery, vigour, contamination, genetic variability and instability (see *section 6*).

9.1 Retardation using low temperature and light

Low temperature and minimal light treatments should be optimized to critical low limits of tolerance and as required best practices should be moderated for different accessions, species and genotypes.

9.1.1 Equipment and materials

- Standard culture medium dispensed into suitable vessels fitted with non-perishable, robust closures previously checked for deterioration during long-term storage (as an alternative, heat sealed vessels i.e. STAR*PACS™ ; AgriStar, Sealy, Texas, could be used).
- Indelible, permanent labels, preferably barcoded.
- Healthy, axenic and bacteriologically indexed cultures.
- A secure, growth environment, accurate to light and temperature specifications.
- Sterile instruments, filter papers.

9.1.2 Procedure

1. Prepare standard culture medium and dispense into storage vessels (an increased volume may be needed for prolonged storage).
2. Select explants from actively growing subcultures, ensuring that no selection occurs, other than discarding contaminated or unhealthy specimens.
3. Transfer to storage medium as *in vitro* plantlets, proliferating shoots, microtubers or nodal cuttings. Optimize selection for MTS based on explant type, size, age and stage of development.
4. Record standard performance indicators of cultures before they are placed in MTS. Use this data as reference criteria for surveillance, quality control and as a comparator for subsequent culture transfers. Digital imaging may help to set a base line for the recording procedure.
5. If necessary, incubate cultures for 1-2 days or longer, under standard growth at optimal light/temperature to confirm health status. Then transfer the cultures to minimal conditions before they start to proliferate excessively or require subculturing or become senescent.
6. Transfer cultures to appropriate conditions (no light, minimal light and low temperature) that have been optimized on a crop and genotype-specific basis (as required). For cold tolerant/temperate species these can range from 4°C to 15°C and for tropical and chill sensitive species from 16°C to 22°C.

7. Optimize light regime for photoperiod (e.g. 16 h light:8 h dark, 12 h:12 h light/dark, total darkness). The reduction of Photosynthetic Photon Flux (PPF) usually varies from ca. > 5 to $50 \mu\text{mol m}^{-2} \text{s}^{-1}$.
8. Monitor cultures on a weekly basis and check for contamination and deterioration.
9. Record culture extension periods and deterioration on a genotype basis for future reference and use this data to determine the logistics for subculturing, regeneration and recovery.
10. Remove cultures from minimal storage conditions.
11. Transfer to fresh culture medium, return to optimal light, and temperature regimes.
12. Test performance and confirm stability (see sections 6, 7 and 10.9).
13. Micropropagate under standard conditions and use or dispatch as required.

9.1.3 Critical points

- *Regular surveillance checklists should be used to monitor status and environmental control specifications for growth rooms, cold rooms and incubators holding germplasm under low light and temperature regimes.*
- *Slow growth environmental chambers should be equipped with an alarm in a secured location.*

9.2 Retardation using osmotica

Osmotic agents minimize growth by the osmotic withdrawal of water across semi-permeable membranes; in effect this process causes growth retardation primarily by water limitation. Osmotic agents can be applied singularly or in combination with other additives.

9.2.1 Equipment and materials

- Culture medium containing osmotic agents at optimized concentration (e.g. 15-60 g/l mannitol or sorbitol and/or 20-60 g/l sucrose and/or at an increased level of agar/gelling agent) dispensed into suitable vessels with non-perishable, robust closures previously checked for deterioration during long-term storage.
- Indelible, permanent labels that are preferably barcoded.
- Healthy, axenic and bacteriologically indexed cultures.
- A secure, growth room environment calibrated to an optimal specification.
- Sterile instruments, filter papers, laminar airflow bench.

9.2.2 Procedure

1. Prepare culture medium incorporating osmotic agent(s) the composition of which should be optimized on a crop/genotype-specific basis (as appropriate) options include mannitol or sorbitol combined with sucrose at growth-limiting levels.
2. Care should be taken in autoclaving media with sugars and sugar alcohols to avoid deleterious heat treatment effects. These include caramelization of sugars, hydrolysis of sucrose to glucose and fructose and carbohydrate breakdown

exacerbated by interactions between different media components, (particularly Fe-NaEDTA). Whilst these effects may occur at lower concentrations, they may become evident in media enriched with higher levels of sugars (Evans et al. 2003, Schenk et al. 1991). The issue of carbohydrate stability during extended autoclave cycles may become significant in media where sugars and polyols are applied for growth retardation and cryoprotective purposes.

3. Dispense the medium into storage vessels, noting that an increased volume may be needed for prolonged storage.
4. Select cultures from actively growing subcultures, taking care that no selection occurs other than discarding contaminated or unhealthy specimens.
5. Record standard performance indicators of cultures before they are placed in MTS. Use this data as reference criteria for surveillance, quality control and for comparisons with subsequent culture transfers.
6. Transfer to osmotica-supplemented medium as either fully developed *in vitro* plantlets, proliferating shoots, microtubers or nodal cuttings. Optimize explant selection for MTS based on explant type, size, age and stage of development.
7. Transfer cultures to the growth room optimized for light regime, photoperiod and temperature.
8. Monitor cultures on a weekly basis and check for contamination and deterioration.
9. Record culture extension periods and deterioration on a genotype basis for future reference and determine logistics for IVAG subculturing, regeneration cycles and recovery.
10. Remove cultures from minimal storage conditions, transfer to fresh culture medium and return to optimal light and temperature regimes.
11. Test culture performance, confirm stability and micropropagate under standard conditions, thereafter dispatch as required.

9.2.3 Critical points

- *For optimization, a range of osmotica should be tested at different concentrations and exposure times for different explant types.*
- *Combinations of growth retarding treatments can also be used, these can comprise minimal light, low temperature and osmotica and they should be tested in their various permutations for enhancing subculture extension.*

9.3 Retardation using inhibitory growth regulators

Physical (light/temperature) and osmotic growth limitation are the usual choices for facilitating slow growth storage. Where these parameters are either sub-optimal or deleterious, alternative treatments may be considered as a substitute, or applied in combination. This does not necessarily exclude the use of alternative treatments for which options include using ancymidol (a growth inhibitor) and/or applying acetylsalicylic as an alternative to mannitol (Chau-um and Kirdmanee 2007).

9.4 Other minimal growth options

Reducing the levels of standard growth regulators and hormones in cold-sensitive genotypes provides an alternative approach for MTS. Nutrient limitation is usually imposed by reducing sucrose and nitrogen in the media (e.g. ½ to ¼ strength).

9.5 Stress amelioration treatments

It may be necessary to alleviate the effects of stress (e.g. hyperhydricity, *in vitro* senescence and phenolic production) and the production of morphological abnormalities induced by slow growth treatments. Plants maintained *in vitro* for long periods accumulate ethylene and care should be taken in using culture vessels that allow gaseous exchange without compromising containment. The production of ethylene during slow growth may be attenuated by applying silver thiosulfate and other ethylene pathway inhibitors (Roca et al. 1984, 1992; Mafla et al. 2004; Chau-um and Kirdmanee 2007). It is cautionary to use minimal growth regimes that do not compromise nutritional status to the extent that severe stress is caused and to prevent this problem it may be prudent to re-formulate culture media. For example (see Chau-um and Kirdmanee 2007), this may be achieved by optimizing iron availability, changing the chelating agent, calcium supplementation and altering macronutrient composition (see *section 9.4*).

9.6 Control of contamination in medium-term storage

Personnel should be fully aware of the special contamination and containment issues that can potentially affect germplasm held in MTS:

1. Greater contamination occurs in explants from soil-derived donors.
2. Explants from perennials often have complex communities of resident flora.
3. Surface sterilized material may be non-axenic due to the presence of covert infections and systemic endophytes.
4. Latent infections can be revealed several weeks or even months or years after culture initiation.
5. Latent infection proliferation is exacerbated by stress treatments.
6. Stresses change the susceptibility of host tissue to microbial spoilage.
7. Symbiotic/commensal and harmless endophytic bacteria can become opportunistic pathogens in stressed tissues.
8. Physiological stress symptoms may be due to systemic infections and not abiotic or sub-optimal treatment factors.
9. Checks should be undertaken for cultures that are asymptomatic for signs of visual infections.
10. Covert and endophytic contaminants can interfere with molecular diagnostic tests.
11. Older shoot cultures seem to be more susceptible to contamination and can also be a source of insect and pest contamination (i.e. mites/thrips)

9.6.1 Critical points

- *An overarching, quality control system is recommended for managing containment and contamination in IVAGs and IVBGs.*
- *This should include routine, spot and process testing of cultures for covert and endophytic contamination using microbial indexing/wide-screening detection media.*
- *For persistent infections, identification of organisms may be necessary to help target strategies for their elimination.*
- *Antibiotics should be used judiciously as they can cause resistance problems.*
- *All contaminated cultures should be removed immediately from MTS.*
- *Contaminated cultures should only be discarded after autoclaving.*

9.7 Choosing minimal slow growth protocols

The principle of slow growth storage is based on optimizing subculture extension without risking germplasm loss and compromising genetic stability through stressful treatments. It is good practice when selecting an MTS protocol to conduct, over time, a cost benefit analysis of savings for resources, time, energy costs and space. This may be equated with maximum-minimum and average recovery ranges for (genebank-significant) numbers of accessions. The process may also be calibrated by exposing different genotypes to several cycles of growth retardants in order to establish robust performance and stability assessment criteria (see *sections 6, 7 and 9.9*). It is important to note that genotype response to growth limiting treatments can be highly variable (IPGRI-CIAT 1994; Pennycooke and Towill 2001). *In vitro* genebanks should therefore carefully consider the economic and efficiency benefits of optimizing standard slow growth regimes for storage-recalcitrant outliers. It is also important to note that low recovery in stress-sensitive genotypes may not be cost effective or good practice in the longer-term because of the risks of selection pressures and germplasm loss. These factors are also compounded by poor regeneration in field regeneration cycles, loss of trueness-to-type and the costs of performing genetic and epigenetic stability assessments (see *section 10.9*).

9.8 Recovery, rejuvenation and recycling

Recovery procedures for germplasm maintained in slow growth are critically dependent upon monitoring the health of stored accessions and their timely regeneration and rejuvenation. Timing of recovery is dependent on the species, accession, explants stored, media used and/or environmental conditions of storage. The processes of recovery, rejuvenation and recycling can help to reduce the risks of irretrievable losses, genetic instability and selection pressures.

9.9 Stability

Careful selection of donor germplasm and stringent optimization of storage conditions are required to minimize stress and selection pressures, therefore personnel should be aware of the precautions that minimize genetic and epigenetic changes, they include:

1. An awareness that some genotypes and accessions of asexually (clonally) propagated species are more likely to display genetic variation that is generated via tissue culture (i.e. as SCV) than are seed propagated species (Scowcroft 1984; Harding 2004) and that specific clonal genotypes may have a propensity for producing off-types and variants.
2. Optimizing subculture intervals before field regeneration is important as prolonged culture increases frequencies of chromosomal aberrations, SCV and epigenetic changes (Scowcroft 1984).
3. The possibility that *in vitro* selection pressure can potentially generate mutants.
4. Recovery responses need to be sufficiently high to offset the problem of selection.

9.9.1 Critical points

- *Minimal growth treatments can exacerbate abnormal morphogenetic responses.*
- *Stability assessment is an important quality control measure.*
- *Many different methods are currently employed for stability assessments (phenotypic, chromosomal, cytological, epigenetic, and genetic molecular tests, RAPD, DNA methylation, RFLP, AFLP) therefore careful choice and testing of analyses is required.*
- *Molecular diagnostics mainly screen a very small part of the genome and some changes may not be detected.*
- *Maintaining shoot and plantlet regeneration through preformed (original) meristems (apical and axillary buds) and avoiding adventitious shoots will greatly aid the maintenance of trueness-to-type and genetic stability and offset the risks of SCV.*
- *Field trials are recommended to test for trueness-to-type and authenticate germplasm identity, particularly for genotypes that have a propensity for instability/off-types being generated.*

9.10 Problems, trouble-shooting and improvements

The main factors limiting successful conservation in MTS are variable genotype responses, aberrant morphogenetic behaviour, hyperhydricity, necrosis, polyphenolic oxidation, covert, delayed and endophytic (systemic) contamination, poor rooting, *in vitro* senescence and stress-induced morbidity and mortality. Troubleshooting for causal factors should be a routine practice and based on timely use of performance indicators, methodical recording and careful interpretation of monitoring data. The interpretation of which should pinpoint those procedures and treatments that are the most limiting with the objective of optimizing or changing the strategy of growth limitation. Where possible, factors (e.g. temperature stress, nutrient deficiency, ethylene production) that predispose cultures in slow growth to genetic instability should be identified. Thereafter, risk management strategies and best practices should be applied to minimize the risks of instability and genetic selection (Scowcroft 1984).

10. Generic technical guidelines for long-term storage: cryopreservation

The objective of cryopreservation is the long-term storage of viable plant germplasm in LN, to a minimal temperature of -196°C in the liquid phase, or in the vapour phase. It is important to note that the temperatures of vapour phase LN can vary considerably (see Benson 2008 for a review) dependent upon the position of the sample in the storage vessel inventory system, the type, size and capacity of the cryotank, filling and top-up regimes and the ambient conditions in which the storage vessel is located. Hunt and Pegg (1996) advise that storage under liquid phase LN assures a constant temperature of -196°C , whereas they report differential *subzero* gradients of approximately 100°C for vapour phase storage. In the case of plant germplasm conserved in vapour phase storage it is important to ensure that a minimum temperature is achieved to enable the long-term stabilization of samples; this is highlighted by Walters et al. (2004) in their long-term study of cryopreserved plant germplasm. The criticality of vapour phase storage temperature is particularly important for vitrified samples (Angell 2002; Volk and Walters 2006; Benson 2008). Some manufacturers of cryogenic storage equipment recommend that the storage of cells must be performed below the water recrystallization temperature of -130°C (Nunc™ 2005, 2008). With the objective of preventing detrimental episodes of rewarming, that risk the stability of samples stored in vapour phase LN. The GPG2 project's CCTF propose that the vapour phase should be maintained at a minimum of ca. -170°C .

These technical guidelines pertain to the different methods of cryopreserving shoot meristems from clonal crops (Schäffer-Menuhr 1996; Escobar and Roca 1997; Escobar et al. 1997; Panis et al. 2005; Sakai and Engelmann 2007; Benson 2008; Engelmann et al. 2008; Keller et al. 2008; Reed 2008a, b; Sakai et al. 2008; Panis 2009). Detailed technical guidelines should be produced on a crop-specific basis. Minimal requirements used for MTS are also applicable to cryostorage (see *section 9*); specialist cryogenic equipment and facilities are highlighted.

This section has been numerated to four hierarchical levels (e.g. 1.1.1.1) to aid the readers' navigation and cross-referencing to different sequences and combinations of cryostorage procedures.

10.1 Cryopreservation terminology consensus

Increasingly, different terms and their permutations are used to describe plant cryopreservation protocols and developing best practices for cryopreservation requires common consensus on storage terminology as used by different genebanks. Common meaning and use of terms is important for quality systems as it prevents misinterpretation and underpins: (1) safe, accurate and meaningful knowledge exchanges; (2) development of standard operating procedures; (3) inter-operability of

IT systems, knowledge bases, genebank inventories and tracking systems; (4) robust technology transfers/validation exercises and (5) facilitates training programmes.

10.2 Sample type

Inventories should be in place to document the type (wild species, cultivar, landrace, breeding lines) and nature of material used for cryobanking. For these guidelines, the explant usually comprises: (1) meristematic shoots from apical/axillary shoots of *in vitro* propagated cultures; (2) *ex vitro* tuber sprouts; (3) surface-sterilized shoot cuttings from field, shade or glasshouse-grown plants, and more generally, (4) dormant buds from field-grown woody species and somatic and zygotic embryos (e.g. Citrus).

10.2.1 Shoot selection criteria

Meristem type (i.e. apical, axillary, dormant bud, species morphology, size, number of leaf primordia) influences successful storage outcomes following exposure to LN. Genetic instability can arise *in vitro* and selecting original shoot meristems generated via direct development is preferred to using those of adventitious origin. Ideally, the same type of meristem should be selected systematically, unless it is proven that meristem origin has no significant effect on post-storage recovery. However, this option can be constrained by technical problems, efficiency and cost issues, in which case, proliferating adventitious meristems may, out of necessity be used. If this is the case, confirmation of genetic stability is prudent. The number of replicates processed per cryopreserved accession should anticipate expected recovery responses, regeneration frequency, potential for germplasm loss and the use of cryobanked materials. Where probabilistic tools based on known performance metrics for viable recovery are used (see Dussert et al. 2003), it is cautious to factor in potential losses incurred by contamination, delayed onset declines in recovery, genotype variability and a potential preponderance for instability and off-types.

10.2.2 Physiological status

Developmental and physiological status and source (i.e. field, glasshouse, shade house) of *ex vitro* donor plants can influence culture initiation, micropropagation and recovery after cryopreservation. For *in vitro* donors, culture age, subculture cycle, position of meristem, and shoot size can also affect recovery. Therefore, testing the effects of physiological condition on survival after cryopreservation is suggested, particularly for recalcitrant genotypes. Knowledge of cold hardiness is helpful for those species that naturally cold acclimate and seasonal screening for hardiness and freeze tolerance is recommended in order to determine which part of a life cycle provides buds that are more amenable to cryostorage.

10.2.3 Basic assumptions

Cryopreservation of clonal crops is critically dependent upon optimizing *in vitro* culture before and after cryogenic storage, therefore, culture regimes should be thoroughly tested before proceeding to cryopreserve germplasm. Recovery after storage may require different to standard culture media to facilitate survival and shoot

regrowth. Donor explants and *in vitro* cultures should be thoroughly checked for health status as survival is compromised if tissues are already stressed, for example as may be the case for ageing cultures or materials that have been maintained under sub-optimal growth conditions (see *sections 6 and 7*). It is assumed cultures and donor materials entering a cryopreservation process have been through stringent phytosanitary procedures. Covert contaminants and systemic, endophytic infections can become highly problematic following cryogenic treatment; bacteriological indexing before cryobanking is thus recommended.

10.3 Cryopreservation procedures and protocols

Cryopreservation methods are compiled as generic guidelines on which to develop crop-specific best practices. These are presented in the following sections (see *sections 10.3 to 10.5*) as the basic sequential components (e.g. equipment/material requirements, pretreatments, meristem dissection techniques, cryoprotection regimes) which are applied in various combinations in specific protocols (e.g. ultra rapid/controlled rate cooling/freezing, droplet freezing/vitrification, Plant vitrification solution number 2 (PVS2) vitrification, encapsulation combined with dehydration, evaporative desiccation and vitrification). These are described in *section 10.6* in full, sequential technical detail, including cryostorage, rewarming and recovery. Critical points are highlighted for each procedure that comprises the different protocol steps.

10.3.1 Basic materials and equipment

Personal protective clothing and equipment (PPC and PPE) are required during LN handling which should be performed in a safe and regulated environment. Tissue culture preparation, cryoprotection and recovery should be undertaken aseptically in a laminar airflow bench, aseptic techniques should be applied throughout all procedures. The following checklist provides basic requirements for most cryopreservation protocols; critical points concerning the use of different materials and equipment are also highlighted.

10.3.1.1 Chemicals, media and reagents

Standard culture media for micropropagation, meristem excision, pretreatments, loading solutions, cryoprotectants, unloading solutions and post-storage recovery are normally prepared in either liquid or solid culture media. They are usually dispensed into aliquots of ca. 25-50 ml.

Media

- Standard culture (micropropagation) media.
- Media for pretreatments, pregrowth and preconditioning media (see *sections 10.3.2.2 to 10.3.2.4*).
- Loading and unloading solutions used for vitrification.
- Media for sequential adjustment of osmotica on thawing/rewarming.
- Recovery media.

Cryoprotectants and additives

These comprise different types, described as follows:

- Colligative: e.g. DMSO, glycerol (permeability is species dependent).
- Osmotic: mannitol, sorbitol, sucrose, ethylene glycol, polyethylene glycol.
- Specialist: additives/agents/cryoprotectants used to confer enhanced tolerance or survival after cryogenic storage and/or applied in combination with other cryoprotective strategies; examples include proline, plant growth regulators, trehalose, bovine serum albumin (BSA), antioxidants, calcium-alginate beads, heat-activated silica gel.

10.3.1.2 Cryogenic safety equipment

- Personal protective clothing: lab coat, safe (closed-toe) footwear, LN-resistant face shield, glasses, goggles, gloves and aprons.
- Personal protective equipment: oxygen monitor with audio/visual alarm.
- Cryotanks: automatic filler, low-level LN monitor with audio/visual alarm.

10.3.1.3 Cryogenic equipment

- Safe/secure LN supply, (supplied by external contractor or by in-house generator).
- Standard refrigeration: 4°C to 0°C; freezers -20°C, -80°C.
- Cold acclimating rooms/growth chambers/incubators, usually minimal 4°C to 0°C or high sub-zero temperatures as required.
- Dewars/tanks LN-approved resistant: 1-2 L capacity for airflow bench work, LN supply Dewars/tanks and pressurized supply tanks for automatic cryogen dispensing, long-term storage Dewars/tanks and inventory system.
- Computer-controlled programmable freezer, passive cooling devices (Mr Frosty®).
- Cryovials, cryocanes, cryoracks, cryodrawers, cryoboxes, cryosleeves.
- 15 mm x 5 mm sterile aluminium foils for droplet-freezing/vitrification.
- LN-equipment, pressure systems: spares, replacement supplies of essential parts.

10.3.1.4 General supplies and equipment

- Pasteur pipettes.
- Hypodermic needles and syringes.
- Autoclave.
- Filters for sterilization (e.g. Millipore®).
- Petri dishes e.g. 90 mm and 50 mm.
- Sterile filter papers, forceps, scissors, scalpels, micro-scalpels.
- Heated magnetic stirrer.
- Binocular dissecting microscope.
- Water bath.
- Thermometer, thermocouple and data logger.
- pH meter.

- Gilson or Eppendorf Pipettes, standard range (20, 50, 100, 500, 1000, 5000 μ l) or similar, branded, automatic pipettes, with sterile tips.
- Media bottles, dispensing bottles, culture bottles.
- Sterile distilled (or sterile) filtered water.

10.3.1.5 Critical points

- *Vitrification-based protocols do not usually require programmable freezers.*
- *If LN supply is ensured, nitrogen generators are not essential; however, larger scale-genebanks that have a high demand may wish to consider purchasing a generator for extending capacity and safeguarding cryobank security.*
- *For safety purposes, cryogenic facilities and equipment require regular, expert maintenance.*
- *It is a regulatory (sometimes statutory) health and safety requirement in some countries for pressurized LN equipment to be checked annually by both internal and external auditors in order to comply with government health and safety regulations.*
- *For convenience a small Dewar (e.g. of 1 L capacity) can be used for the initial cryopreservation step (i.e. plunging in LN). For example, small Dewars may be located in the laminar airflow bench (for vitrification protocols) or near to the programmable freezer to allow rapid transfer from the intermediate hold temperature. Dry shippers may be used to transport safely vials to long-term storage cryotanks located in other parts of a facility.*
- *It is important that vials are plunged immediately into LN at an appropriate point e.g. following cryoprotection or after they are removed from the programmable freezer/Mr Frosty™ unit.*
- *The uncontrolled rewarming of samples during cryo-protocol transfer stages or transportation is detrimental to cryopreserved tissues.*

10.3.2 Basic procedures

These comprise procedures that are common to most shoot meristem cryopreservation protocols; variations may be required for certain crops, species and genotypes.

10.3.2.1 Meristem dissection

The objective of this procedure is to excise shoot meristems of an appropriate size and structure that are capable of surviving cryostorage, growing new shoots and forming a stable plant via non-adventitious development. Shoot meristems are usually excised: (a) directly from untreated plants; (b) from plants that have been pretreated, acclimated or preconditioned or (c) from nodal stem cuttings that have been pretreated, acclimated or preconditioned.

Equipment and materials

See section 10.3.1.4 General supplies and equipment

Procedure

1. Meristem dissection is best achieved on a moistened, sterile filter paper (or other suitable sterile surface) that is placed in a 9 cm Petri positioned on the viewing platform of a dissecting microscope (x 20-40 magnification) that is placed in a

laminar airflow to maintain sterility.

2. Remove shoots from the culture vessel and place on the sterile surface.
3. Trim away any large leaves and roughly locate the meristematic area of the bud or shoot.
4. With the aid of two hypodermic needles and/or micro-scalpels remove larger expanded leaves from the shoot tip region taking care not to damage the meristem
 - a. It is important to be cautious at this stage to retain any partly expanded leaf primordia and a small amount of subjacent meristem tissue, before proceeding to dissect too vigorously as this will injure the meristem.
5. Carefully progress with the meristem dissection to an optimal crop-specific size:
 - a. Minimally dissected shoot meristems usually comprise a partially exposed translucent apical dome, surrounded by 1-3 unexpanded leaf primordia.
 - b. Maximally dissected shoot meristems usually comprise the apical dome, several leaf primordia, partially expanded leaves and tissue subjacent to the meristem region.
6. Trim the meristem to an optimal size [ca. 0.5 to 5 mm] dependent upon genotype/species.
7. Immediately following dissection transfer shoot tips to one of the following:
 - a. A holding solution, usually liquid medium or pretreatment solution.
 - b. Filter paper bridges soaked with the holding solution in a Petri dish.
 - c. A cryoprotective loading solution (LS).
 - d. A post-excision recovery medium, this can include 1-5% (v/v) DMSO that is applied to improve recovery following dissection injury.

Critical points

- *Standardize excision technique and optimize shoot tip/nodal cutting size by using meristem controls at all precryo steps before proceeding to test cryogenic treatments.*
- *Shoot tip size and origin (apical, lateral, axillary) can influence post-storage survival.*
- *Due to technical problems associated with excising original (i.e. non-adventitious) meristems, for some crops (e.g. *Musa*) adventitious, proliferating shoot cultures are used (Panis 2009).*
- *Shoot tips rapidly dehydrate during dissection, therefore this manipulation is best performed on a moist surface; similarly, excised meristems rapidly succumb to desiccation and require immediate transfer to a suitable holding medium. This should be of an appropriate osmotic potential e.g. isotonic relative to the pregrowth/treatment/culture medium from which the shoots were originally removed.*
- *Placing excised meristems in water or hypotonic medium during the dissection and post-dissection process can negate protection afforded by osmotica applied during pretreatment or pregrowth.*
- *Meristems may also be transferred to colligative or osmotic cryoprotectants and vitrification loading solutions (dependent upon crop specific method).*

- *It is important to note that once the excised meristem is exposed to cryoprotectant solutions the process or loading sequence is already initiated and should be timed from this onset.*
- *To standardize the cryoprotectant process, meristems should be excised in batches that are timed in known intervals from first (start) to last (completion) batch samples.*
- *It may be necessary to determine if the timing of the meristem excision process and subsequent holding times are critical to survival and if they affect the reproducibility of a cryopreservation protocol. Meristems can be excised the day before cryopreservation and incubated overnight on sterile filter papers infiltrated in 50 mm Petri dishes with an appropriate culture or pretreatment medium.*
- *Wounding of dissected meristems can sometimes be exacerbated by cryogenic stress. Pretreatment for 16-48 h in 1-5% (v/v) DMSO applied in a medium of appropriate osmotic potential can significantly enhance the recovery of meristems from some species that are especially vulnerable to physical dissection injury (Benson et al. 2007).*
- *Personnel should demonstrate proven competence in achieving meristem survival following dissection before proceeding to use excised meristems for cryogenic purposes.*
- *Meristem dissection is a highly skilled procedure; personal aptitude should be tested on a no-blame basis as not all staff acquires the required competency even after training.*

10.3.2.2 Cold acclimation

Cold acclimation (cold hardening) is a natural process in which exposure to low temperatures enhances resistance to much lower temperatures and freezing. Several options are available for acclimating plants, explants and shoot meristems prior to cryogenic treatments, these mainly exploit natural acclimation, in which case meristems are excised directly (see *section 10.3.2.1*) from cold acclimated buds. Alternatively, acclimation can be artificially simulated *in vitro*; the objective of both procedures is to enhance survival after cryopreservation.

Equipment and materials

See *section 10.3.1.4*

Procedure(s)

Option 1. Natural acclimation

1. Optimize the protocol for natural field-acclimation by selecting mother plants/shoots at different stages of the seasonal life cycle, this is usually concomitant with maximum acclimation.
2. Apply appropriate phytosanitary/surface sterilization procedures to the explants.
3. Excise shoot tip meristems (see *section 10.3.2.1*).
4. Test meristem survival through sequential *in vitro*/cryopreservation treatments.
5. Select the optimal acclimation stage of the life cycle.

Option 2. Cold storage acclimation

1. Optimize cold storage for donor material using refrigerator conditions.
2. Apply phytosanitary and surface sterilization procedures as appropriate (e.g. for donor explants that have not been introduced *in vitro* prior to cryopreservation treatments).
3. Excise shoot tip meristems (see *section 10.3.2.1*).
4. Test meristem survival through sequential *in vitro* and cryopreservation treatments.
5. Select the optimal acclimation regime for temperature and storage duration.

Option 3. In vitro cold simulated acclimation

1. Grow shoot cultures on a standard medium and transfer to cold-acclimating conditions in a suitable incubator at ca. midpoint in their previous subculture cycle or, at a suitable stage of development.
2. Optimize cold acclimation procedure for the cultures. As a general guide, plants normally maintained *in vitro* at 20-25°C may be tested for a 1-4 week cold acclimation treatment using 8 h days at 15-22°C and 16 h nights at -1 to 4°C. Different alternating temperatures may also be tested.
3. Excise shoot tip meristems from acclimated donor cultures (see *section 10.3.2.1*).
4. Test meristem survival through sequential *in vitro* and cryopreservation treatments.

Option 4. In vitro simulated acclimation using additives

1. *In vitro* shoot cultures are grown on standard medium and transferred to a medium containing a suitable additive. Transfers are undertaken ca. midpoint in their previous subculture cycle or at a suitable stage of development when the shoots are healthy and not senescent due to prolonged subculture; nodal stem segments can also be used.
2. Optimize the procedure for axenic *in vitro* shoot cultures or nodal cuttings.
3. Cold acclimation can be substituted for, or simulated (see Dumet et al. 2000) by sugars, for example sucrose applied within the range ca. 0.3 to 0.8 M, either as a single or stepwise treatment. In the latter, sucrose is sequentially increased to an appropriate concentration that is applied over 1-4 weeks.
4. Excise meristems from acclimated cultures/nodal cuttings (see *section 10.3.2.1*).
5. Test meristem survival through sequential *in vitro* and cryopreservation treatments.

Critical points

- *Sucrose-simulated acclimation can be applied to chill sensitive genotypes of naturally cold acclimating species; abscisic acid, mannitol and polyols are also used in acclimation.*

- *Different species and genotypes exhibit variable cold hardiness responses.*
- *In the absence of a specialist facility it is possible to improvise cold hardening by using manual transfers between a refrigerator at the lowest setting, and a growth room.*
- *Once acquired, cold hardiness is transient and acclimated donor materials should be maintained under acclimating conditions until they are required for meristem excision.*
- *Removing donor plants/cultures from the acclimation cycle for extended periods during processing can negate the positive effects of the cold hardening treatment and contribute to variable recovery responses.*
- *Logistically it is useful to programme the diurnal acclimation cycle for workday meristem processing to take place during the warm-light part of the acclimating sequence.*

10.3.2.3 Preconditioning

This term can be used interchangeably with cold acclimation and may also describe the simulation of natural cold acclimation *in vitro*, by the application of sucrose, abscisic acid and other agents. Preconditioning treatments also involve: (1) changing light (high, low; dark/light) as well as temperature conditions prior to cryopreservation; (2) selecting plants at specific points during subculture and (3) manipulating cultures to produce plants/excised meristems at a specific developmental stage or morphogenetic type (e.g. proliferating shoots). For technical manipulations follow the same basic format as outlined in *section 10.3.2.2* and adjust/optimize preconditioning treatments on a crop or genotype-specific basis.

10.3.2.4 Preculture, pregrowth and pretreatment

These measures enhance survival but do not usually confer total protection; generally pretreatment, preculture and pregrowth confer cumulative resistance to LN when used in conjunction with cryoprotectants. They are also used to enhance tolerance to cryoprotection stress, particularly by increasing resistance to dehydration and desiccation. The terms pretreatment, preculture and pregrowth are often used interchangeably, sometimes inclusive of preconditioning treatments. Currently there is no consensus as to their definitive use in different applications; for clarification in these guidelines preculture and pregrowth infer protective treatments applied to still-growing *in vitro* plants or shoots. The sequential application of osmotic agents administered in the subculture cycle before cryopreservation is an example of a preculture or pregrowth treatment. In the literature, pretreatment is a more general term used to either: (a) encompass pregrowth and preculture, or (b) describe short treatments for which new growth is limited or ceases. It is helpful to qualify the use of terms when developing best practices in a specific context, facility or project.

Equipment and materials

See *section 10.3.1.4*

- Cultures optimally selected for cryopreservation.
- Media comprising pretreatment additives dispensed as single or multiple additives of increasing concentrations/levels, selecting from:

- Osmotica: sucrose, mannitol, sorbitol (ca. 0.1 to 1 M or 1-10% [w/v]) applied singularly or at increasingly cryoprotective concentrations.
- Colligative cryoprotectants: DMSO (ca. 1-5% v/v); glycerol (0.1 - 0.5 M) at non-cryoprotective levels.
- Growth regulators: abscisic acid (e.g. ranges of 1-10 mg/L; 1-75 μ M); GA₃ (to stimulate shoot expansion during recovery); cytokinins (to stimulate shoot development during recovery) or other additives as required.
- Anti-stress compounds that are known to enhance protection against injuries and stresses incurred during cryopreservation treatments (e.g. proline, antioxidants). These are applied at appropriate concentrations which have been tested prior to their routine use.

Procedure

1. Various plant materials are transferred to pretreatment media as appropriate:
 - a. shoot cultures, midpoint subculture or at a required stage of development;
 - b. nodal stem segments;
 - c. excised shoot meristems (see *section 10.3.2.1*).
2. Optimize the pretreatment based on: (a) choice and combination of additive; (b) concentration and sequential applications of increasing additive concentrations and (c) duration of exposure.
3. Select optimal treatment.
4. Test the effects of the optimal treatment on survival after sequential cryopreservation treatments.
5. Select the treatment that confers the highest level of protection and recovery.

Critical points

- *In the context of these guidelines, pretreatments are generally defined as specialized media containing protective additives that are applied a few days before (24-72 h) or just prior (1-2 h) to cryopreservation.*
- *Pretreatment additives/solutions are usually prepared in standard semi-solid or liquid culture medium. In the case of osmotic additives, the cumulative level of any sucrose added to media (e.g. as an energy source in standard medium) should be confirmed.*
- *It is advisable to check medium pH after incorporating pretreatment additives.*
- *Heat labile pretreatment additives should be filter sterilized.*
- *Additives applied in combination can act synergistically and reduce the toxicity of other additives.*

10.3.2.5 Encapsulation

The objective of encapsulation is to encase excised shoot meristems in calcium alginate beads (Fabre and Dereuddre 1990; Benson et al. 1996; Engelmann et al. 2008). The procedure is undertaken either on untreated meristems, or on meristems excised from cold acclimated (preconditioned) and pretreated (pregrown, precultured) shoot cultures and nodal cuttings. When used in combination with other cryoprotective

strategies encapsulation can precede various manipulations, including loading with colligative and osmotic cryoprotectants, evaporative desiccation, and vitrification solution applications (Sherlock et al. 2005; Benson 2008; Engelmann et al. 2008). Encapsulation greatly facilitates the handling of individual, excised shoot tips.

Equipment and materials

See *section 10.3.1.4*

- Excised shoot meristems.
- Calcium-alginate solutions:
 - a. *Alginate solution*: calcium-free, liquid culture medium, containing 3% (w/v) sodium alginate (SIGMA™, 2% low viscosity, 250 cps, sodium salt derived from sea Kelp) dispensed into small bottles as 20-30 ml aliquots (slowly dissolving alginate while stirring over time might be helpful before autoclaving the solution).
 - b. *Calcium solution*: liquid culture medium containing 100 mM CaCl₂ dispensed as 20-30 ml aliquots in 50 ml conical flasks or beakers or culture bottles.
- For dispensing alginate solutions use either: (a) 5 ml Gilson "Pipetteman" (or similar automatic pipette) fitted with a sterile replaceable tips or (b) 3 ml disposable plastic pastettes or similar Pasteur pipette. When using pipette tips, it is advisable to cut the tip at an appropriate place to allow a single drop of alginate to form a bead of an optimally-sized diameter.
- A 250 - 500 µm mesh sieve.
- Sterile tissues or pieces of filter paper.

Procedure

1. Transfer shoot tip meristems to 3% (w/v) alginate solution; gently swirl to assist tissue immersion, taking care not to form air bubbles.
2. Using a 5 ml Gilson "Pipetteman" (or similar automatic pipette) or 3 ml plastic pastette, place the sterile tip in the alginate solution and gradually withdraw ca. 2 ml solution, concomitantly capturing ca. 3-5 meristems, taking care to avoid air bubble formation.
3. Hold the pipette/pastette vertical, to ensure spherical drops are formed, by delivering droplets at ca. one-second intervals into 0.1 M CaCl₂ solution; gentle swirling of the CaCl₂ solution helps to prevent the beads from sticking together.
4. The alginate will form spherical 45-50 µl beads that encapsulate the meristems and with care, beads containing single shoot meristem can be easily obtained. The usual bead size is ca. 4 mm, although this can vary dependent upon the physiology, structure and size of the meristem, shoot and leaf primordia.
5. Allow beads to polymerize in the calcium solution for ca. 15-20 min after the formation of the last bead.
6. Pour the calcium solution and beads into a sterile sieve or Petri dish.
7. Retrieve beads containing shoot tips.

8. The empty beads can be retained for moisture content (MC) assessments.
9. Use the encapsulated shoots as required in cryopreservation protocols.

Critical points

- *Solubilizing and autoclaving alginate to produce uniformly dispersed solutions can be technically challenging, therefore test the following options: (1) add the alginate to liquid medium on a magnetic stirrer, heat to boiling whilst agitating vigorously, dispense the alginate into appropriate vessels, and autoclave; (2) prepare the alginate solution by vigorously agitating (at ambient temperature) on a stirrer, autoclave in bulk, dispense and autoclave. Discard any solutions that contain flocculated 'clumps' of partially solubilized alginate after autoclaving.*
- *Ensure alginate solubilisation is uniform, as variable concentrations will have variable cryoprotective properties and behave non-uniformly during dehydration and desiccation.*
- *Prepare calcium-free standard liquid medium (Ca²⁺ salts of vitamins accepted) and add 3% (w/v) sodium alginate (SIGMA™ 2% low viscosity kelp alginate, as the sodium salt).*
- *Uniform bead size is critical for reproducible bead dehydration and desiccation so it is important to use an alginate dispenser that produces beads of a consistent size.*
- *It is possible to adjust pipette tip diameters using a hot scalpel to cut the tips to accommodate larger structures; this standardizes the pipette used for aperture size, noting that manufacturers produce pipettes with different-sized apertures.*
- *If undertaking technology transfers and validation exercises with other laboratories, it is advisable to ensure that an identical or equivalent type of pipette is used.*
- *Size differences in beads leads to non-uniform desiccation, which can affect cryoprotective properties and reduce reproducibility; this variability can influence validation exercises.*
- *The way in which the alginate drop is dispensed into the CaCl₂ solution is important, delivering too low to the surface tends to give flat beads, whilst dispensing too high above the surface produces tear-dropped shaped beads. Keeping a uniform distance of the pipette above the CaCl₂ and gentle swirling of the calcium solution helps to form uniform beads.*
- *Osmotica and additives (activated charcoal) can be applied in the alginate mixture.*
- *The brand and type of sodium alginate can have an influence on drying rates.*

10.4 Evaporative desiccation

The objective of this procedure is to: (1) reduce the amount of water available for ice formation and (2) increase cellular viscosity to a critical point that ensures a stable glass is formed. Evaporative treatments are applied to non-encapsulated, naturally cold hardened buds and cuttings, and to alginate-encapsulated meristems after they have been osmotically dehydrated (see section 10.6.3). Desiccation is achieved in a sterile airflow or over activated silica gel. For desiccation tolerant germplasm, evaporative desiccation may be the only cryoprotective treatment required when it is combined with an effective pretreatment; it is unsuitable for dehydration/desiccation sensitive (i.e. recalcitrant) germplasm.

10.4.1 Air desiccation

Airflow desiccation can be achieved passively, for example under low relative humidity (RH) conditions in a cold room used to store non-axenic acclimated bud cuttings. Alternatively, it can be achieved using the sterile air of a laminar airflow cabinet (for alginate-encapsulated meristems, see *sections 10.3.2.5 and 10.6.3*) described as follows:

10.4.1.1 Equipment and materials

See *sections 10.3.1.4 and 10.3.2.5*

- Osmotically pretreated encapsulated meristems.
- Laminar airflow bench (horizontal airflow direction).
- Sterile 50 mm or 90 mm Petri dishes.

10.4.1.2 Procedure

See *sections 10.3.2.1 and 10.3.2.5*

1. Remove alginate encapsulated meristems from the pretreatment solution.
2. Blot beads dry on sterile filter papers to remove any surface moisture.
3. Transfer the beads to the inner or outer surface of an open sterile Petri dish. In some laminar airflow systems using the inner surface of Petri dishes allows the rims to create air turbulence which can enhance the chances of non-uniform desiccation. The appropriate surface can be selected by checking the uniformity of bead desiccation in a particular airflow using MC status assessments.
4. Locate the Petri dish (open to the air) at the back of the airflow bench.
5. Air desiccate for an optimal period of time and use the beads as required.

10.4.1.3 Critical points

- *Reproducibility is dependent upon uniform bead size and evaporation rates.*
- *Ensure surface moisture is removed from beads before they are placed in the airflow.*
- *Ensure beads are evenly spread in the Petri dish and that they are not touching each other, as this can cause irregular desiccation.*
- *Desiccation profiles may vary between laminar airflow benches and/or laboratories.*
- *For quality control, technology transfers and validation exercises record the temperature and RH in airflow bench at the onset and end of the desiccation period. This will help to ensure parity between cryo runs.*
- *Where RH and ambient temperatures are variable it is advisable to substitute silica gel based desiccation and use empty 'blank' beads as controls to gravimetrically measure the water loss using an appropriate balance and drying oven (see Section 10.4.3).*

10.4.2 Silica gel desiccation

Silica gel desiccation (Sherlock et al. 2005) is preferentially used under conditions where RH, airflow and temperature are variable, it is usually applied to alginate-encapsulated meristems (see *section 10.3.2.5*) as follows:

10.4.2.1 Equipment and materials

See *sections 10.3.1.4 and 10.3.2.5*

- Osmotically pretreated encapsulated meristems.
- Sterile 50 mm or 90 mm Petri dishes.
- Heat activated silica gel.

10.4.2.2 Procedure

1. Dispense activated silica gel at a consistent weight into sterile glass 90 cm Petri dishes (e.g. 20 g/dish); alternatively metal boxes or mini-desiccators can be used.
2. Dry heat-sterilize the system in an oven if the materials are heat resistant.
3. Concomitantly this will heat-activate the silica gel.
4. Remove the desiccator (or homemade system for containing silica gel) from the oven and seal air-tight with tape or a cap.
5. Transfer the alginate encapsulated meristems into a sterile 50 mm Petri dish.
6. Overlay the open dish on the silica gel layer, this provides a support and a sterile surface on which the encapsulated meristems can be placed.
7. Desiccate for an optimal time and use as required e.g. for cryopreservation or moisture content (MC) calibration (see *section 10.4.3*)

10.4.2.3 Critical points

- *Reproducibility is dependent upon uniform bead size and evaporation rates.*
- *Ensure surface moisture is removed from the beads before they are placed in the desiccator.*
- *Ensure beads are evenly spread in the Petri dish and not touching each other.*
- *Desiccation profiles may vary if the silica gel has not been effectively heat activated.*
- *Use a standardized system for type of container, quantity of silica gel and number of beads/container.*

10.4.3 Bead moisture calibration

The objective of evaporative desiccation is to achieve a critical minimal MC that: (a) stabilizes glasses during cryopreservation, (2) ensures viability and (3) permits the regrowth of normal shoots. Ambient temperature, RH, variable laminar airflows and silica gel activation heat treatments all critically influence bead desiccation rates and thus the final MC achieved after the treatment. Calibrating bead MC profiles is important for quality control, validation and protocol optimization.

10.4.3.1 Equipment and materials

See *section 10.3.2.4* for pretreatments, general supplies and equipment.

See *section 10.3.2.5* for encapsulation supplies and equipment.

See *sections 10.4.1 and 10.4.2* for evaporative desiccation (airflow and silica) supplies and equipment.

- Two 'series' of experimental materials for MC-viability calibrations:
 1. empty alginate beads for MC determinations;
 2. alginate beads containing meristems to assess recovery.
- Batches of empty, dehydrated beads exposed to sequential concentrations of osmotic solutions e.g. 0.5 to 1.0 M sucrose for different durations of time (usually 16 to 72 h).

- Batches of empty, osmotically dehydrated beads, evaporatively desiccated for different durations of time (usually 16 to 72 h) in either: (a) sterile airflow or (b) silica gel (usually for 3-8 h).
- Duplicate bead batches containing meristems exposed to treatments (for recovery).
- Non-treated controls (empty or meristem-containing beads) to determine MC status.

10.4.3.2 Procedures

Meristem preparation

1. Excise meristems as described in *section 10.3.2.1*.
2. Encapsulate meristems as described in *section 10.3.2.5*.

Calibration of MC and survival profile

This calibration compares MC status and meristem recovery at each stage of the treatment:

1. Prepare (as above) batches of alginate beads with and without meristems.
2. Select an appropriate number of replicates (5-10 beads) for each protocol step.
3. Transfer bead batches to sucrose dehydration media (e.g. 0.3 M, 0.5 M, 0.75 M, 1.0 M).
4. Incubate for various times in the dehydration-media concentration series (e.g. overnight for 16, 24, 48, 72 h).
5. Remove dual batches of replicate beads sequentially for:
 - a. immediate MC determination;
 - b. transfer to evaporative desiccation stage.
6. Remove dual batches of beads from sucrose solutions.
7. Blot the beads dry on filter papers.
8. Desiccate, using sterile airflow/silica gel over a suitable time course (see *sections 10.4.1 and 10.4.2*).
9. Withdraw bead samples for MC determinations.
10. In parallel, repeat treatments for beads with meristems.
11. Transfer the meristems encapsulated in beads to recovery media at each treatment stage.

Moisture content determination

1. Remove beads from treatments.
2. Blot dry the beads on filter papers to remove residual surface water.
3. Record bead fresh weight.
4. Transfer the beads to an oven set at an appropriate temperature (e.g. 85°C to 105°C).
5. Monitor weight loss due to water, to a constant weight to ensure consistency.
6. Calculate the empty bead MC on the basis of:
 - (a) % Residual MC

$$\text{Residual moisture (\% fresh weight)} = \frac{\text{fresh weight (g)} - \text{dry weight (g)}}{\text{fresh weight (g)}} \times 100$$

- (b) g H₂O g dry weight⁻¹

MC calibration for optimal survival

1. Plot MC profiles against survival of shoot meristems recovered via the same process.
2. Select the optimal conditions for dehydration/desiccation and maximum survival.
3. Proceed to test these conditions for an optimal cryoprotective strategy.

10.4.3.3 Critical points

- *In humid environments, transfer of samples to and from drying ovens should be undertaken over activated silica gel in a desiccator.*
- *MC profiles can be constructed in various ways, by residual MC and/or sequential calculation of water loss at each individual stage of the process sequence from osmotic dehydration to evaporative desiccation*
- *The final MC is usually ca. 20-25% on a fresh weight basis; or ca. 0.2 to 0.4 g water dry weight⁻¹ dependent upon sucrose loading of alginate and the concentration of alginate.*
- *Some protocols apply alginate at 5% (w/v) and supplement the alginate medium with sucrose (0.5 to 0.75 M); nutrient medium may be used at reduced or half strength or ammonia-free in both the alginate and CaCl₂ solutions. Modification in the composition of alginate may alter the thermal, dehydration, desiccation and cryoprotective properties of the beads.*

10.5 Cryoprotectant solutions

Following appropriate acclimation and pretreatments excised and/or encapsulated meristems normally require further cryoprotection before they can be successfully recovered from cryostorage. Cryoprotection usually involves applying solutions of single or multiple additives of which there are several types:

- *Colligative*: penetrating chemicals that act as agents that protect cells from cryoinjuries caused by intracellular ice, dehydration, deleterious cell volume changes and damaging solution effects.
- *Osmotic*: non-penetrating (non-colligative) chemicals that osmotically reduce the amount of water available for ice formation.
- *Combinations of colligative/osmotic additives*: mixtures that act with synergistic effects.
- *Vitrification*: different permutations of colligative and osmotic chemical cryoprotectants, encapsulation and evaporative desiccation/dehydration treatments that are applied to achieve a critical cell viscosity which on exposure to low temperatures cause a glassy state to be formed. This is characterized by the glass transition temperature (T_g).

10.5.1 General preparation of cryoprotectants

These are generic methods for pretreatments, cryoprotectants, loading and unloading solutions.

10.5.1.1 Equipment and materials

See section 10.3.1

- *Standard cryoprotectants*: dimethyl sulphoxide (DMSO), glycerol, mannitol, sorbitol, sucrose, ethylene glycol.
- *Special components*: sodium alginate and calcium chloride solutions.
- *Occasional cryoprotectants and protective additives*: polyethylene glycol (various molecular weights), BSA, proline, abscisic acid, antioxidants.
- Appropriate semi-solid media used as a base and diluent for the cryoprotectant.
- Appropriate liquid culture media used as a base and diluent for the cryoprotectant.
- Millipore/micropore filters.
- Sterile 20-50 ml bottles or similar small vessels used for dispensing aliquots of cryoprotectants and their associated solutions.

10.5.1.2 Procedures

Dispensing

1. Select, on a crop-specific basis an appropriate diluent usually: (a) standard or basal liquid medium and/or (b) standard or basal semi-solid culture medium.
2. Prepare cryoprotective/loading solutions using an appropriate procedure:
 - a. For (%) -based formulations dispense:
 - i. *volumetrically (v/v)* by volume; or adjust volume for specific gravity (w/v);
 - ii. *gravimetrically (w/v)* using scales to weigh both liquids and solids.
 - b. For molar-based solutions dispense on a formula/molecular weight basis.
3. Check pH after adding cryoprotectants.
4. Dispense solutions as 10-50 ml aliquots into suitable vessels:
 - a. bottles, 25-50 ml conical flasks for solutions;
 - b. 50 mm or 90 mm Petri dishes or culture vessels for semi-solid medium treatments.

Sterilizing

1. Check additives for heat sensitivity.
2. Filter-sterilize heat labile constituents.
3. For heat tolerant additives, prepare in standard liquid or solid media, dispense as 10-50 ml aliquots in suitable vessels (McCartney bottles, 25-50 ml flasks or similar) and autoclave.
4. For heat sensitive additives, prepare standard or basal liquid or solid medium, dispense as 10-50 ml aliquots into a suitable vessel autoclave and cool to a safe temperature (minimal critical temperature for heat labile substances).
 - a. Add the filter sterilized additives, mix thoroughly and dispense into previously sterilized bottles, 25-50 ml flasks Petri dishes or culture vessels.
5. Label and store appropriately.

10.5.1.3 Critical points

- *Cryoprotectants and pregrowth additives should be of the highest purity.*
- *Spectroscopic grade or similar is recommended for DMSO as lower grades can contain harmful contaminants.*
- *See manufacturers' Material Safety Data Sheets for information concerning cryoprotectant additive recommended storage and sterilizing temperatures.*
- *Take care when interpreting protocols that use sucrose as a cryoprotective additive; the solution is made to the final concentration/level of sucrose advised for cryoprotective purposes i.e. not the cryoprotectant loading of sucrose plus the sucrose normally used for culture/growth purposes, unless otherwise specified.*
- *Check medium pH after incorporating additives and before autoclaving (if possible); this may not be feasible when applying filter sterilized additives.*
- *Cryoprotectant mixtures should be filter sterilized, when this is technically difficult e.g. for high viscosity solutions, autoclaving is the only practical option.*
- *Lengthy autoclave hold times after the sterilization cycle should be avoided to prevent heat-degradation and caramelization of sugars.*
- *It is recommended that cryoprotectant mixtures are freshly made and used without prior storage, particularly those containing DMSO and complex biochemical additives (e.g. abscisic acid, BSA, proline, antioxidants).*
- *Thermal and cryoprotective properties may change after a prolonged shelf life.*

10.6 Cryopreservation protocols

This section describes how the various generic procedures and methods described in the previous section(s) are combined in specific cryopreservation protocols. Contemporary plant cryopreservation comprises various permutations of different pretreatments and cryoprotection strategies, these can be considered in terms of freezing, colligative cryoprotection and vitrification as follows:

- *Freezing with colligative and/or osmotic cryoprotection:*
 - *Ultra rapid freezing:*
 - *in cryovials,*
 - *in (μ l) droplets of cryoprotectants (termed droplet freezing) dispersed on aluminium foils.*
 - *Controlled rate freezing:*
 - *in cryovials and using a computer-controlled programmable freezer (noting that some models do not use computer-based systems),*
 - *in cryovials and using a passive cooling unit "Mr Frosty®."*
- *Vitrification by various strategies:*
 - *osmotic dehydration/evaporative desiccation of non-encapsulated or encapsulated meristems,*

- using ‘cocktails’ of osmotic and colligative cryoprotectants at high concentrations, administered in 1-2 ml volumes in cryovials or in micro (μ l) droplets (termed droplet-vitrification) dispersed on aluminium foils,
- applying cocktails of osmotic and colligative cryoprotectants applied at high (Molar) levels to encapsulated meristems.

Colligative cryoprotection is combined with controlled rate or ultra rapid freezing; this procedure involves the formation of extracellular ice, albeit some of the intracellular material may become partially vitrified. Vitrification is the creation of a metastable amorphous glassy state in highly viscous solutions; this is characterized by the glass transition temperature (T_g) which is the thermal point at which a glass is formed. Vitrification is a useful alternative to controlled rate freezing and it has the advantage that tissues remain ice-free; these protocols support the direct immersion of samples in LN without the need for programmable freezing equipment. However, glasses are thermally unstable and glass relaxation and devitrification can occur on rewarming and these events may risk ice formation. Therefore, glass stabilisation is critical, this is achieved by manipulating different cryoprotective additives, (e.g. glass-stabilizing sugars) and the careful control of rewarming regimes.

10.6.1 Ultra rapid freezing

The ultra rapid freezing of shoot meristems can be achieved by direct immersion in LN of the colligatively/osmotically cryoprotected materials contained either in cryovials or cryoprotectant droplets.

10.6.1.1 Droplet freezing

This protocol was first devised by Kartha et al. (1982) for cassava shoot tips and was later adapted for potato by Schäffer-Menuhr et al. (1996). These guidelines provide a generic droplet freezing method based on the technical protocol of Keller et al. (2008) and Schäfer-Menuhr et al. (1996) as developed for potato.

Equipment and materials

See sections 10.3.1, 10.3.2.4 and 10.5.1

- Optimally pretreated, excised meristems (see section 10.3.2.2 and 10.3.2.3).
- Freshly prepared, filter sterilized 10% (v/v) DMSO (see section 10.5).
- Sterile aluminium foil strips of 0.6 x 1.5 cm dimensions.
- Cryovials.
- Polystyrene float with holes of a suitable size to support the diameter of cryovials.
- Polystyrene box for LN.
- Water bath.
- Recovery medium comprising 1% (w/v) agarose dispensed into liquid culture medium.
- Standard culture medium for shoot propagation.

Procedures

Cryoprotection

1. Dispense ca. 2-3 ml 10% (v/v) DMSO into a Petri dish containing a filter paper overlay.
2. Transfer excised shoot tips onto the surface of a DMSO-soaked filter paper:
 - a. with the aid of a hooked hypodermic needle/forceps,
 - b. avoid causing physical damage to the meristem (*see section 10.3.2.1*).
3. Cryoprotect for an optimal duration (ca. 30 min to 3 h) during which time prepare the following materials:
 - a. label the cryovials ensuring that the ink/surfaces are LN-resistant/indelible,
 - b. place the aluminium foils on a sterile Petri dish,
 - c. fill the polystyrene box with LN,
 - d. open the cryovials and place in the holes of the polystyrene float on the LN surface,
 - e. fill the cryovials with LN.
4. Dispense 5 x 2 µl droplets of 10% (v/v) DMSO onto the sterile aluminium foils.
5. Transfer a single meristem to each 10% (v/v) DMSO droplet.

Freezing

1. Transfer the foils supporting the droplets to the open cryovials and plunge directly into LN.
2. Submerge the foils for a few seconds until they have reached LN temperature.
3. Refill the open cryovials with LN.
4. Repeat the process to complete the batch of meristems.

Storage

1. Close the cryovials, optionally transfer to cryo-canes, enclosed in a plastic LN-resistant cryo-sleeve before transferring to:
 - a. 1 L bench top Dewar used to transfer them to the cryotank,
 - b. transfer to the cryotank directly,
 - c. store appropriately in inventories, in either the vapour or liquid phase of LN.

Thawing

1. Warm agarose medium in a 70°C water bath, after it has melted set the bath to 50°C, to ensure that the agarose does not set during subsequent manipulations.
2. Label the liquid culture medium for each cryovial.
3. Fill the polystyrene box with LN.
4. Retrieve the cryovials from the cryostorage tank and place them in the holes of the polystyrene float which is overlaid on the surface of the LN in the polystyrene box.

5. Remove the lids from the cryovials and using forceps retrieve the foils/meristem-droplets, transfer immediately to bottles containing liquid culture medium.
6. The meristems will thaw rapidly and float into the liquid culture medium.
7. Repeat the process for the remaining meristems.
8. To avoid cross-contamination use separate bottles for each cryovial.

Recovery

1. Dispense 5 drops of agarose solution onto the surface of an empty sterile 50 mm Petri dish.
2. Retrieve the meristems from the thawing solution and place one in each agarose solution droplet.
3. Repeat the process for the remaining meristems.
4. To avoid cross-contamination, use a separate Petri dish for each recovery bottle.
5. Allow the agarose to solidify for ca. 30-60 min.
6. Dispense 2.5 ml of liquid culture medium in Petri dishes with agarose/meristems and seal the Petri dishes with Parafilm® or cling film.
7. Recover under species-specific growth room conditions.
8. Assess recovery for up to 6-12 weeks, dependent upon species/genotype.
9. Once new shoots have developed transfer to standard culture medium.

10.6.1.2 Critical points

- *Optionally, to improve survival after dissection injury and shoot regeneration after cryogenic storage 1-5% (v/v) DMSO may be added to the pretreatment solution and the meristems incubated overnight before they are cryopreserved (Benson et al. 2007).*
- *The droplet freezing protocol may contravene health and safety regulations for some countries and institutions. This is because the use of cryovials outside manufacturers' safety recommendations (i.e. purposeful filling and sealing of vials with LN); non-LN resistant vessels such as polystyrene boxes may not be compliant with the safe use of LN.*
- *Due to the direct exposure of meristems to LN, this procedure is not contained and strict precautions should be taken to ensure that the germplasm has been through a stringent phytosanitary process and is confirmed free of covert or endophytic microbial infection.*

10.6.2 Controlled rate freezing

Controlled rate freezing is used synonymously with the terms controlled rate cooling (using passive or computer-based cooling methods) and two-step freezing, for which there are a range of protocols. These guidelines present a generic method that is based on several standard procedures (Escobar et al. 1997; Benson et al. 2007; Reed and Uchendu 2008). Controlled rate freezing is usually preceded by cold acclimation, pregrowth and pretreatment of the donor tissues and/or their excised meristems.

10.6.2.1 Equipment and materials

See sections 10.3.1, 10.3.2.4 and 10.5.1

- Excised meristems (see sections 10.3.2.1, 10.3.2.2, 10.3.2.3 and 10.3.2.4).

- Freshly prepared sterilized cryoprotectants (see *section 10.5.1*) examples include:
 - Single colligative additives*
 - 10% (v/v) DMSO (Benson et al. 2007)
 - Multiple additives: colligative and osmotic cryoprotectants*
 - 15% (v/v) DMSO and 3% (w/v) sucrose (Kantha et al. 1982)
 - 1 M sorbitol, 10% (v/v) DMSO and 4% (w/v) sucrose (Escobar et al. 1997)
 - 10% (w/v) each of polyethylene glycol (MW 8000) glucose and DMSO (Reed et al. 2001).
- Ice, ice bath or bucket.
- Controlled rate cooling apparatus:
 - Option 1. Computerized programmable freezer (see *section 10.6.2.1*)
 - Option 2. Passive freezer Mr Frosty™ (see *section 10.6.2.2*)
 - Option 3. Stirred methanol bath with cooling and heating elements.
- Water bath set at 40-45°C.
- Bottles containing sterile-distilled water.
- Antibacterial wash or 70% (v/v) alcohol.
- Recovery medium.
- Standard culture medium for shoot propagation.

10.6.2.2 Procedure(s)

See sections *10.3.2.1, 10.3.2.2, 10.3.2.4 and 10.5.1*

Cryoprotection

1. Clearly label cryovials ensuring marker inks are LN-resistant and indelible.
2. Dispense 1-2 ml of sterile cryoprotectant solution into a batch of 2-3 ml cryovials.
3. Transfer shoot meristems as manageable batches to the cryovials.
4. Choose one of two options for applying the treatment:
 - a. cryoprotection on ice/ice-slush (0-4°C), which is the more usual option.
 - b. cryoprotection at ambient temperatures (ca. 20-25°C) for germplasm that is sensitive to chilling.
5. Cryoprotect for an accurately timed, optimal period (usually 30 min to 2 h).

10.6.2.3 Controlled rate freezing using a programmable freezer

A programmable freezer is usually a computer-controlled instrument comprising: (1) a freezing chamber with a thermal event recording unit and thermo-couples for monitoring sample and chamber temperature; (2) a computer and printer and (3) a cooling system supplying the sample chamber which uses pressurized LN as the cryogen; in older instruments solvents are used as the coolant. A programmable freezer can be programmed with multiple options and it can usually store eight or more programmes dependent upon the manufacturers' specifications.

Procedure(s)

Programming

1. Preprogramming a computer controlled rate freezer according to the manufacturers' instructions is optimized for a crop-specific programme, some examples of parameters are:
 - The start temperature which is appropriate to the cryoprotection incubation temperature:
 - 0-4°C or 20-25°C.
 - A 5 min hold time to load the cryovials.
 - Cooling rate (-0.25 to -5°C/min)
 - As required, an optional "ice seeding" stage is programmed, usually within a range of -5 to -15°C, followed by a hold of 5-20 min to allow for extracellular ice nucleation/initiation of the excursion of intracellular water.
 - Ice seeding can be achieved manually by touching cryovials with forceps chilled in LN vapour or, by the automated control of the programmable freezer (this is optional dependent upon the manufacturer).
 - A resumed cooling ramp to a terminal transfer temperature.
 - The terminal transfer temperature, usually at or around the temperature of homogeneous ice nucleation (ca. -40°C).
 - A hold time ca. 30 to 45 min, usually programmed to allow the transfer of cryovials to LN from a constant temperature.
2. Programmes are checked for entry accuracy by using the pre-run programme facility of the computer-controlled freezer unit.
3. Programmes are saved and a print out is retained as a copy for reference and quality control purposes.
4. Following manufacturer's specific safety and operational instructions the LN is connected to the cooling line and the pressurized LN dispenser to the freezer unit. In the case of solvent-cooled freezers this step does not apply.
5. The unit is pressurized to the manufacturer's recommended level and the apparatus is checked for LN leaks. In the case of solvent-cooled freezers this step does not apply.
6. The lid of the freezing chamber is checked for security.

Basic freezing protocol

1. Place a reference cryovial containing cryoprotectant in the freezer chamber.
2. Insert the instrument's sample-temperature probe into the reference vial (this records sample temperature, ice nucleation and thermal events during the run).
3. Transfer remaining cryovials (containing the meristems) to cryocanes or freezer racks and insert in the programmable freezer (this takes ca. 5-10 min).
4. Complete the loading process before the end of cryoprotection incubation.

5. Ensure the lid of the freezing chamber is secure.
6. Once the cryoprotection period is completed, press *RUN*.
7. Check the real time progress of the programme on the computer and/or printout.
8. On completion retain a hard and digital copy of programme prints out for quality control.
9. On completing the programme an alarm will normally sound.

Storage

1. Remove the freezer chamber lid and quickly transfer the cryovials to a small LN-filled Dewar.
2. This should be located by the side of the freezer to enable safe transfer, this procedure reduces the risks of un-controlled rewarming of the cryovials.
3. Optionally, rapidly enclose the cryocanes in a plastic LN-resistant cryosleeve before transferring to:
 - a. 1 L bench top Dewar used to transfer them to the cryotank,
 - b. the cryotank directly,
 - c. store as appropriate in inventories in either the vapour or liquid phase of LN.

Thawing

1. Transfer the bottles containing sterile-distilled water to the 40°C to 45°C water bath and allow a few minutes for them to stabilize at that temperature.
2. Transfer the cryovials from the cryotank to a small LN-filled Dewar and transfer the Dewar to the side of the water bath.
3. Rapidly transfer the cryovials to bottles containing sterile-distilled water (one vial/bottle), after the ice has melted remove them immediately to avoid over-heating.
4. To remove excess and potentially contaminating water from the water bath, wipe the outside of the cryovial with a tissue soaked in an antibacterial/sterilizing solution or 70% (v/v) alcohol.
5. Transfer the cryovials to a laminar airflow bench.

Recovery

1. Expel the vial contents onto sterile filter papers contained in a 50 or 90 mm Petri dish; the cryoprotectant can be removed by several options. These are described by Reed and Unchendu (2008), who comment that in the case of shoot tips exposed to cryoprotectants and controlled rate cooling it is possible to rinse with liquid medium. They also remark that for several protocols it is usual for standard liquid culture medium to be used to dilute and remove cryoprotectants. This may be due to the fact that additives applied in controlled rate cooling are colligative and penetrating, and as such do not expose the germplasm to the same degree of osmotic stress as non-penetrating cryoprotectants. However, if osmotica are applied it may be necessary to take precautions in the rinsing stages and gradually reduce the osmotica or wash

with a medium of similar osmotic strength (Benson et al. 2007), for example by:

- a. capillary action of the filter papers,
 - b. rinsing in liquid culture medium,
 - c. rinsing in a washing solution (e.g. sucrose) of the same osmotic strength as the cryoprotectant mixture.
2. Transfer the shoot tips to recovery medium dispensed in Petri dishes and seal the Petri dishes with Parafilm® or clingfilm.
 3. Recover meristems under species-specific growth conditions.
 4. Assess recovery for up to 6-12 weeks dependent upon genotype-specific rate of recovery.
 5. Once new shoots have developed, transfer them to standard culture conditions.
 6. Morphologically normal shoots are regenerated prior to the final score of a successful outcome in order to confirm positive and sustained recovery.

10.6.2.4 Controlled rate freezing using Mr Frosty™

Mr Frosty™ is the trade name for a small (ca. 86 x 117 mm) portable freezing unit comprising a polyethylene/polycarbonate “cryochamber”. It holds 18 to 20 cryovials (1 to 2 ml size) in foam insert. The tank, once filled with isopropanol cools at a controlled, but fixed rate of -1°C/min (Reed and Unchendu 2008). The terminal transfer temperature of the samples placed in the vials is dependent upon the freezer specifications (usually -20°C, -70 or -80°C). The rate of cooling in Mr Frosty™ is usually linear over the range of cooling from -10°C to -15°C, after which the *sample* temperature profile loses its linear cooling rate as it cools more slowly to reach equilibrium at -20°C (see Nalge Nunc International 2003). Plant materials, equipment, cryoprotection, thawing and recovery procedures are the same as those described for the programmable freezer protocol (see *section 10.6.2.1*)

Equipment and materials

See *sections 10.3.1, 10.3.2.4 and 10.5.1*

- Nalgene™ Cryo 1°C freezing container or a Mr Frosty™.
- Cryovials Nunc 377267 CryoTube™ 1.8 ml Nunc internal Starfoot round (50/bag450/case) available from Nalgene or equivalent.
- Isopropanol (C₃H₈O) sometimes abbreviated to “IPA”.
- Thermocouple or electronic thermometer (data logger) capable of accurately measuring and recording temperatures to -80°C and monitoring the stability of refrigerator/freezer temperatures (e.g. when applied overnight).
- Cryoprotectant mixture.

Procedure

See *sections 10.3.2.1, 10.3.2.2, 10.3.2.4 and 10.5.1*

Temperature calibration test

1. Fill the Nalgene™ Cryo 1°C freezing container or Mr Frosty™ unit with clean isopropanol (C₃H₈O) "IPA" to the 250 ml line on the freezing unit.
2. Transfer the cryovials with cryoprotectant to the cooling unit.
3. Insert a thermocouple into one of the cryovials and close the lid of the apparatus.
4. The lead of the thermocouple should be sufficiently narrow to allow door closure.
5. The temperature data logger should be placed external to the freezer.
6. Place the Mr Frosty™ unit in a -70°C or -80°C freezer.
7. The Mr Frosty™ unit will passively cool at -1°C/min.
8. Record the timing of the thermal nucleation events on the temperature logger.
9. Repeat the procedure at different places in the freezer.
10. Identify any local temperature effects, changes in cooling/nucleation profiles and the timing of the thermal events.
11. Calibrate cooling profiles of the Mr Frosty™ unit and determine consistency between freezing runs.
12. Retain information for quality control and validation purposes.

Freezing

1. Fill the Nalgene™ Cryo 1°C Freezing Container or a Mr Frosty™ unit with clean isopropanol (C₃H₈O) "IPA" to the 250 ml line on the freezing unit.
2. Transfer the cryovials to the cooling chamber.
3. Insert a thermocouple in one of the cryovials, this is designated a surveillance vial which is used to check for temperature changes.
4. Close the Mr Frosty™ unit (as above).
5. Cool at -1°C/min and record the thermal events on the thermocouple/temperature logger.
6. Based on the calibration information, cool to an optimized terminal transfer temperature/time.
7. Hold at the terminal temperature (usually around the temperature of homogenous ice nucleation, -40°C)
8. Based on calibration information of real time temperature, remove the unit.
9. Rapidly transfer the vials to cryocanes or a suitable inventory system.
10. Plunge into LN in a 1 L Dewar located next to the freezer.
11. Then transfer to the cryotank.

Proceed to thawing and recovery stages as for Programmable freezer *section 10.6.2.1*

10.6.2.5 Critical points

Critical cryogenic factors specific to controlled rate freezing are highlighted below; see previous and following sections for critical factors that are more generally relevant to other

(e.g. vitrification and ultra rapid freezing) cryopreservation protocols and their associated procedures.

- Excessive freeze-induced dehydration can lead to colligative damage that is caused by the harmful concentration of solutes and cell volume changes.
- In the case of controlled rate freezing a penetrating colligative cryoprotectant(s) is thus required and is usually applied in combination with osmotic additives and pretreatments.
- Colligative cryoprotectants are optimally used to reduce harmful solution effects, deleterious volume changes and osmotic stress.
- Initiation, “seeding” or nucleation, of extracellular ice is a critical factor as it creates a water vapour deficit between the inside and outside of the cell, thus causing intracellular water to move outside the cell.
- Nucleation thus results in cryoprotective freeze-induced cellular dehydration.
- Optimized controlled rate cooling of tissues to an intermediate sub-zero freezing temperature helps to ensure that sufficient intracellular water moves to the outside of the cell, thereby reducing the water that is available to form ice inside the cell.
- A “hold” is programmed at a fixed time and terminal transfer temperature, usually at, or around, the point of homogeneous ice formation, which is ca. -40°C .
- Additional optional cooling ramps/hold times can be incorporated to optimize the freeze-induced excursion of water from the cell.
- Programmable freezers consist of a freezing chamber cooled by liquid nitrogen, temperature probes (connected to the sample and the chamber) are used to monitor precisely thermal history and by means of computer programming it is possible to investigate a wide range of cooling/freezing rate and hold time parameters.
- With an appropriate output device (e.g. chart recorder/PC) the temperature at which extracellular ice is formed (the latent heat of crystallization) may be determined with reasonable accuracy. Albeit this is not always possible as it is dependent upon the capabilities of the freezing unit.
- Ice nucleation can mark the onset of intracellular dehydration and, as such, it can be an important factor in developing a controlled-rate cooling method.
- In the absence of external intervention, it is possible for extracellular ice nucleation to occur randomly (i.e. via heterogeneous ice nucleation), however, this may be problematic and can lead to variable freezing responses between different programmable freezers.
- Some programmable freezers are fitted with a device, which initiates nucleation by mechanically agitating the cryogenic samples. It is also possible to induce nucleation by touching the outside of the cryovial with a liquid nitrogen-chilled instrument.
- It is important that vials are plunged immediately into LN after they are removed from the programmable freezer or Mr Frosty™ unit; rewarming of the samples during this transition is detrimental to the tissues. For convenience a small Dewar (e.g. 1 L capacity) can be located near the programmable freezer and used to transport cryovials to the long-term storage site.
- Qualified service engineers should service programmable freezers annually.

- *Mr Frosty™ provides a simple and cost effective alternative.*
- *Used singularly Mr Frosty™ is not efficient for large batches of samples as only a few samples can be processed and using one rate of freezing. However, several Mr Frosty™ units may be used at the same time increasing the number of samples that can be batch-processed.*
- *It is recommended that between each new freezing run IPA is decanted from the Mr Frosty™ chamber which should be washed, dried, and stored at room temperature.*
- *Manufacturers recommend that alcohol should be replaced after every fifth use of Mr Frosty™.*

10.6.3 Encapsulation-dehydration

Fabre and Dereuddre (1990) first developed this cryoprotective method for *Solanum phureja*, it involves encapsulating shoot meristems in a calcium-alginate bead (see section 10.3.2.5) followed by osmotic dehydration in sucrose, evaporative desiccation in sterile airflow, or over silica gel (see section 10.4.2) followed by direct plunging in LN. Encapsulation-dehydration is dependent upon achieving a critically low MC which supports the vitrification of the alginate bead and the entrapped meristem. This generic protocol is based on the method developed for potato by Fabre and Dereuddre (1990) and adapted by Benson et al. (1996), Keller et al. (2008) and Sherlock et al. (2005) for silica gel. Crop and genotype-specific modifications may be required when applying the method to a wider range of species (Engelmann et al. 2008).

10.6.3.1 Equipment and materials

See sections 10.3.1, 10.3.2.4, 10.3.2.5 and 10.4

10.6.3.2 Procedure

Pretreatments and preparative steps

1. For meristem excision and bead preparation see section 10.3.2.1
2. For encapsulation see section 10.3.2.5
3. For sucrose pretreatments see section 10.3.2.4
4. For desiccation see sections 10.4.1, 10.4.2 and for MC calibration see section 10.4.3

Basic osmotic dehydration and evaporative desiccation steps

1. Place encapsulated meristems into a 0.75 M sucrose solution prepared in liquid medium.
2. Incubate in a 50 ml conical flask overnight (16 h) on a reciprocal shaker.
3. Remove, beads, blot dry on sterile filter paper.
4. Desiccate in a laminar airflow or over silica gel for 3-8 h or for an extended duration which is dependent upon achieving an optimal exposure time.

Cryopreservation

1. Remove beads, transfer to cryovials and dispense 5-25 beads/vial.
2. Directly immerse the cryovials into LN in a cryotank.

Rewarming

1. Remove cryovials from the cryotank.
2. Warm the cryovials at ambient temperatures for 20-30 min.
3. Wipe the exterior of the cryovials with a sterilant solution.
4. Remove beads and either transfer to solid recovery medium or re-hydrate in standard liquid culture medium for 10-20 min (Reed 2008b).

Recovery

1. Transfer the beads to standard culture medium.
2. Monitor for recovery for 6-8 weeks or longer as required.

10.6.3.3 Critical points

- *Desiccation profiles may vary between laminar airflow benches and laboratories and alginate brands.*
- *To monitor quality control between cryoruns record the temperature and RH in the airflow bench at onset/end point of desiccation period.*
- *Where RH and ambient temperature are variable use silica gel based desiccation.*
- *Some protocols recommend rewarming beads at 45°C for approximately 1 min.*
- *Transferring beads to fresh medium during the first 1-3 days can aid recovery.*
- *Some protocols recommend excising meristems from the beads during recovery (e.g. after one week, this involves cutting the bead in half with the cut on one side of the shoot tip and carefully excising the shoot meristem, alternatively the exposed shoot grows out of the cut end of the bead.*
- *Encapsulation-dehydration has a proven efficacy for several crops/species that are unresponsive to controlled rate cooling, but it has the disadvantage of not being applicable to desiccation intolerant germplasm.*

10.6.4 Vitrification using PVS2

Vitrification is the solidification of a liquid without crystallization and it is achieved by applying cryoprotectants that are sufficiently concentrated to create an amorphous glassy state on cooling. This is a thermal event punctuated by the glass transition temperature (T_g). Steponkus et al. (1992) describe plant vitrification in five steps:

1. **Loading:** equilibration in a solution of permeating (colligative) cryoprotectants (e.g. DMSO, ethylene glycol and glycerol, albeit permeability may be cell specific).
2. **Dehydration:** dehydration in a concentrated, osmotically active cryoprotectant(s) (e.g. sucrose).
3. **Rapid cooling:** the germplasm is vitrified by direct plunging into LN.
4. **Warming:** the germplasm is rewarmed either rapidly or by stepwise increases in temperature.
5. **Unloading:** vitrification solution removal by dilution and/or washing.

In practice, loading and vitrification solutions comprise mixtures of penetrating and non-penetrating cryoprotectants; several have been used for plant germplasm, (Steponkus et al. 1992). **PVS2** was devised by Sakai and colleagues; Sakai and Engelmann (2007) and Sakai et al. (2008) provide a history and summary of the different range of protocols that use PVS2 and other derivative methods that are termed PVS protocols. Sakai et al. (2008) report **PVS3** as an alternative vitrification solution comprising (w/v) each of 40% sucrose and 40% glycerol (w/v) prepared in liquid medium. In the wider literature (Reed 2008a), PVS3 is reported to comprise 50% (w/v) sucrose and 50% (w/v) glycerol. PVS2-based protocols are the most commonly used vitrification methods applied to the mandate crops for which these multi-crop guidelines are designed. The following generic method is described as a basic protocol, it may however require optimization for specific crops, genotypes and different types of germplasm.

10.6.4.1 Equipment and materials

See sections 10.3.1, 10.3.2 and 10.5.1

10.6.4.2 Procedure

1. For meristem excision, see section 10.3.2.1
2. For pretreatments, see section 10.3.2.4

Cryoprotectant preparation

Using the methods described in section 10.5 prepare the following solutions in liquid culture medium. Sucrose is added to the final concentration required for cryoprotection.

- **Loading solution:** usually 2 M glycerol and 0.4 M sucrose.
- **PVS2:** 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (final level/concentration).
- Optionally prepare a PVS2 dilution series: ca. 50, 60, 70, 80 % (v/v) PVS2/liquid culture medium as sequential loading of increasing concentrations may be required to avoid toxicity.
- **Unloading solution:** 1.2 M sucrose (final concentration) in liquid culture medium.

Loading

Prepare/excise meristems (section 10.3.2.1) apply a pretreatment as appropriate.

1. Transfer meristems to 2 M glycerol and 0.4 M sucrose loading solution for 20-25min at 25°C.

PVS2 toxicity test

Construct a toxicity trial for PVS2 based on testing the effects of solution concentration and duration of exposure on germplasm survival, as follows:

1. Treat meristems with ice-chilled (PVS2 can for some species also be used at room temperature) 1 ml aliquots of PVS2 within the range of 50-80% (v/v) to a specific concentration and exposure time (e.g. 1-20 min).

2. Remove almost all the vitrification solution, replace with intermediate increasing % solutions, stepwise to higher % levels of PVS2.
3. Gradually increase the level of PVS2 until tissues are in a 100% solution.
4. Remove the 100% PVS2.
5. Replace with "unloading" solution containing 1.2 M sucrose in standard liquid medium.
6. Wash 2-3 times in fresh unloading solution and maintain in this solution for up to 30 min.
7. Expel vial contents onto a sterile filter paper to soak away the liquid excess.
8. Transfer meristems to recovery medium.
9. Select the treatment which permits maximum survival and tolerance to cryoprotection.

Cryopreservation

1. Apply optimal PVS2 treatments on ice as determined by the toxicity test applied to meristems (as described above).
2. Once in 100% PVS2, transfer the meristems to cryovials (5-25/vial) to which 0.5-1 ml of fresh PVS2 has been added.
3. Directly plunge the cryovials in LN contained in a 1 L Dewar.
4. Transfer the vials to a cryotank for storage.

Rewarming

1. Transfer bottles containing sterile-distilled water to a 45°C water bath and allow a few minutes for them to stabilize at that temperature.
2. Transfer the cryovials from the cryotank to a small LN-filled Dewar and transfer the Dewar to the side of the water bath.
3. Proceed to rewarm using one of two options:
 - a. rapidly transfer the cryovials to bottles containing sterile-distilled water (one vial/bottle) to the 45°C water bath and agitate for 1-2 min, remove to avoid over-heating,
 - b. hold the cryovials for 1 min at ambient (20 to 25°C) to allow for glass relaxation and to avoid fractures, then proceed to transfer to a 45°C water bath and proceed as in (a).
4. To remove excess and potentially contaminating water, wipe the outside of the cryovial with a tissue soaked in an antibacterial/sterilizing solution or 70% (v/v) alcohol.
5. Transfer the cryovials to a laminar airflow bench.

Unloading

1. Remove the 100% PVS2 and replace with 1.2 M sucrose unloading solution.
2. Perform 2-3 washes in fresh unloading solution.
3. Maintain in the final rinse solution for 20 to 30 min.
4. Transfer the meristems to a sterile filter paper and allow the solution to drain away

Recovery

1. Place a sterile filter paper over the surface of a Petri dish containing solidified culture medium.
2. Transfer the meristems onto the surface of the filter paper.
3. After one day, transfer the meristems to a new filter paper/Petri dish with fresh medium.
4. Continue this process for 1-3 days after which transfer the meristems to medium only.
5. Monitor recovery regularly for 6-8 weeks or longer as required.

10.6.4.3 Critical points

- *Ensure PVS2 contains sucrose at a final level of 0.4 M.*
- *Applying (w/v) DMSO, glycerol and ethylene glycol additives to liquid culture medium already containing 0.4M sucrose does not result in a final level of 0.4 M sucrose which volume per volume is diluted out by the other cryoprotectants.*
- *A simple check test: on exposure to LN, correctly prepared PVS2 solution should be completely clear i.e. “glassy”, some glass fractures (cracks) may be observed.*
- *If the PVS2 becomes white/opaque on exposure to freezing temperatures it has not been prepared correctly as opacity indicates ice formation. In this event, the preparation of PVS2 should be re-checked.*
- *PVS2 is very viscous and takes some time to dissolve, therefore the gradual addition of cryoprotectants during vigorous agitation on a magnetic stirrer is recommended.*
- *Adjust the pH of PVS2 after formulation.*
- *Filter sterilization of small volumes of PVS2 is recommended.*
- *Make fresh PVS2 for each use.*
- *Pretreatments (pregrowth, preculture), acclimation and preconditioning are often critical to successful cryopreservation outcomes using PVS2.*
- *Applying loading solutions prior to PVS2 improves survival of germplasm, most probably by enhancing tolerance to dehydration.*
- *Optimizing the sequential application, dehydration and incubation steps of the PVS2 protocol is similarly critical to successful outcomes.*
- *Shoot tips may not survive direct (i.e. immediate) exposure to 100% PVS2, stepwise addition of an increasing concentration, e.g. in a range of 50-100% (v/v) of the cryoprotectant is best optimized by exposing to different time durations for different concentrations (e.g. usually 5-20 min, but can be longer).*
- *Adding chilled PVS2 solutions on ice may reduce its toxic effects.*
- *It may be possible to expose tissues to higher concentrations of PVS2 if applied at 0°C.*
- *Due to glass relaxation events, larger volumes of PVS2 may fracture on rewarming, if this is harmful to meristems, a two-step rewarming procedure may be tested. This involves a brief 30-60 sec rewarming on initial removal from LN (either in vapour phase LN or at ambient [ca. 25°C]) temperatures to allow for safe glass relaxation, followed by rapid rewarming at 45°C. In these cases it is important to take care that the glass does not*

crystallize during devitrification; the process should proceed directly from glass to liquid without an intervening ice nucleation and thawing event.

- *Following vitrification, the shoots are initially recovered in an "unloading" solution which prevents osmotic shock by replacing the vitrification solution with 1.2 M sucrose.*
- *PVS3 is an alternative vitrification solution comprising (w/v) each of 40% sucrose and 40% glycerol (w/v) prepared in liquid medium (Sakai et al. 2008). Panis (2008) reports the use PVS3 for *Allium* meristem cryopreservation, albeit based on the original formulation of 50% sucrose and 50% glycerol (w/v). PVS3 is similarly applied (as for PVS2) but in conjunction with an osmoprotection solution of 2 M glycerol and 0.4 M sucrose prepared in standard culture medium.*
- *PVS2 vitrification has proved a very effective cryoprotectant for a wide range of crops and is particularly useful for cryopreserving tropical and recalcitrant germplasm.*

10.6.5 Droplet vitrification

This approach is a modification of both the PVS2 protocol (above) and the droplet freezing protocol (see *section 10.6.1.1*) in which DMSO is replaced by PVS2 as the cryoprotectant. Pennycooke and Towill (2000) first tested droplet-vitrification for the cryopreservation of sweetpotato meristems; subsequently Panis et al. (2005) developed the method for *Musa*. Droplet-vitrification has been successfully applied to a range of genotypes and species (Sakai and Engelmann 2007). A generic protocol is presented, based on Panis (2008) and Panis et al. (2005). However, the basic method will require pretreatment, preculture and pregrowth optimization (see *section 10.3.2.4*) on a crop-specific basis.

10.6.5.1 Equipment and materials

See *sections 10.3.1 and 10.3.2.4*

- Vitrification solutions as prepared as described in *section 10.5*.
 - Loading solution: 2 M glycerol and 0.4 M sucrose
 - PVS2: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M (final level) of sucrose in standard liquid culture medium.
- Ice, ice bath or bucket.
- Small 1L bench top Dewar, cryostorage tank or Dewar.
- Recovery medium.

10.6.5.2 Procedure(s)

Pretreatments and preparative steps

1. For meristem excision, see *section 10.3.2.1*
2. For sucrose pretreatments, see *section 10.3.2.4*

Loading

1. After excision, transfer the meristems to a loading solution of 2 M glycerol and 0.4 M sucrose in standard culture medium.
2. Incubate for a minimum of 20 min.

Cryoprotection

1. Remove the unloading solution and replace with ice-cooled 100% PVS2.
2. Cryoprotect in PVS2 on ice for 30-60 min.
3. During which time, dispense 5-8 15 μ l droplets of chilled PVS2 solution onto the surface of sterile aluminium foils placed in a sterile 50 mm or 90 mm Petri dish (5 x 20 mm).
4. Transfer the foils/Petri dish to ice in a polystyrene icebox.
5. Five to ten minutes before the end of PVS2 cryoprotection, transfer meristems (one per droplet) to the droplets on the surface of the aluminium foils.

Cooling

1. Pre-prepare labelled cryovials, ensuring the ink/surfaces are LN-resistant and indelible.
2. Place aluminium foils on a sterile Petri dish.
3. Fill a polystyrene box with LN, open the cryovials, place them in the holes of the polystyrene float, and place on the surface of the LN.
4. Fill the cryovials with LN.
5. With the aid of forceps, transfer the foils and droplets to the open cryovials and plunge them directly into LN, submerging the foils for a few seconds until they have reached LN temperature (i.e. the vapour has stopped boiling).
6. Refill the open cryovials with LN.
7. Repeat the process to complete the batch of meristems.

Storage

1. Close the cryovials.
2. Optionally transfer to a cryocane and enclose in a plastic LN-resistant cryosleeve.
3. Transfer to a 1 L bench top Dewar before transfer to the cryotank.
4. Alternatively, transfer to the cryotank directly.
5. Store in appropriate inventories in either the vapour or liquid phase of LN.

Rewarming and unloading

1. Remove lids from cryovials, using forceps retrieve the foils/meristems in droplets.
2. Plunge directly into bottles containing 1.2 M unloading solution; incubate for ca. 15 min at ambient temperature.
3. The meristems rapidly float into the solution.
4. Repeat the process for the batch of meristems.
5. To avoid cross-contamination use separate bottles of unloading solution for each cryovial.

Recovery

1. Place a sterile filter paper over the surface of a Petri dish containing solidified medium.

2. Transfer meristems onto the surface of the filter paper.
3. After 1-2 days transfer to a new filter paper/Petri dish with medium.
4. After which transfer the meristems to medium only and culture under standard conditions.
5. Monitor for recovery regularly for 6-8 weeks or longer as required.

10.6.5.3 Critical points

Also see critical factors for *section 10.6.4*

- *This protocol has proved effective for cryopreserving meristems from species that have not responded to the conventional PVS2 method.*
- *Optimization of meristem excision/pretreatments may be required, particularly for problematic genotypes.*
- *This protocol may contravene health and safety regulations for some countries and institutions for which the use of cryovials outside manufacturer's safety recommendations i.e. purposeful filling of vials with LN and use of non-LN resistant vessels e.g. polystyrene boxes are not compliant with the safe use of LN. Small Dewars with larger openings are more effective containers.*
- *Due to the direct exposure of meristems to LN, this procedure is not contained and strict precautions should be taken to ensure germplasm has been through a stringent phytosanitary process, and is confirmed axenic and free of covert microbial infection.*

10.6.6 Encapsulation-vitrification

This method usually combines encapsulation with the PVS2 vitrification protocol, although other vitrification solutions may be used. The original PVS2 protocol was developed to increase the technical efficiency of the standard encapsulation-dehydration protocol (see Sakai and Engelmann 2007). It involves encapsulation followed by treatment with PVS2 solutions and uses the basic preparative and bead-making procedures for alginate and PVS2 (see *sections 10.3.2.5, 10.5, 10.6.3 and 10.6.4*).

10.6.6.1 Equipment and materials

- For basic materials, see *sections 10.3.1*
- For PVS2 solutions, see *sections 10.5 and 10.6.4*
- For meristem excision and pretreatments see *sections 10.3.2.1 and 10.3.2.4*
- For encapsulation see *section 10.3.2.5*

10.6.6.2 Procedure(s)

Loading and cryoprotection

1. Encapsulate meristems in calcium alginate as described in *section 10.3.2.5*.
2. Transfer encapsulated meristems to a loading solution (see *section 10.6.4*) of 0.4 M sucrose, or 2 M glycerol and 0.4 M sucrose (usually prepared in standard culture medium) and incubate for 1 h at 25°C (this may require optimization for temperature and exposure time).

3. Remove encapsulated shoot tip meristems and place in a 50-100 ml flask containing 100% PVS2 solution (see sections 10.5 and 10.6.4).
4. Place flask on a reciprocal shaker optimized incubation time (e.g. 60-100 rpm) for up to 1 h for different durations.

Cooling

1. Put the PVS2 into the cryovial first and then add the beads, this ensures that the beads are always immersed in PVS2 from the start to finish of the procedure.
2. Remove beads, place in 1.8-2.0 ml cryovial (5-25 beads/vial) to which previously 1 ml of PVS2 has been added.
3. Directly immerse the beads and the vials into LN and transfer to a cryotank.

Rewarming and unloading

1. On retrieval from storage, rewarm the vials in a water bath at 35-45°C for ca. 1 min.
2. Swab the cryovials with a disinfectant.
3. Drain away the PVS2, replace with 1.2 M sucrose unloading solution, and incubate for 10 to 30 min or optimize for different durations.

Recovery

1. Remove the beads and blot dry on filter papers to remove excess solution.
2. Place on recovery medium.
3. Monitor for recovery regularly for 6-8 weeks.

10.6.6.3 Critical points

- *Optimization of acclimation, pretreatments and PVS2 incubation, loading, and unloading may be required on a crop-specific basis.*
- *Initial recovery on ½ strength solidified medium for 1-2 weeks may aid regrowth.*
- *The encapsulation-vitrification protocol is technically easier than the traditional encapsulation-dehydration protocols as it offsets the need for prolonged osmotic dehydration and evaporative desiccation of the beads.*
- *This method has proved particularly successful for species/genotypes that are not amenable to either the traditional PVS2 or encapsulation-dehydration (Sakai et al. 2008).*
- *Some studies indicate that the encapsulated shoot tips of certain species and genotypes can withstand up to 2 times longer PVS2 exposure than non-encapsulated shoot tips.*
- *Encapsulated shoots may require longer pre- and unloading solution exposure times.*

10.7 Control of contamination in long-term storage

Personnel should be aware of the special phytosanitary treatments, contamination and containment issues affecting germplasm held in LTS (Benson 2008) most of which are common to those for active *in vitro* culture and slow growth. Specific cryogenic contamination risks are associated with: (1) sterilization of cryoprotectants, particularly those that are filter-sterilized; (2) cryovial containment and the risks of contamination; (3) cross-infections during laborious cryogenic manipulations;

(4) accumulation of adventitious microbial flora in long-term cryobanks during LN tank filling and (5) contamination on rewarming or when thawing in water baths. Morris (2005) provides experimental evidence for the accumulation of contaminants in cryotanks.

10.8 Recovery

Recovery of germplasm from cryobanks will need to be addressed on a crop and species basis, dependent upon the overall success of the protocol and its applicability across genotypes. Potential treatments that can improve recovery include: (1) optimization of unloading after vitrification (see *section 10.6.4*); (2) optimization/phasing of plant growth regulators to avoid adventitious development and callogenesis and encouraging normal shoot regrowth; (3) sequential and gradual removal of osmotica by frequent transfers to media with decreasing levels of additives; (4) initial recovery in low or no light to reduce photooxidation and (5) recovery in special media containing antioxidants and ethylene inhibitors (Mafla et al. 2004; Benson 2008).

10.9 Stability

In vitro genebank practices must ensure biological resources maintain their unique characteristics and that these are unchanged by tissue culture and cryogenic manipulations. Genetic variability is cautioned due to risks of *in vitro*-generated instability proceeding through somaclonal variation (SCV) which is defined as heritable genetic variability in plants generated by tissue culture (Scowcroft 1984). The genetic consequences of SCV are more significant for clonally propagated genetic resources as they manifest in the asexually regenerated plant. Ameliorating the potential risks of SCV resulting from *in vitro* manipulations connected to cryopreservation is thus important. Scowcroft (1984) recommends care should be taken in making critical technical decisions as to how *in vitro* germplasm is manipulated and maintained to avoid practices that increase genetic variation.

Preferred practices are: (1) avoidance of germplasm propagated *via* dedifferentiated and adventitious routes of development and (2) selecting germplasm from young cultures as SCV increases and totipotency decreases during prolonged culture. Careful selection of donor plants and germplasm and the optimization of cryopreservation protocols are also required to minimize stress and selection pressure; these precautions are similar to as described for MTS. Mode of regrowth and regeneration is important for clonal crops that have a propensity for somaclonal variation. It is advisable that recovery, shoot regrowth and regeneration proceeds via normal routes of primary meristematic development. Stress-induced selection pressures may become problematic for low surviving genotypes.

Stability testing after cryogenic storage should be a quality control measure and prioritized for species, crops and genotypes that: (1) have a known tendency to generate somaclones, off-types and are prone to genetic and epigenetic variability;

(2) low surviving genotypes; (3) cultures that have been maintained *in vitro* for extended periods before they are cryopreserved; (4) plants recovered via dedifferentiated or adventitious routes and (5) plants with abnormal characteristics.

Stability assessments may be conducted at the cytological (ploidy/chromosomal assessments), genetic, epigenetic and phenotype levels and field regeneration studies permit true-to-type assessments using crop descriptors. Although a result may not be indicative of genetic instability or stability and molecular-based testing is best supported by trueness-to-type evaluations (Harding 2004). Testing can include: molecular, genotypic and phenotypic assessment of stability, developmental competence and field performance of clonal crops regenerated from *in vitro* storage. Specific procedures may comprise: long-term field performance trials, biometric analysis of phenotypes and their developmental competencies, microsatellite (SSRs) analysis, AFLPs, RAPDs, RFLP-ribosomal RNA genes, DNA methylation, biosynthetic stability, secondary metabolite production, flow cytometry, ploidy assessments and evaluations of transgene stability, as well as the assessment of morphological and genetic stability in progeny.

10.9.1 Critical points

- *Quality control stability assessment should be considered at phenotypic, epigenetic/genotypic levels of assessment.*
- *Methods currently employ phenotypic, chromosomal, cytological and molecular tests (e.g. RAPD, DNA methylation, RFLP, AFLP).*
- *Molecular diagnostics mainly screen a very small part of the genome.*
- *Field trials should be considered to assess trueness-to-type this procedure can run concurrent with germplasm authentication.*

10.10 Longevity

Currently there is no notable evidence for cryopreserved germplasm deteriorating with time in storage *per se*. However, constantly maintained LN levels and carefully controlled deposits and retrievals from cryotanks are critical to good cryobanking practice. Inadvertent and unregulated warming of stored samples (particularly those that are vitrified) can put at risk their longevity. Periodic assessment of cryobanked germplasm and the use of sentinel cryovials may be prudent measures to build into standard genebank quality control operations.

10.11 Problems, troubleshooting and improvements

The main factors limiting successful cryopreservation are variable genotype responses, arrested shoot development, limited regrowth of surviving meristems, delayed decline in recovery, poor rooting in survivors, covert and endophytic contamination. One of the main limitations is developing cryostorage protocols that are applicable to wide a range of genotypes and the inefficiency of optimizing protocols. Applying the probability equations of Dussert et al. (2003) is a useful and necessary approach as they calculate the lowest recovery rates that allow a target number of regenerants to survive

cryopreservation. It is important to caution that these equations do not take into account losses from contamination, genetic instability and selection pressures in low survivors. Improvements to cryopreservation protocols can be achieved by the optimization of generic protocols and the development of new approaches for recalcitrant genotypes based on both fundamental (e.g. proteomics) and applied research. Improved technology transfers and validation exercises that use critical point analyses will facilitate these approaches. Implicitly, this also means that the development of improved procedures and protocols in IVGBs will be facilitated by the continued development of quality systems and their associated best practices.

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See Appendix I for an expanded bibliography.

TECHNICAL TERMINOLOGY

In vitro conservation

Base collection: as defined by Genebank Standards (1994) for seed germplasm, base collections are a set of genetically distinct, different accessions as close, as is possible to the sample originally procured to establish the collection. The base collection is: (a) preserved for the long-term future and (b) not normally distributed from directly to users. Ideally, the base collection represents a comprehensive genepool of the crop or species. Because of the potentially extreme longevity of germplasm held in LN, cryobanks have become synonymous with base collections.

Biorepository: a body/institute/laboratory/genebank that receives, stores, processes and disseminates organisms, organs, tissues, cells, cultures and other constituent biological materials. The term embraces infrastructures, documentation and all processes involved in the safekeeping stored biological resources and their dispatch to clients, end users and beneficiaries.

Colligative cryoprotectant: the term 'colligative' refers to the properties of solutions and it is characterized by the amount of solutes that are dissolved in the solution. A colligative cryoprotectant is an additive that is able to penetrate the cell, it is applied to reduce or prevent the damage caused by excessive cell volume changes and the toxic concentration of solutes, both these injurious effects occur during cryopreservation. Colligative cryoprotectants also cause freezing point depression which is also protective, as when ice nucleation does occur, it is not as injurious as would be the case for ice formed at higher sub-zero temperatures.

Controlled rate cooling: a cryopreservation protocol that controls the rate at which cryoprotected germplasm is cooled before it is finally plunged into LN (also known as 2-step or stepwise cooling). The process involves various cooling rates, hold times and a terminal transfer temperature(s). Synonymous with controlled rate freezing.

Controlled rate programmable freezers: computer-operated machines that deliver pressurized LN into a freezing chamber *via* a solenoid valve that is controlled by a thermal device responsive to the difference between the actual and desired temperature. The computer control unit is programmed with the desired temperature gradient, and holding points which allow the samples to be placed in, and withdrawn from the chamber. Controlled rate cooling requires the sample to be 'seeded' (the process by which ice nucleation is induced), some programmable freezers contain devices that allow automatic sample seeding.

Core collections: a limited set of accessions, representing, with minimum replication, the genetic diversity of a crop species and including its wild relatives. A contemporary definition is an optimally representative range of genetic resources that meets the needs and requirements of the end users and genebank curator.

Cross-contamination: the transfer of one part of a sample to another, or cross-infection with a sample that is contaminated by a microbial agent, pest or pathogen.

Cryopreservation: the conservation of living cells, tissues, organs and organisms at ultra low temperatures, usually in LN or its vapour, to a minimum temperature of -196°C.

- Cryoprotectant:** an additive, mixture of additives or a process that allows living cells, tissues, organs and organisms to survive exposure to cryogenic temperatures (usually in LN and its vapour to a minimum temperature of -196°C).
- Cryoprotective dehydration:** the removal of water by osmotic or evaporative means to allow survival after cryopreservation.
- Cryostorage tank:** a specialist vacuum-insulated LN tank used to store cryopreserved germplasm; these tanks have the advantage of being independent of electrical power so long as the LN level is maintained.
- Droplet freezing:** a cryopreservation protocol in which meristems are suspended in micro-droplets of DMSO solution on aluminium foils and cooled at ultra rapid rates by direct exposure to LN. On rewarming, the foils/meristems are placed in unloading solutions and passively rewarmed at ambient temperatures.
- Droplet-vitrification:** a cryopreservation protocol in which meristems are suspended in micro-droplets of vitrification solution on aluminium foils and are cooled at ultra rapid rates by direct exposure to LN. On rewarming, the foils/meristems are placed in unloading solutions and passively rewarmed at ambient temperatures or at 45°C in a water bath.
- Dry shipper:** a special IATA, safety-approved transport container used to transport cryopreserved germplasm at the temperature of LN vapour (see liquid nitrogen) by means of LN being adsorbed into a special material which prevents risks of spillage.
- Encapsulation-dehydration:** a cryoprotective process in which meristems are encapsulated in calcium-alginate beads, osmotically dehydrated, evaporatively desiccated in a sterile airflow or, over silica gel, directly cooled in LN, and passively rewarmed at ambient temperatures.
- Encapsulation-vitrification:** a cryoprotective process in which germplasm is encapsulated in calcium-alginate beads, osmotically dehydrated and/or loaded with cryoprotective additives, exposed to vitrification cocktails such as PVS2 and cooled in LN, placed in unloading solutions and either passively rewarmed at ambient temperatures or at ca 45°C in a water bath.
- GPG2:** the Collective Action for the Rehabilitation of Global Public Goods Phase 2. The Consultative Group on International Agricultural Research (CGIAR) project of the CGIAR-System-wide Genetic Resources Programme (SGRP). The practical aim of the project is the upgrading of genebank operations and facilities and guiding the CGIAR's contribution in developing a global, secure plant genetic resources system.
- Ice nucleation:** also termed 'seeding' is the point at which ice crystals are first initiated in a cryopreserved sample, usually during controlled rate cooling.
- In Vitro Active Genebank (IVAG):** an *in vitro* collection of germplasm comprising accessions held in conditions (low temperatures, light, high osmotica) that limit culture growth in order to reduce costs and increase efficiency by extending subculture intervals. The process is also termed Medium Term Storage (MTS). The cyclic flow of material is the essential feature of the IVAG which is maintained by successive subculturing and recovery, allowing renewal of cultures for use and distribution.
- In Vitro Base Genebank (IVBG):** as defined by Genebank Standards (1994) a base collection is preserved for the long-term future in cryobanks and not normally distributed to users. Because of the extreme longevity of germplasm in cryobanks, the process is called Long-Term Storage (LTS) and is synonymous with cryopreservation.

In Vitro Genebank (IVGB): a genebank dedicated to the conservation of genetic resources *in vitro* under active growth slow growth and cryopreserved conditions.

Liquid Nitrogen (LN): a cryogenic coolant which is used to cool and store germplasm in either the liquid phase (at a temperature of -196°C) or vapour phase at variable temperatures (depending on how far the sample is from the liquid phase). See **Liquid phase storage** and **Vapour phase storage**.

Liquid phase storage: cryopreservation in the liquid phase of LN at -196°C.

Long-term storage (LTS): cryopreservation (cryobanking) usually in *in vitro* base genebanks. Also see **In Vitro Base Genebank (IVBG)**.

Medium-term storage (MTS): preservation using slow or minimal growth usually in *in vitro* active genebanks. Also see **In Vitro Active Genebank (IVAG)**.

PVS2: Plant Vitrification Solution number 2, comprising 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose, developed by Akira Sakai and colleagues.

PVS3: Plant Vitrification Solution number 3, PVS3 is an alternative vitrification solution comprising (w/v) each of 40% sucrose and 40% glycerol (w/v) prepared in liquid medium (Sakai et al. 2008). Panis (2008) reports the use PVS3 for *Allium* meristem cryopreservation based on the original formulation of 50% (w/v) sucrose and 50% (w/v) glycerol.

Seeding: the process by which ice nucleation is initiated in a cryopreserved sample, usually during controlled rate cooling.

Vapour phase storage: cryopreservation in the vapour phase of LN at ca. temperatures of -130°C to -190°C (Hunt and Pegg 1996; Benson 2008).

Vitrification: A process which increases cell viscosity to a critical point at which an amorphous glassy state is formed at a critical glass transition temperature, called the T_g (see T_g).

Best practices, quality and risk management

Accident: an event or occurrence deviating from a best practice or regulated procedure that may potentially, or will deleteriously affect personnel, germplasm and quality genebank systems (e.g. diagnostics, storage, authentication) and the subsequent use of the genetic resource. Accidents should be formally recorded and investigated to ensure any deleterious outcomes are obviated, corrected or limited.

Accreditation: the process by which an external auditor which is itself accredited by formal standards (endorsing the auditor is competent to undertake accreditation by quality assurance) awards a certificate of competency and procedures.

Adverse outcome: a detrimental outcome as a result of an accident or incident.

Aseptic technique: the special processing of materials, explants and germplasm in such a way that the potential for contamination with microorganisms and cross-contamination *via* other samples is prevented.

Audit: a formally assessed documentation of personnel, procedures, records and protocols used to evaluate adherence to **Best practices (BPs)** and **Standard operating procedures (SOPs)** regulations and statutes.

Authentication: proof and verification of correct identity.

- Best Practice (BP):** a protocol, technique, method or process that is the most effective in producing a particular outcome compared to any other approach that is used. The BP must operate within a process that has been: (1) properly checked and tested against compliance with specific and desired outcome(s) and (2) is achieved without complications, changes to the protocol or unforeseen problems. Best Practices are the most efficient (in terms of time, resources, facilities, costs) and effective (producing the best possible result) approach to undertaking a task. A BP is usually and ideally proven and tested by others such that it is capable of being repeated by others (also see **Technology transfer, validation and verification**).
- Biohazard:** an organism, pathogen or substance that is a threat or harmful to personnel, germplasm, other organisms, crops and the environment.
- Biorepository core responsibilities:** (1) purity: freedom from contaminating organisms; (2) authenticity: correct identity; (3) stability, including functionality and fitness for purpose.
- Clean room:** a room especially equipped to maintain a clean environment, for aseptic processing, usually with restricted entry, working at positive pressure, fitted with air filters to remove dusts, particulates, microorganisms and operating to specific containment conditions and specifications (also see **Controlled areas**).
- Cold chain:** a supply chain of a product, material or germplasm that must be maintained at a controlled low temperature defined by critical specific limits to preserve its stability (also see **Glass transition temperature, T_g**).
- Community of practice:** a term used to describe shared activities that embrace people and their institutions with shared/common goals and that require coordinated interactions to strive for and satisfy these goals; the term is associated with biorepository sectors.
- Consignee:** an institute or member of staff that takes receipt of materials and germplasm and who is responsible for their dispatch, storage in transit, tracking and deposition in genebanks.
- Containment:** the complete demarcation or enclosure of a process or sample so that it does not come into contact with a boundary or border that puts it at risk to contamination or infection by means of direct, adventitious or cross-contamination (also see **Double bagging**).
- Controlled areas:** restricted areas coded with a defined level of security, only accessible to signed in, authorized personnel; they are usually alarmed and can be placed under camera surveillance.
- Critical point factor:** a factor or issue that affects the successful outcome of a procedure or protocol.
- Custodian:** the person responsible for the management of the germplasm held in the genebank and who works in cooperation with the donors, recipients, stakeholders, end users and beneficiaries. The custodian is responsible for ensuring the overall technical, regulatory and safe management of the genebank according to guidelines and best practices.
- Deviation:** an unintended or intended departure from a normal or best practice, standard operating procedure or regulatory requirement that requires formal explanation, justification and documentation.
- Donor:** the source of the biological material, explant or germplasm that can be designated as an institute, genebank, person or mother plant.

Double bagging: the secure, double-containment of germplasm stored in cryobanks to prevent leakage of LN into cryovials and cryobags, thus minimizing the risks adventitious contamination and cross-infection from other samples held in the cryobank.

Environmental monitoring system: an automated surveillance and data logging system that monitors a controlled environment within critical specifications and that triggers an alarm if the environmental control system goes off limits or malfunctions.

Error: a departure from a best practice or standard operating procedure involved in any aspect of the IVGB activities that may be due to: technical and documented mistakes, miscalculations, mislabelling and that requires formal and documented corrective action to mitigate against current and potential deleterious consequences and may require re-call of samples and the re-writing of best practices.

Exemplar: in the context of these guidelines, exemplar is used (in preference to the use of 'example') to mean a typical (or generic) illustration of a process, technique, protocol or procedure (including reporting procedures).

Hazard: anything or a process or procedure that can cause harm.

Hazard analysis and critical control points: a systematic preventative approach used in food safety and the pharmaceutical industry to identify and control hazards based on the principles of: (1) hazard analysis; (2) identification of critical control points; and establishing (3) critical limits for each control point; (4) critical control point monitoring requirements; (5) stabilizing corrective actions; (6) record keeping procedures and (7) formulating procedures for ensuring a process is working properly.

Incident: an unplanned deviation from a best practice, or safety protocol affecting a standard operating procedure (SOP) and which may put at risk personnel carrying out the procedure, the germplasm/sample/process and consequently requiring instigation of an incident response plan and report documentation (see also **Accident**).

Institutional review: a panel, review board or committee formally appointed to review periodically the activities of an institute and a process that can be undertaken as part of an overarching quality review programme.

International Air Transport Association (IATA): the regulatory body that oversees the safe aviation transport and the organization that requires IVGB complicity with its regulations for the safe shipment of germplasm and biological materials (see **Dry shipper**).

International Treaty on Plant Genetic Resources: the objectives of the Food and Agriculture Organization's Treaty are the conservation and sustainable use of plant genetic resources for food and agriculture and the fair and equitable sharing of the benefits arising out of their use, in harmony with the Convention on Biological Diversity (CBD), for sustainable agriculture and food security.

ISO: the logo and designation of The International Standard for quality management systems of the International Organization for Standardization, a body composed of representatives from a wide range of national standards organizations, setting standards of practices.

Material Safety Data Sheets (MSDS): information sheets provided by equipment and chemical manufacturers and suppliers providing physical/chemical/biological properties; information on the hazards of their product with ratings related to their handling risks: first aid and fire fighting measures, accidental release contingencies,

exposure controls, Personal Protective Equipment (PPE) and Clothing (PPC), handling and storage controls, stability and reactivity, toxicological and ecological information, transport and disposal information and regulatory controls and procedures.

Material Transfer Agreement (MTA): an agreement that controls the transfer of germplasm between two organizations, which specifies how the recipient will use the genetic resource or biological material. The MTA defines the rights and obligations of the donor/provider/recipient regarding germplasm use. The prefix **standard** specifically refers to the standard **MTA (CGIAR-FAO)** devised for plant germplasm.

Preservation: the process by which germplasm, physical and biological materials, chemicals, and reagents are stored to ensure that they do not deteriorate, usually within a specific period notified by an expiry date, or as defined by long and short-term storage.

Procedure: a series of consecutive steps designed to result in a specific outcome.

Process(es): any of all the procedures in the IVGB involved in the overall security, safe management storage, dispatch and use of its genetic resources.

Quality: confirmation of compliance with specific, predetermined standards.

Quality Assurance (QA): Evidence of action confirming that work is done effectively using systematic accounting of actions related to the work, so that they give sufficient confidence that a process, product or service meets given requirements for quality. **QA** normally involves all activities in the process; including documentation and assures that the processes are fit-for-purpose. **QA** also includes failure testing, to anticipate a weakness or critical point in the process.

Quality Assurance (QA) IVGB-specific: an integrated system for managing all aspects of a genebank's activities to ensure that they are performed to the appropriate and required standard in full compliance with all regulatory obligations.

Quality Management System (QMS) IVGB-specific: as for **Quality Assurance**.

Risk: the chance, high or low of harm being caused by a hazard.

Risk assessment: identification of a risk, usually placed into unavoidable and avoidable categories, the former being outside operator control no matter what safety measures are put in place. These risks include '*Force Majeures*', natural climatic disasters, armed conflict and terrorism. Avoidable risks are potential hazards resulting from routine and/or research operations that are not conducted with due care and vigilance. These are identified in a risk assessment and measures are taken to prevent them from happening. In the event that they can potentially occur, risk assessment includes protocols and procedures obviating or limiting their deleterious impacts and dealing with their consequences as effectively as possible. Risk assessment constitutes a systematic, recorded operation rationally foreseeing and mitigating against potentially adverse or damaging incidents. The assessment is usually based on information from a number of sources including: national regulatory authorities and it is administered in a no-blame culture.

Risk management IVGB-specific: the processes and procedures involved in assessing and mitigating against hazards and risks in order to ensure the safety of personnel, samples, germplasm and genebanks and their freedom from harm.

Seeding: an automatic or manual process that induces the formation or nucleation of ice crystals, during colligative controlled rate cooling protocols this is achieved manually,

by touching the cryovials with a cold instrument or, using a programmable freezer seeding programme.

Shipping log: a formal written, processing, dispatch and receipt log for the safe, regulated shipment of germplasm and biological samples.

Shipping manifest: a formal written description of the contents of a package shipped from the genebank.

Slow growth: the process by which the growth of *in vitro* cultures is retarded to increase the length of the subculture cycle to increase the efficiency and reduce costs of their maintenance, it is usually applied in the **IVAG**.

Standard Operating Procedure(s) (SOPs): a portfolio of standard procedures, methods and protocols that are technically detailed and formally validated and documented and that comprise the procedures used in common by all the genebank personnel in a repository. SOPs are an integral part of a **Quality Management System**.

Sterility: an *absolute term* which is defined as the complete absence of detectable, viable, contaminating microorganisms; it is usually achieved by validated, testing procedures that confirm the absence of microbial flora.

Sterilization: an *absolute process* undertaken by chemical, filtration or heat treatments that is validated (i.e. fit-for-purpose) to destroy microorganisms.

Storage: the preservation of biological and non-biological samples and specimens under specific conditions for their future use in the medium- (minimal, slow growth) or long-term (cryopreservation) designated as **MTS** and **LTS** respectively.

Technology transfer: the process by which the outcomes of scientific research are applied in practice, usually involving technology acceptance and implementation on a routine basis by its practitioners.

Tg: the glass transition temperature of vitrified, cryopreserved material.

Tolerance limits: the limits that define the maximum and minimum range of acceptable values that are used to test the efficacy of a procedure or process or storage method, which if exceeded needs the implementation of corrective or emergency actions.

Traceability: the ability to locate a germplasm sample or an accession during any stage of the biobanking/genebanking process, it is synonymous with the term 'tracking'.

Validation: a process by which a procedure is checked against a specific criterion, confirming that it meets the intended needs of users; it can also mean a declaration of fitness-for-purpose. Validation may need to satisfy specific criteria that are regulated and defined by external standards. Validation is an indicator of how robust a protocol is within the constraints of its operational parameters.

Validation exercises: a formal testing by different validators in a community of practice, of a protocol, procedure, analytical technique or diagnostic method so that it is demonstrated to consistently produce the expected results or outcomes based on performance indicators within predetermined and precise specifications.

Verification: usually an internal quality process, ensuring a protocol, test or procedure is compliant with its required performance standard(s).

USEFUL WEBSITES

Accreditation

ISO-International Organization for Standardization

<http://www.iso.ch/iso/en>

Benefit sharing

General bibliography

<http://www.kew.org/conservation/access-benefit.pdf>

Leipzig Declaration

<http://www.fao.org/FOCUS/E/96/06/more/declar-e.htm>

The Convention on Biological Diversity, Bonn Guidelines

<http://www.cbd.int/doc/publications/cbd-bonn-gdls-en.pdf>

The International Treaty on Plant Genetic Resources for Food and Agriculture

<http://www.planttreaty.org/>

Best practices

CABRI

<http://www.cabri.org/guidelines.html>

ISBER

<http://www.isber.org/bp/index.cfm>

OECD Guidelines for Best Practices for Security in Biological Resources Centres

<http://www.oecd.org/dataoecd/6/27/38778261.pdf>

OECD Guidelines for Best Practices in Biological Resources Centres

<http://www.oecd.org/dataoecd/7/13/38777417.pdf>

National Cancer Institute Best Practices for Biospecimen Resources

http://biospecimens.cancer.gov/global/pdfs/NCI_Best_Practices_060507.pdf

Biorepositories and culture collections (General)

European Culture Collection Organization (ECCO)

<http://www.eccosite.org>

UK National culture collections

<http://www.ukncc.co.uk/>

International Society for Biological and Environmental Biorepositories

www.isber.org

World Federation for Culture Collections

<http://www.wfcc.info/>

The OECD Biological Resource Centres

<http://www.oecd.org/dataoecd/55/48/2487422.pdf>

Biosafety and biotechnology

Cartagena protocol

<http://www.cbd.int/biosafety/>

SGRP Biosafety

<http://www.cbd.int/doc/external/mop-04/cgiar-bs-en.pdf>

Consultative Group on International Agricultural Research

www.cgiar.org

CGIAR Virtual Information Centre

<http://www.cgiar.org/vic/index.html>

<http://www.cgiar.org/publications/secretariat.html>

Convention on Biological Diversity

www.biodiv.org

Food and Agriculture Organization of the United Nations

www.fao.org

Genebanks (CGIAR's clonal crop)

Bioversity International (*Musa*)

<http://www.bioversityinternational.org/>

<https://sites.google.com/a/cgxchange.org/musanet/musa-collections/international-transit-center-itc>

International Potato Centre (potato, sweetpotato)

<http://www.cipotato.org/>

(http://www.cipotato.org/publications/program_reports/99_00/20clonverif.pdf).

International Center for Tropical Agriculture

<http://www.ciat.cgiar.org/>

International Institute for Tropical Agriculture

<http://www.iita.org/>

Genetic resources

Common Access to Biological Resources and Information (CABRI)

<http://www.cabri.org>

Global Action Plan for Plant Genetic Resources

<http://www.fao.org/ag/AGP/AGPS/GpaEN/gpatoc.htm>

Global Crop Diversity Trust

<http://www.croptrust.org/main/>

Guidelines: Technical crop-specific

Musa (Bioversity)

<https://sites.google.com/a/cgxchange.org/musanet/documentation/technical-guidelines>

Material Transfer Agreements (Standard)

International Treaty on Plant Genetic Resources Plant Material Transfer Agreement

<http://www.planttreaty.org/>

Organization for Economic Cooperation and Development

www.oecd.org/home/

Plant protection

International Plant Protection Convention (IPPC)

<https://www.ippc.int/IPPC/En/default.jsp>

Plant Protection Health and Safe Movement of Germplasm

http://www.musalit.org/pdf/IN960084_en.pdf

WTO

http://www.wto.org/english/tratop_e/sps_e/sps_e.htm

Quality assurance

OECD Guidelines for Quality Assurance in Molecular Genetic Testing

<http://www.oecd.org/dataoecd/43/6/38839788.pdf>

Regulations, codes and treaties

Budapest Treaty

http://www.wipo.int/treaties/en/registration/budapest/trtdocs_wo002.html

Leipzig Declaration

<http://www.fao.org/FOCUS/E/96/06/more/declar-e.htm>

The Convention on Biological Diversity, Bonn Guidelines

<http://www.cbd.int/doc/publications/cbd-bonn-gdls-en.pdf>

The International Treaty on Plant Genetic Resources for Food and Agriculture

<http://www.planttreaty.org/>

Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)

<http://www.cites.org/>

Research projects (cryoconservation)

Cost Action 871 CryoPlanet

<http://www.agr.kuleuven.ac.be/dtp/tro/cost871/Home.htm>

CRYMCEPT

<http://www.agr.kuleuven.ac.be/dtp/tro/crymcept/CRYMCEPT.htm>

http://ec.europa.eu/research/agriculture/projects/qlrt_2001_01279_en.htm

CRYSTAL

<http://www.crystal-eu.org/>

EURALIVEG

<http://euralliveg.ipk-gatersleben.de/home.php>

Safety and risk management

Control of Substances Hazardous to Health (COSHH)

<http://www.hse.gov.uk/coshh/>

Risk Assessment Guide

<http://www.hse.gov.uk/pubns/raindex.htm>

<http://www.hse.gov.uk/pubns/indg136.pdf>

<http://www.hse.gov.uk/pubns/bioindex.htm>

Guidance for Hazard Determination

<http://www.osha.gov/dsg/hazcom/ghd053107.html>

Personal Protective Equipment

<http://www.hse.gov.uk/pubns/ppeindex.htm>

Nunc™ 2008. *Safety First: Nunc™ Cryopreservation Manual*. 20.4.2008

<http://www.nuncbrand.com/en/frame.aspx?ID=608>.

BOC Industrial Cryogenic Gas Safety

http://www.bocindustrial.co.uk/bocindustrial/product_information/Cryogenic_Products/safety/index.asp

System-wide Genetic Resources Programme (SGRP)

<http://www.sgrp.cgiar.org/>

Transport

International Air Transport Association (IATA)

<http://www.iata.org>

International Civil Aviation Organization: International Transport Regulations

<http://www.icao.int/>

APPENDIX I. BIBLIOGRAPHIES

Appendix I comprises a series of bibliographies to support these guidelines, they each include a compilation of historical and contemporary information collated over the time course of the GPG2 project up to 2009. The general bibliographic section includes some representative literature relating to the development of quality systems and best practices across the wider biorepository and biobanking sector, including examples from medical and microbial communities of practice. The rationale for including these elements is based on the 'lessons learnt' approach that underpins GPG2 Activity 1.2 and the system-wide philosophy for managing genetic resources. The *in vitro* crop conservation bibliography is a collation of general and specific crop-by-crop literature pertaining to the *in vitro* conservation of CGIAR'S clonal crops.

General bibliography

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APPENDIX II

Examples of *in vitro* genebank equipment calibration and maintenance

Examples are specific to the equipment and facilities required for *in vitro* storage using slow growth and cryopreservation, they do not include specialist phytosanitary, diagnostic and molecular equipment. The frequency of tests and calibrations are indicated where they generally advised by manufacturers, otherwise tests are undertaken based on the utility and frequency of use.

(A) Safety and risk management

Equipment	Use	Maintenance	Tests & calibration	Procedures
O ₂ Monitors cryogenic oxygen deficiency hazard (ODH) for personnel asphyxiation risks/safety	Audio-visual alarms alert personnel to safety risks by monitoring oxygen depletion. These alarms are calibrated to critical minimal lack of O ₂ asphyxiation levels. They are used in closed or poorly ventilated areas and atmospheres holding larger volumes of LN		In house testing and re-calibrations using test gases (every 3-6 months)	Personnel risk management
Liquid Nitrogen low level alarms	Audio-visual alarms alert low levels of LN in cryotanks and cryogen supply vessels.	Manufacturer service/local checks	Calibration of alarms with critical LN levels	Sample risk management
Pressurized LN vessels and gauges	LN Supply tanks, programmable freezers	Manufacturers' services and local checks; in some countries 3 rd party Health and Safety checks are mandatory/statutory legal obligations and cryogenic vessels have a designated finite lifetime after which they cannot be used	Calibration of pressure gauges may be required Calibration of pressure gauges for automatic-fill vessels; calibration of LN burn-off	Personnel and sample risk management Personnel and sample risk management
LN Dewars	Sample storage and supply			
Dry Shippers	Safe transport of samples in LN	Local checks for vessel-LN charging and cleaning	Calibration of LN burn-off, contamination checks	Personnel and sample risk management
Personal Protective Clothing/ Equipment (PPC) (PPE)	Personnel protection	Manufacturers' service and local checks of fume hoods, extraction equipment, PPC PPE cleaning.	Fume hood-extractor airflow calibration particle count	Personnel risk management

(B) Media and reagent preparation

Equipment	Use	Maintenance	Tests & calibration	Procedures
Balances	Weighing of reagents, cryoprotectants and media		In-house testing and calibration of balances (annual or after re-location)	Accurate dispensing of gravimetric weights and measures
Gilson pipettes	Volumetric dispensing of reagents, cryoprotectants and media		In-house testing and calibration of automatic pipettes (annual)	Accurate dispensing of volumetric measures
pH Meters	pH is critically important for biological processes and can be changed by cryoprotective and slow growth additives	Manufacturer and/or local service	Test against manufacturers' standard test solutions (weekly)	Preparation of media, buffers, reagents and cryoprotectants
Water purification	Production of purified and/or distilled water	Manufacturer and/or local service of stills, ion exchange units, replacement of water purification filters	Manufacturer-recommended standard tests for conductivity (weekly)	Preparation of reagents, cryoprotectants and media
Washing machine	Washing of glassware	Manufacturer and/or local service	Test for efficient cleaning and rinses with purified water	Preparation of clean glassware for media preparation

(C) Sterilization and aseptic procedures

Equipment	Use	Maintenance	Tests & calibration	Procedures
Autoclaves	Sterilization of media, cryoprotectants and equipment	Manufacturer and/or local service checks in some countries 3 rd party Health and Safety checks are mandatory and autoclaves have a finite life	In-house testing and calibration of temperature and pressure gauges, thermal indicator tests and contamination checks	Sterile <i>in vitro</i> operations; media and cryoprotectant preparation
Laminar flow cabinet	Aseptic manipulations		In-house testing and calibration of airflow, particle count and microbial plate testing	Sterile <i>in vitro</i> operations and autoclaved media dispensing
Clean rooms	Positive air pressure environments	Manufacturer and/or local service	In-house testing and calibration of airflow and particle counts	Clean room for <i>in vitro</i> operations

(D) Refrigerators and freezers

Equipment	Use	Maintenance	Tests & calibration	Procedures
-4°C, -80°C and -20°C refrigerators and freezers	Storage of thermo-labile reagents, biological samples	Temperature check, defrosting and cleaning	Testing and calibration of temperatures and at different locations in the freezer	Cold storage
-80°C freezers	Passive controlled rate cooling using Mr Frosty™ units		Testing and calibration of temperatures and at different locations in the freezer	Cryopreservation
Ice machines	Production of ice for cryoprotection addition	Defrosting and cleaning	Contamination checks	Cryopreservation

(E) Thermal equipment

Equipment	Use	Maintenance	Tests & calibration	Procedures
Incubator	Pregrowth and acclimation treatments, testing for microbial contaminants	Manufacturer and/or local service, regular cleaning	Testing and calibration of temperatures and at different locations in the thermal incubator/temperature controlled unit	Cryopreservation Microbial culture Thermotherapy
Water bath	Thawing and rewarming of cryo-stored materials			Cryopreservation
Ovens	Dry weight determination for critical point desiccation tolerance. Equipment sterilization			Cryopreservation Sterilization

(F) Temperature controlled environments

Equipment	Use	Maintenance	Tests & calibration	Procedures
Growth rooms	Plant culture	Manufacturer and/or local service	Testing and calibration of temperatures, humidity, light and alarms and checks at different locations in the environmental control facility	General culture maintenance, slow growth, recovery after medium-term and long-term storage
Cold rooms	Cold storage			Storage of cold acclimated/hardened propagules and storage organs (tubers, bud cuttings)
Air-conditioned laboratories	Controlled operations			Cryopreservation, general analytical procedures

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