Collective Action for the Rehabilitation of Global Public Goods Phase 2

Refinement and standardization of storage procedures for clonal crops

Global Public Goods Phase 2:
Part I. Project landscape and general status of clonal crop *in vitro* conservation technologies

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, the GPG2 Project Milestone 1.2.1 "Review *in vitro* protocols applied to clonal crops"; and it concomitantly assists the delivery of Sub-activity 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."

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Abbreviations and acronyms

ACOI	Universidade de Coimbra, Culture Collection of Algae, Portugal
AFLP	Amplified fragment length polymorphism
BA	6-benzyl adenine
BP	Best practice
CABRI	(European) Common Access to Biological Resources and Information
CBD	Convention on Biological Diversity
CCALA	Culture Collection of Algal Laboratory, Czech Republic
CCAP	Culture Collection of Algae and Protozoa
CCP	Critical control point
CCTF	Clonal crop task force
CFU	Colony forming unit
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
FDA	Fluorescein diacetate
GBRCN	Global Biological Resources Centres Network
GLP	Good laboratory practice
GPG2	Global Public Goods Phase 2
HACCP	Hazard analysis critical control point
HFEA	Human Fertilization and Embryology Association (of the UK)
HSE	Health and Safety Executive (of the UK)
IAA	Indole acetic acid
IARC(S)	International Agricultural Research Centre(s)
IBPGR	International Board for Plant Genetic Resources
IFAD	International Fund for Agricultural Development
IITA	International Institute of Tropical Agriculture
INIBAP	International Network for the Improvement of Banana and Plantain
IPGRI	International Plant Genetic Resources Institute
IPK	Institut für Pflanzengenetik und Kulturpflanzenforschung, Germany
ISB	Institute of Soil Biology, Collection Czech Republic
ISBER	International Society for Biological and Environmental Repositories
ISO	International Standard Organization
ISSR	Inter simple sequence repeats
ITC	(INIBAP) International Transit Centre
ITPGRFA	International Treaty of Plant Genetic Resources for Food and Agriculture
IVAG	*In vitro* active genebank
IVBG	*In vitro* base genebank
IVGB	*In vitro* genebank

LN	Liquid nitrogen
LTS	Long-term storage
MDG	Millennium Development Goals (United Nations)
MSDS	Material safety data sheet
MTA	Material transfer agreement
MTS	Medium-term storage
NARS	National Agricultural Research Services
NERC	Natural Environment Research Council (of the UK)
OECD	Organization for Economic Co-operation and Development
PCR	Polymerase chain reaction
PVS	Plant vitrification solution
PVS2	Plant vitrification solution number 2
RAPD	Randomly amplified polymorphic DNA
RAF	Randomly amplified polymorphic DNA fingerprinting
RFLP	Restriction fragment length polymorphism
RTD	Research training and development
SAG	Sammlung von Algenkulturen (Universität Göttingen, Germany)
SCV	Somaclonal variation
SGRP	System-wide Genetic Resources Programme
SINGER	System-wide Information Network for Genetic Resources
SMTA	Standard material transfer agreement
SSR	Simple sequence repeats
Td	Devitrification temperature
Tg	Glass transition temperature
Tm	Melt temperature
TR	Translational research
UKNCC	UK National Culture Collections
UN	The United Nations
UNDP	United Nations Development Programme
USDA	United States Department of Agriculture
VNTR	Variable number tandem repeat
WHO	World Health Organization

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 (Biodiversity, 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 cryo-response 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.

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1. Introduction

The mission of the Consultative Group on International Agricultural Research (CGIAR) is: "to achieve sustainable food security and reduce poverty in developing countries through scientific research and activities in agriculture, fisheries, policy, and the environment." To facilitate this process, the System-wide Genetic Resources Programme (SGRP) of the CGIAR (http://sgrp.cgiar.org/) unites the collective efforts of its individual institutes. The System-wide Information Network for Genetic Resources (SINGER) is an information product of SGRP; this is an infrastructure that provides CGIAR partners with easy access to information about diversity, germplasm conservation as well as crop-related knowledge. In support of CGIAR's mission, SGRP created the Global Public Goods (GPG) project to upgrade the management of its in-trust collections. The collective action for the rehabilitation of Global public goods system: Phase 2 (GPG2) specifically aims to enhance the security and stewardship of crop genetic resources held in-trust by the CGIAR's genebanks, these collections comprise >650,000 samples of plant genetic resources. The GPG2 Project and its associated knowledge base (http://cropgenebank.sgrp.cgiar.org/) were implemented under the aegis of SGRP in order to provide a comprehensive, system-wide work programme for upgrading the crop genebanks and the practices used to manage their collections. This will ensure that the CGIAR centres meet their in-trust commitments, manage their collections efficiently and sustainably and facilitate access by users. The GPG2 Project positions CGIAR's genebanks to play a leading role in building a comprehensive global system for conserving, managing and exchanging plant genetic resources for food and agriculture.

1.1 High standard stewardship for clonal crop *in vitro* conservation

The GPG2 project involves upgrading genebank operations and facilities and guiding CGIAR's contribution in developing a global, secure plant genetic resources system. Implicitly, high standards of stewardship require collective activities consolidated by common, cross-cutting best practices and quality systems and to achieve this, GPG2 conforms to Logframe Based Management embodied in six outputs and outcomes. It has the development goal "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 six issues that define the overarching activities of the project membership are shown in Figure 1, noting that in the wider context trust collections are international public goods and their use is not restricted to developing countries.

1.2 Aims

An overarching objective of GPG2 is to build upon the existing competencies of CGIAR's centres, to develop new modes of collaboration and maximize integration and sharing of standards and methods. This document specifically concerns Activity GPG2 1.2: "Refinement and standardization of storage procedures for clonal crops. In addition, Sub Activity 1.2.1 provides a "Review of *in vitro* protocols applied to clonal crops" in the context of GPG2's cross-cutting themes of best practice development, risk management and performance measurement. The milestones associated with Activity 1.2 require an appraisal of clonal crop *in vitro* conservation status and the formulation of multi-crop guidelines. The work plans associated with these milestones include a CGIAR Clonal Crop Task Force survey, a workshop and literature review.

Figure 1. Distillation of the six cross-cutting activities of the Collective action for the rehabilitation of global public goods in the CGIAR genetic resources system: Phase 2 project. Culminating in the development goal (centre box) and implemented under the aegis of the CGIAR System-wide Genetic Resources Programme (SGRP).

The overarching rationale of these activities is to apply the information to develop collectively, multicrop guidelines for the conservation of clonal crops. This involves technology transfer and knowledge exchange to validate best practices for preserving the *in vitro* germplasm of Andean root and tuber crops, cassava, *Musa*, potato, sweetpotato, and yam. Three outputs have been compiled to aid this process and ensure compliance with the delivery of GPG2 Activity 1.2 milestones, they comprise:

- Part I - Global Public Goods Phase 2 (GPG2) - Project landscape and general status of clonal crop *in vitro* conservation technologies.
- Part II - *In vitro* conservation status of potato, cassava, *Musa*, yam, sweetpotato, Andean root and tuber crops.
- Part III - Multi-crop guidelines for developing *in vitro* conservation best practices for clonal crops.

These outputs are also intended to facilitate *in vitro* preservation by the wider plant conservation community of practice, therefore, Part I introduces the GPG2 project within the CGIAR landscape and overviews the status of *in vitro* plant conservation subsequent to the IPGRI-commissioned report of Ashmore (1997).

2. GPG2 project road map and landscape

The CGIAR's global membership comprises developing and industrialized countries, private foundations, regional and international organizations each cooperating to provide strategic direction, technical assistance and funds in support of CGIAR's mission. Co-sponsorship is provided by the Food and Agriculture Organization (FAO) of the United Nations (UN), The United Nations Development Programme (UNDP), and the International Fund for Agricultural Development (IFAD) and the World Bank.

2.1 Overview: CGIAR's plant genetic resources conservation landscape

Of the 15 CGIAR research centres, the following four are involved with conserving clonal crops *in vitro*: Centro Internacional de Agricultural Tropical (CIAT), Centro Internacional de la Papa (CIP), International Institute of Tropical Agriculture (IITA) and Bioversity International. Most activities of the group are common to all CGIAR centres and they are mandated to respond to GPG2 by:

- Collective programmatic action to increase the overall impact of Centre activities on poverty alleviation.
- Collective institutional action to increase Centre impact efficiency.

In vitro conservation presents challenges not encountered in other modes of germplasm management, consequently, CIAT, CIP, IITA and Bioversity comprise a specialist community maintaining the international genebanks, which preserve and distribute the *in vitro* genetic resources of clonal crops. These centres cooperate as the 'Clonal Crop Task Force' (CCTF) and they have a common mission to conserve germplasm from Andean root and tuber crops, cassava, *Musa*, potato, sweetpotato and yam. Their operations are shaped by:

- The Global Plan of Action, for the Conservation and Sustainable Utilization of Plant Genetic Resources for Food and Agriculture (FAO 1996).
- The International Treaty of Plant Genetic Resources for Food and Agriculture (FAO 2002).
- The System Priorities for CGIAR Research (CGIAR Science Council 2005).

2.2 The Global Plan of Action

Critical issues pertinent to *in vitro* conservation in the Global Plan of Action are:

- Sustaining existing *ex situ* collections.
- Regenerating threatened species accessions.
- Supporting the collecting of plant genetic resources for food and agriculture.
- Expanding *ex situ* conservation activities.

These are targeted because genebanks and their *ex situ* holdings increased substantially during the 1970-1980s in response to increased threats to global genetic resources security. The need to upgrade and rehabilitate infrastructures was identified as most countries had limited long-term storage facilities and focused support was required to enable *ex situ* conservation by rationalizing activities across genebanks. The Global Plan of Action also recognized the need to conserve under-utilized species and recommends low-cost technology development suited to local conditions, but it cautions that some technologies transferred from temperate climates may not be appropriate for tropical countries and *vice versa*.

2.3 International Treaty of Plant Genetic Resources for Food and Agriculture

The objectives of the International Treaty are aligned with the Convention on Biological Diversity (CBD) and the FAO of the United Nations. They concern the conservation and sustainable use of plant genetic resources for food and agriculture and the fair and equitable sharing of the benefits arising from their use. Supporting components are the Global Plan of Action Articles 14 and 15 for *ex situ* collections of plant genetic resources for food and agriculture held by the International Agricultural Research Centres (IARCs). They concur with other international institutions and agendas related to plant conservation (Ashmore et al. 2007b) and sub-sections specify that the facilities supporting the *ex situ* collections remain under the authority of the IARCs. These undertake their management and administration in accordance with internationally accepted standards endorsed by the FAO Commission on Genetic Resources for Food and Agriculture (Genebank Standards 1994).

2.4 The system priorities for CGIAR research

A process of system-level priority setting was undertaken in 2005 by the Science Council and the SGRP, culminating in the identification of a research projection to 2015 (CGIAR Science Council 2005). The aim being to develop a more cohesive, focused and high quality research programme for alleviating poverty, hunger and malnutrition. CGIAR's Science Council prioritized:

- Achieving a greater impact through a more consolidated research focus.
- Avoidance of research dispersion.
- Rationalization of project funding to maximize the core strength of the CGIAR as a supplier of research pertaining to global public goods.
- More effective mobilization of research capacity across the CGIAR system.
- Strategic engagement in multi-pronged research involving different commodities, themes and disciplines.
- Enhancement of coordination and cooperation across CGIAR's centres.
- Research focused on well-defined system priorities to develop more effective partnerships with National Agricultural Research Systems (NARS) and advanced institutes in the north and south.
- Well defined and consolidated research approaches identifying clear routes to poverty alleviation to enhance the participation of stakeholders in priority setting and assist donor resource allocation, thereby leading to greater impacts.
- Setting System priorities to enhance accountability.

Five CGIAR System priority areas, each defined by four priorities, were targeted by CGIAR's Science Council for action in the period 2005-2015; Priority Area 1 is central to the remit of this review:

- Priority Area 1: Sustaining biodiversity for current and future generations.
- Priority Area 1A: Promoting conservation and characterization of staple crops.
- Priority Area 1B: Promoting conservation and characterization of underutilized plant genetic resources.

2.5 Implementing system-wide priorities in a cooperative landscape

Undertaking a 'system priorities' approach to CGIAR's 2005-2015 activities under the auspices of SGRP presents new elements that will focus on capacity building, conservation, and genetic enhancement activities. These are linked to germplasm and crop usage and research is undertaken for development by matching appropriate technologies to projects. With respect to research management, priorities maintain a system-wide focus for which the cross-cutting theme of poverty alleviation underpins all endeavours. Future interdisciplinary connectivity is intimated, for example, by the successful establishment of in-trust collections of plant germplasm being followed by conserving animal and fish genetic resources. Emphasis is placed on collective research management, review and analysis. This involves creating a framework to optimize collaboration across CGIARs' clonal genebanks, and centres are expected to make evolutionary not revolutionary changes to their scientific programmes. The Science Council and SGRP highlighted CGIAR's need to translate new priorities into coherent research programmes that bridge and synchronize the efforts of the centres and those of their partners.

3. Collective action for the rehabilitation of global public goods

The SGRP's GPG2 Project was devised to facilitate CGIAR centres in meeting their in-trust commitments for managing collections efficiently and sustainably, now and in the future. The Project's overarching remit is to facilitate building a comprehensive global system, for conserving, managing and exchanging plant genetic resources for food and agriculture, it comprises two phases.

3.1 Phase 1 - Springboard for sustainability

The first phase (2003-2006) addressed the most urgent needs identified by each CGIAR centre and SINGER. These included, upgrades in capital items, technical activities and dealing with backlogs in essential conservation operations; for genebanks conserving clonal crops, Phase 1 involved the Clonal Crops Task Force in the CGIAR's upgrading exercise. Consequently reported achievements were: improved storage facilities, alleviating processing accession backlogs, safety-duplication; improved plant health facilities, processing of plant health backlogs, new molecular identification facilities and enhanced accession characterization. Hardware and software upgrades were important outcomes, including barcoding instalment, enhanced SINGER operations and functions and data quality improvement. Phase 1 showed the proven impact of investment quantified as improved infrastructures and capacity building, thereby enhancing the safety and security of global public goods.

3.2 Phase 2 - Consolidation, research and leadership

The second phase of GPG2, and the one to which these documents (Parts I, II and III) pertain, builds upon the efforts of Phase 1. Implemented in January 2007 for a period of three years it focuses on optimizing the CGIARs' contribution to global conservation and the use of its genetic resources held in trust. The centres benefit by enhancing their own facilities, operations and capacities, making them better equipped to serve stakeholders and beneficiaries. The core practical and operational benefits of their collective actions make for a more accessible, cost effective, efficient and secure stewardship of their in-trust collections. Benefits are captured through collective activities (Figures 1-2) targeted at improving the security of crops held in common, through knowledge sharing, cohesive risk management and best practices development. The definitive beneficiaries of the GPG2 project will be the poor farmers of the developing world, as well as national agricultural research stations, public and private plant breeding organizations and seed producers. As a result, there will be a greater confidence in the security of genetic resources in a world increasingly impacted by the challenges of climate change, environmental erosion and conflict. Collective action for the rehabilitation of genebanks in Phase 2 also supports a greater access to crop diversity held in-trust by the CGIAR.

3.3 Optimizing genebank operations for clonal crop germplasm

CGIAR's Science Council 2005 review conveyed the intent to link priority setting to monitoring, evaluation and performance measurement. This intercalation was deemed vital for research efficiency and meeting the CGIAR's commitment to the UN Millennium

Development Goals (MDG). Linking these priorities (see Figure 2) aids high standard stewardship through collective genebank experience. Concomitantly, this will improve the quality of and access to information related to CGIAR's germplasm collections and improve streamlined ordering. On completion of the GPG2 project, the Knowledge Base will provide a communications hub (http://cropgenebank.sgrp.cgiar.org/) and a one-point access to information on best practices, policies, risk management, inventory systems, performance indicators, crop information, guidelines and training materials.

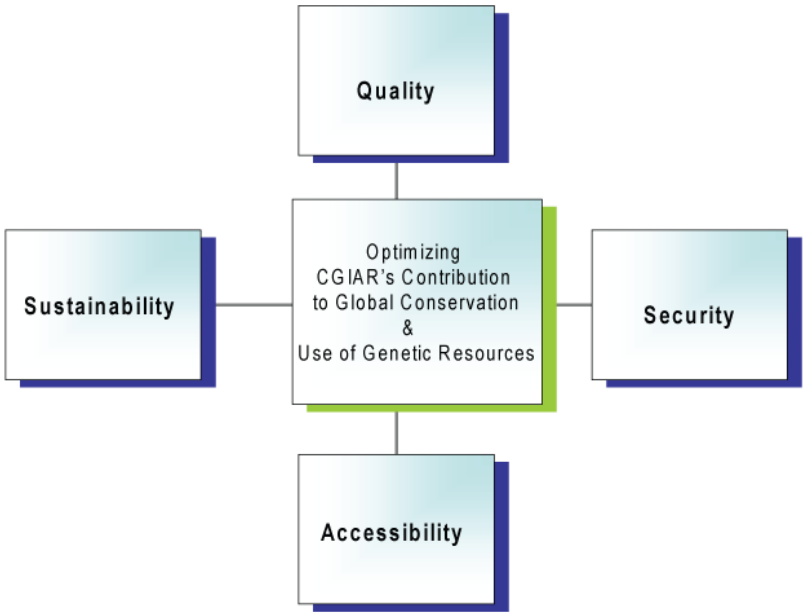

Figure 2. Targets in GPG2 for optimization of genebanking standards and attainment of high standard stewardship within the CGIAR centres.

3.4 *In vitro* genebank standards

The long-term objective of GPG2 is to raise plant genebanking standards worldwide. This requires compliance with regulations concerning germplasm acquisitions, material transfer agreements, ownership and phytosanitary legislation, all of which are drivers for quality management and best practice development. With respect to technical issues, priority areas are: protocol optimization, validating best practices and undertaking risk assessments between cooperative partners. However, attaining high standard stewardship across the CGIAR's genebanks will progress differently as some standards are generic (quality, sustainability, security and accessibility) and cross-cutting (Figure 2) whereas, others are technically specific, albeit overarching standards are common (see Genebank Standards 1994).

3.4.1 The significance of standards in quality storage systems

A standard is a level of quality accepted as the norm, or a means by which attainments are judged, they are important targets for individuals, groups and organizations to aim for, and they are central to developing quality systems across federated genebanks. Commonly held standards also provide cohesion across their communities of practice and they help to build

consensus in meeting collective compliance with regulations, good practices and codes of conduct (von Versen et al. 2000). However, Genebank Standards (1994) offer two cautionary points: (1) limitations of fixing of standards in a point in time and (2) inability of some institutions to attain standards. Problems can arise when existing standards limit advancements that are in step with future technological developments. It is therefore important that the CGIAR's global genebank networks do not become fixed at any one level, this concern is addressed in force by the GPG2 project and its motivation for best practice development. Intrinsically more problematic, are constraints in institutions, for which standard attainment will remain aspirational whilst their capacity building progresses (OECD 2007). Genebank Standards (1994) specify two standards:

- **Acceptable standard**: in many cases minimal, but adequate in the short term.
- **Preferred standard**: a higher and consequentially safer standard.

The CGIAR has progressively developed standards and technical guidelines for *in vitro* germplasm collections (Withers 1985; IBPGR 1986; IPGRI-CIAT 1994; Panis and Thinh 2001; Engels and Visser 2003; Reed et al. 2004a; Rao et al. 2006; Panis 2009). These contemporary standards have added value when working towards and sustaining accreditation, such as ISO certification via an external body at CIP. However, once awarded there is a need for a long-term financial and service commitment to maintain accreditation status. Where resources and infrastructures are limited, curators need to be pragmatic, as although prevailing conditions may be less than ideal, collections should not be placed in jeopardy (Genebank standards 1994). It is critical to perform risk assessments when setting standards, for germplasm held *in vitro*, these may need to be more risk averse to compensate for the potential loss of essential infrastructures such as liquid nitrogen (LN) supply. The management and stewardship of clonal genetic resources maintained in culture and cryogenic storage has some commonality with orthodox seed banking (Genebank Standards 1994; Engels and Visser 2003; Rao et al. 2006) but there are some critical differences between these two modes of conservation. For *in vitro* conservation, long-term, sustainable efforts are best supported by preferred standards due to the (very) long-term security and safety requirements of specialist infrastructures.

4. *In vitro* conservation: safeguarding against loss of clonal crop diversity

Seed storage is the preferred conservation method, but it is not feasible for germplasm from crops that are either clonally propagated and/or that do not produce seeds. For some genotypes, elite genetic combinations are only preserved through clonal means as their conservation is dictated by breeding strategy, this is because heterozygosity does not permit the maintenance of desired characteristics. Clonally propagated plants thus require special conservation approaches. Options include maintenance in field genebanks and the conservation, in cold stores of dormant vegetative propagules (Reed 2001), however, these methods have limitations regarding efficiency, costs, security and long-term maintenance. *In vitro* conservation is preferentially applied to clonal crop germplasm as it also supports safe germplasm transfers under regulated phytosanitary control (IBPGR 1988).

4.1 Principles of the *in vitro* genebank (IVGB)

Conservation in IVGBs combines tissue culture and cryopreservation for medium-term (MTS) and long-term (LTS) storage respectively (Figure 3). For MTS, subculture intervals are extended, reducing processing costs by arresting growth using reduced temperature treatments and/or growth retardants. For LTS, germplasm (usually shoot tip meristems) from *in vitro*-propagated plants is cryobanked for long-term storage in liquid nitrogen (LN) to a minimal temperature of -196°C in the liquid phase.

4.1.1 The *in vitro* base genebank (IVBG)

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 samples originally procured that are used to establish the collection (Figure 3). Key attributes of germplasm in the base collection are: (a) it is preserved for the long-term and (b) it is not normally distributed directly to users. A base collection ideally represents a comprehensive genepool of the crop or species; for security purposes this is dispersed and managed across different institutions. Since the germplasm they hold is stored for extended periods, IVBGs are usually designated as cryobanks (IBPGR 1986; IPGRI-CIAT 1994). Different cultures, explants and germplasm types are used for conserving plant genetic resources. Examples include: pollen, seeds, embryonic axes, excised zygotic embryos, somatic embryos, dormant and/or acclimated bud material, shoot meristems, nodal cuttings, callus and cell suspensions (Day and Stacey 2007; Reed 2008). Cryopreservation has been applied to a wide range of crops and other socio-economic plant groups, including, clonal forestry and agroforestry species, horticultural plants, biotechnologically significant, secondary metabolite producing cell lines and transformed plant germplasm and cultures (Benson 2008a). This report is mandated to place emphasis on CGIAR's genebanks; as such focus is given to the *in vitro* cryopreservation of shoot meristems derived from clonally propagated crops.

4.1.2 The *in vitro* active genebank (IVAG)

As defined by Genebank standards (1994) an active collection comprises accessions immediately available for multiplication, distribution and use and based on the principles similar to those developed for seed banks, the IVAG was created (Withers 1989). The cyclic flow of material is the key feature of IVAGs, which maintain by successive subculturing, the

renewal and distribution of their cultures (Figure 3). Within IVAGs, cultures are maintained under conditions that slow or retard growth (IPGRI-CIAT 1994); this increases the efficient use of resources and staff time and offsets selection risks and contamination.

4.2 *In vitro* technologies and the safe movement of germplasm

The IVGB supports the safe movement of germplasm across international borders providing assurance that pathogens (including viruses, viroids, phytoplasmas, microorganisms, pests) are not transmitted, this is facilitated by:

Phase 1: Germplasm import, *in vitro* pathogen testing, eradication, and indexation.

Phase 2: *In vitro* storage of certified pathogen/disease-free germplasm.

Phase 3: Germplasm export, shipment from the *in vitro* conservation laboratory.

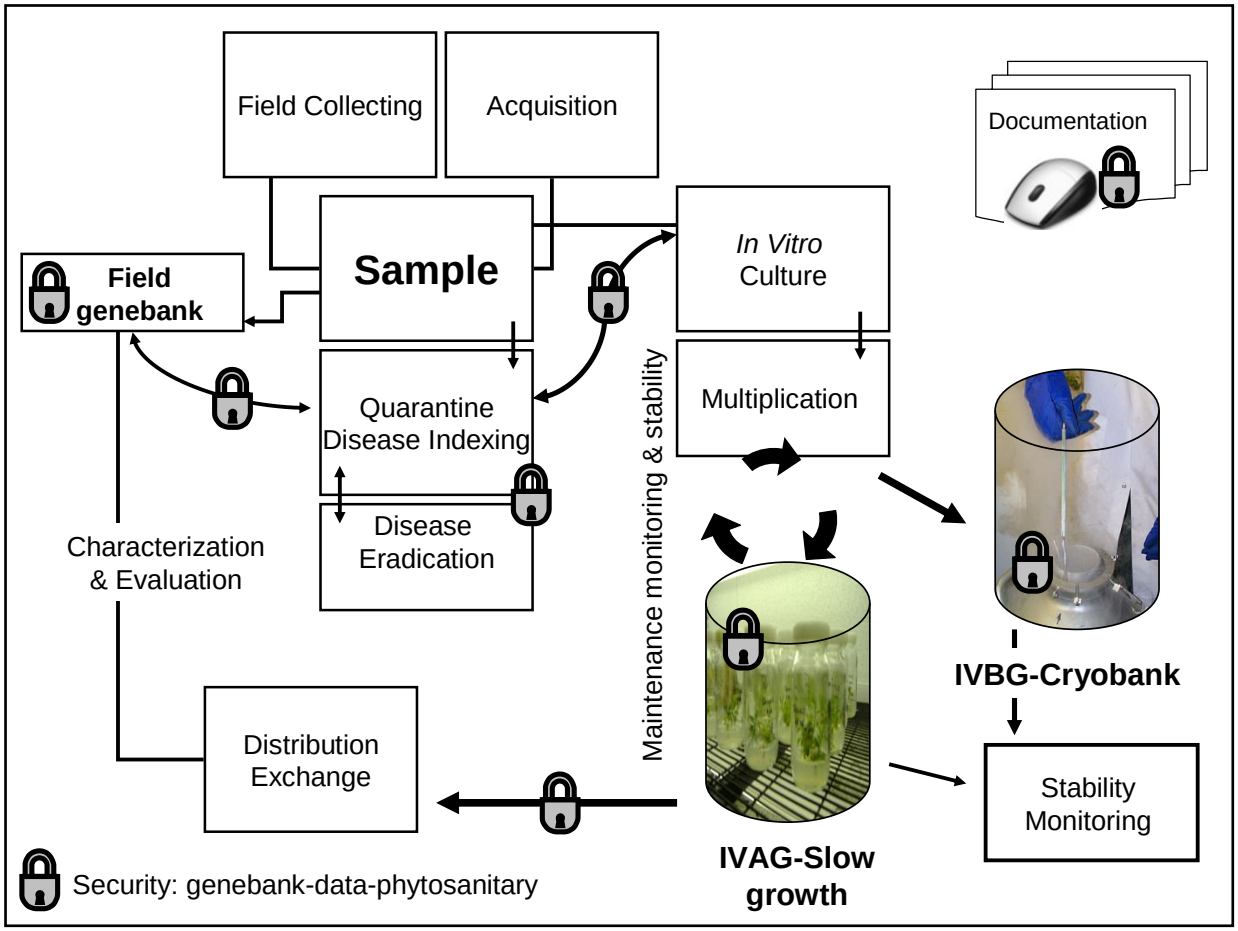

Figure 3. Principles of the *in vitro* genebanks and their relationships with other operations (based on IBPGR 1986).

IVAG = *In vitro* active genebank, utilizing slow growth for medium-term storage (MTS)
IVBG = *In vitro* base genebank, utilizing cryostorage for long-term storage (LTS).
Critical points of security are indicated.

Regulated quarantines and inspections take place in Phases 1 and 3, on collection and distribution. The timing of pathogen indexing is critical and may require *in vitro* quarantine, dependent on whether initial indexing and virus elimination have been performed before, or

after germplasm is placed in culture (Reed et al. 2004a), as would be the case for germplasm collected using *in vitro* techniques (Pence et al. 2002). Virus and pathogen testing and elimination may be undertaken at any stage, no plants should be distributed until testing is complete and delineation of *in vitro* collections into tested, certified disease-free and untested components is advisable.

4.2.1 Containment, quarantine and testing

In vitro containment, in combination with disease indexing and transfer of cultures maintains a high level of phytosanitary control (IBPGR 1988; Reed et al. 2004a), although this is only upheld if critical conditions and requirements are met:

1. **Documentation:** robust record keeping and the use of tracking systems throughout, so that the phytosanitary status of a sample is known at any time, optimally by using bar-coded electronic inventories and data loggers.
2. **Quarantine:** *in vitro* containment is not a substitute for quarantine and at appropriate points (e.g. entry of materials into the collection) stringent isolation procedures should be adhered to in compliance with quarantine regulations.
3. **Timing of pathogen testing:** separation and safe containment of different collections into the safe storage of material, prior to therapy and by the segregation of indexing into 'pathogen tested' and 'pathogen untested' and the control of flow through collections as materials progress through the various stages of phytosanitary treatment. This includes materials infected with more than one pathogen and those that have not completed a therapeutic cycle or been certified free from pathogens.
4. **Testing containment:** comprising totally contained pathogen-testing systems.
5. **Phytosanitary treatments:** visual inspection, pest/pathogen testing and treatment, virus indexing and elimination (meristem culture, thermotherapy, chemotherapy, cryotherapy) leading to disease-free status certification.
6. **Technology updates:** keeping pace with new technologies for pathogen testing.
7. **Testing *in vitro*:** where virus testing/indexing is undertaken *in vitro*, detection and elimination procedures must be developed for cultured materials as the amount of virus can vary in plants. It is cautioned that *in vitro* plants may not provide adequate inoculum, so maintenance of positive *in vitro* controls in special collections helps to circumvent the risks of false negatives. The potential for tissue culture components (e.g. plant growth regulators) interfering with test outcomes requires clarification and steps should be applied to prevent false negatives.
8. **Safe destruction and disposal:** safe removal and disposal of infected materials ensures that a pathogen or pest is not released into the environment.
9. **Purposeful retention of infected cultures:** it may be desirable to maintain some infected *in vitro* materials under strict containment so they may be used, as reference materials or as positive controls in testing procedures.
10. **Distribution control:** stringent procedures for the distribution of materials that are acceptable to recipients and plant health authorities; *in vitro* processes offer advantages as small volumes of disease-free certified materials can be dispatched more effectively. Stringent virus therapy, and disease indexing are crucial for offsetting the risks of pathogen transmission; unless treatments are robust *in vitro* material cannot be guaranteed virus free (IBPGR 1998).

4.3 Security of *in vitro* germplasm storage

Security measures should be compliant with safety, and ethical authorities, regulations and guidelines; including observance of: (a) the Convention on Biological Diversity, (b) the Standard Material Transfer Agreement (SMTA) for plant genetic resources exchange and (c) the International Plant Protection Convention. Security is required to ensure the core responsibilities of all biorepositories (Stacey and Day 2007) which are:

Purity: freedom from contaminating organisms,

Authenticity: correct identity,

Stability: fit-for-purpose and trueness-to-type.

4.3.1 Purity: freedom from contamination

Tissue culture is central to clonal plant health care, conservation, and safe germplasm movement and risks of containment breach (Figure 4) must be addressed. Following phytosanitary processing there remain three main modes of entry of a potential contaminant that can compromise the *in vitro* conservation cycle by pathogenic or adventitious means: (1) a covert or unknown organism goes undetected; (2) contamination enters from the external environment or as a consequence of operations and (3) cross contamination from another culture, culture vessel or cryovial.

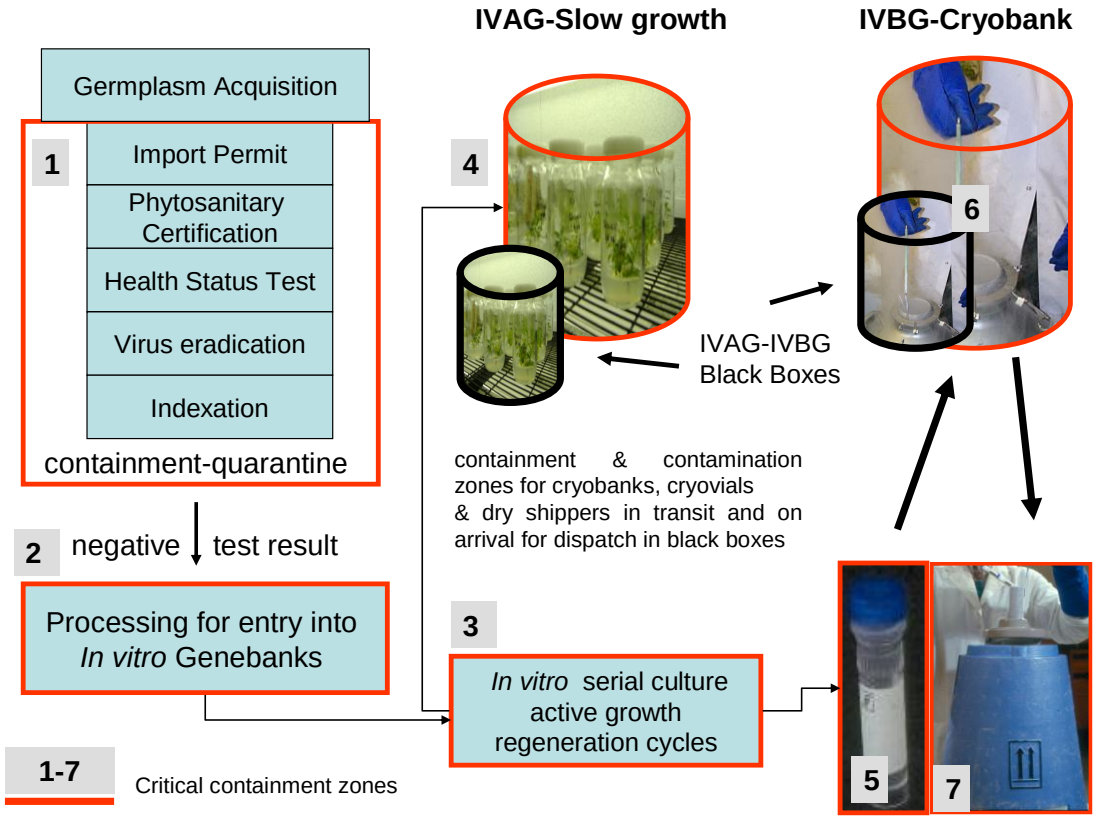

Figure 4. Critical containment zones (1-7) for clonal plant germplasm in an *in vitro* genebank: (1) processing before entry into the genebank; (2) entry after confirmation of negative test result; (3) serial subculture for regeneration, bulking up of germplasm for the IVBG, IVGB and dispatch; (4) IVAG, associated black box; cryogenic containment in: (5) the cryovial; (6) the cryotank and associated black box and (7) the transit Dewar. Breach of containment at any one stage can lead to contamination.

All these factors should be considered a real risk and operators are cautioned to assume their practices ensure, at all times, that germplasm is not and does not become contaminated; yet take precautionary measures because they may be infected (Pegg 1999). Protection of germplasm conserved *in vitro* is reliant on good laboratory practices and asepsis; stringent attention to containment is essential and implicit to IVGB operations is testing for aseptic technique competency.

It is likely a contaminant still residing in germplasm after disease eradication and sterilization procedures is of unknown source and identity. This type of infection can be more problematic to control than a pathogen eradicated by disease indexing before entry to the IVGB. Pernicious contaminants are usually covert, resilient and systemic endophytes and once in the IVGB they can become opportunistic pathogens and pandemic agents, particularly if they are spread by vectors such as mites. Latent infections are challenging as they can go unnoticed for several months. Standard tissue culture media may not support the active proliferation of many bacteria, fungi and yeasts, as certain components attenuate the growth of nuisance microflora leading to undetected false negatives. These are revealed later as positives when cultures become stressed by extended subculture cycles, slow growth, and cryopreservation. Some organisms are opportunistic pathogens, whilst others have beneficial associations with plant materials (Hamill et al. 2005). Any microorganism or its propagule able to grow in culture is a contaminant and even if benign, it may become pathogenic or a nuisance under different conditions. Bunn and Tan (2004) report that any bacterium in a tissue culture can form epiphytic, endophytic, or pathogenic associations. Over 40 different bacteriological genera have been isolated as plant tissue culture contaminants, including both gram negative and positive bacteria. These have been found in similar proportions and commonly include *Bacillus* spp. and *Pseudomonas* spp. (Leifert and Waites 1990); the axenic state is therefore only a presumed and temporary condition. To circumvent risks of contamination from covert, adventitious organisms it is recommended that potential routes and points of entry are identified and measures are taken to block them (Cassels 1991; Bun and Tan 2002; Thomas and Prakash 2004; Thomas 2007). It is prudent to ensure that cultures are indexed and eradicated of nuisance and covert microflora before any germplasm enters the genebank (Tanprasert and Reed 1997a, b).

4.3.2 Authenticity: correct identity

Authenticity is confirmation a genetic resource is what it is assumed to be, it is a process that uses stable phenotypic and genotypic characters as evidence to verify that the identity of germplasm is correct (Müller et al. 2005, 2007; Stacey and Day 2007). Obviating the risks of misidentification and incorrect labelling are key quality control measures in clonal crop genebanks, particularly for those using a multiplicity of procedures and conservation processes. Failure to retain an authentic status has severe consequences and in some sectors, cultures not matching their purported origin and identity have resulted in published research papers being withdrawn and the invalidation of research projects (Stacey 2004). Within CGIAR's genebank operations authentication commences with the verification of documentation associated with germplasm acquisition. This involves confirming evidence with donors concerning the reliability of passport information, followed by the classification of incoming accessions by testing standard markers and descriptors. Informatics tools may be used and incoming accessions are categorized as 'tentative' until they have been characterized. A wide range of molecular techniques can be applied to authenticate germplasm (de Vicente 2004) and their use is evidence that plant genebanks are evolving to

meet the needs, and harness the benefits of molecular technologies; this has advantages in enabling the analysis of holdings and connecting omics technologies (see *Section 6.2*) and research to genetic resources conservation and use. This includes the possible authentication of plant genebank holdings by embracing the 'bar-code-of-life,' a concept which is currently evolving in other bioresources sectors (see Gachon et al. 2007; Williamson and Day 2007). DNA barcoding is a robust technology with multiple uses, in addition to research benefits it helps avoids unnecessary duplication, allows routine checks for genetic authenticity, and helps to ensure a mistaken identity is not perpetuated. However, the technology will require further research before its application in crop genebanks is possible and practical.

Stringent recording and documentation of stored materials within and across MTS and LTS inventories is essential as germplasm is maintained for extended timelines in cryobanks and slow growth. Furthermore, records and documentation processing is effected when staff and modes of record-keeping change. Electronic inventory systems are robust traceability processes as they support retention of authenticated status and help to prevent errors arising from transcribing hand written records. They also help to optimize management practices and as such become cost effective and efficient tools. Electronic barcoding is also a powerful quality assurance tool as it allows instant traceability and provides current information on status at any point in the genebank and its process chain.

4.3.3 Stability: optimal storage

In vitro genebank practices need to ensure their biological resources maintain their special characteristics and are not changed because of storage and associated tissue culture practices. Genetic instability includes the risks of *in vitro*-generated instability termed somaclonal variation (SCV), defined by Scowcroft (1984) as heritable genetic variability in plants generated through tissue culture. Genetic changes can also arise because of epigenetic processes, stress and selection pressure. The consequences of SCV are significant for genetic resources conservation as it is manifest in the regenerated plant; therefore, reducing the potential risk of SCV is necessary. Scowcroft (1984) suggested that *in vitro* storage protocols should avoid practices that increase the risks of genetic variation occurring. These are: (1) avoiding germplasm propagation via dedifferentiated (callus) and adventitious routes; (2) limiting the use of plant growth regulators that increase the possibility of dedifferentiation and adventitious development, and (3) selecting germplasm from young cultures as SCV increases and totipotency decreases during prolonged culture.

Genetic instability arising from tissue culture is particularly significant for clonally propagated crops as compared to sexually propagated species in which chromosomal abnormalities are eliminated by gametogenesis and fertilization (Cassels and Curry 2001). Ideally, germplasm with a higher risk of manifesting genetic instability should be monitored at the genetic level, as recessive genetic changes occurring during the tissue culture of asexually propagated species will have no phenotypic expression. In this context (see Scowcroft 1984), clonal crops may thus be expected to display a potentially higher frequency of SCV than those propagated by seed; however, as off-types can arise in field-grown, clonally propagated plants, some variation may be unrelated to culture conditions. *In vitro* conservation can help safeguard against the genetic changes that occur in field-propagated materials that have a predilection to producing off-types.

The issue of *in vitro*-generated genetic instability and its consequences for clonal crop conservation thus requires very careful consideration and in all probability, on a crop-by-

crop basis. This is the case for banana, which has a tendency to produce off-types and for some genotypes this inclination can be exacerbated by tissue culture (Sandoval et al. 1996; Sahirjam et al. 2003; Ramage et al. 2004; Strosse et al. 2004). A prudent measure may be to conduct a risk management exercise for those crops, species, or genotypes that are known to have a higher propensity for off-type production and instability. This approach would help to allocate the safest conservation strategy by taking practical measures to reduce the risks of any instability occurring. For example, by considered selection of explant type and plant growth regulators, applying regeneration cycles and using quality controls to define the limits of acceptance of variants (de Oliveira et al. 2000; Lakshmanan et al. 2007). Sharma et al. (2007) assessed the stability of potato plants regenerated by various routes and concluded that low-level molecular variation may become apparent on a genome-wide level and, that in the case of somatic embryogenesis this could be attributed to epigenetic changes. In their study, differences in yield and height at the time of harvesting were not significantly different among potato plants propagated through four different routes (axillary bud proliferation, somatic embryogenesis, microtubers and true potato seed). Tyagi et al. (2007) observed no significant variation in shoot cultures of turmeric conserved *in vitro* for one year. In this study, stability was confirmed by comparing 25 primer-RAPD profiles of mother plants with those of *in vitro* conserved plantlets. Ryynänen and Aronen (2005) similarly used RAPD analysis to demonstrate stability in short and long-term tissue cultures of silver birch and for meristems recovered from cryostorage. In contrast, using 44 primer-RAPD profiles, Santos et al. (2008) found variability in micropropagated, ornamental pineapple.

Scowcroft (1984) recommended field performance trials of clonal crops should be extended to two propagation cycles, this is based on the potential for both pre-existing and induced genetic variation becoming manifest in culture-derived plants (Scowcroft 1985). Rani and Raina (2000) caution that variation and instability is not only confined to callus cultures and they present evidence for SCV arising from organized meristems, as revealed by molecular technologies. The finding that SCV occurs in plants derived from organized, meristematic shoot cultures has ramifications for the *in vitro* conservation of clonal crops, although its prevalence is most likely to be crop-specific. Strosse et al. (2004) reported SCV to be widespread in banana plants regenerated from shoot cultures, they found the incidence of this occurring to be cultivar dependent, and that the frequency of instability was amplified by culture-induced factors. Thus, the number of *in vitro* generation cycles affected the rate of variation, whereas standard growth regulators did not.

Growth retardants might impose selection pressures and genetic change with time, and environmental stress could induce mechanisms, which cause genomic modification, particularly at the epigenetic level (Cassels and Curry 2001; Harding 2004). The biological stasis of storage in LN may be expected to offset the risks of genetic instability occurring; although culture practices associated with pre- and post-cryopreservation manipulations may still cause stability problems (Harding 1996). Stability testing of plants recovered from MTS and LTS has been undertaken at cellular, biosynthetic, phenotypic and genotypic levels (Harding 2004; Cha-um and Kirdmanne 2007). A result may not be indicative of genetic instability or stability and trueness-to-type evaluations (Perazzo et al. 2000) thus, crop performance indicators (Martínez-Montero et al. 2002; Medina et al. 2007) may be applied to support molecular testing. Methods include the molecular, genotypic and phenotypic assessment of stability and evaluations of developmental competence and field performance of clonal crops regenerated from *in vitro* storage. Examples are: nuclear and chloroplast DNA

(Harding and Benson 2000); microsatellite analysis (Harding and Benson 2001); long-term field performance trials (Konan et al. 2007); biometric analysis of phenotypes and developmental competency (Benson et al 1996a; Harding and Staines 2001, Harding and Benson 1994); AFLPs (Hao et al. 2001, 2002a, b); RAPDs (Schäfer-Menuhr et al. 1997; Hirai and Sakai 2000; Dixit et al. 2003); SSRs (Perazzo et al. 2000); combined analyses of genotype, phenotype and biosynthetic stability (Ahuja et al. 2002); secondary metabolite production (Dixit et al. 2003); RFLP ribosomal RNA genes (Harding 1991, 1997), RAFs (Randomly Amplified Polymorphic DNA Fingerprinting) (Kaity et al. 2008); DNA methylation (Harding 1994; Harding et al. 2000, Kaity et al. 2008), flow cytometry (Ward et al. 1993) and ploidy status (Benson et al. 1996a) and assessment of transgene stability (Ryynänen et al. 2005). Harding (1996) comments on these methods in assessing the risks of genetic change in plants recovered from *in vitro*-stored germplasm. In this document, these issues are discussed with respect to contemporary conservation developments (see S*ections 6.2, 6.3.4* and *6.4.3*).

5. Risk management and safety in the *in vitro* genebank

In the GPG2 project, risk management is core to all activities and has a wider significance in anticipating and avoiding any threat to the security and sustainability of in-trust collections. In the GPG2 project risk management has two main contexts:

1. Internal risk management concerning technical, physical and biological risks to which the collections and their related information are exposed.
2. External risk management concerning factors threatening project objectives.

The risk of a potential hazard occurring, or a threat to safety and security to achieving a desirable outcome requires evaluation across different operational activities these are described as follows.

5.1 Links between risk management, best practices and safety

Effective management of risk is essential for creating and maintaining a safe *in vitro* storage environment, including the safety of personnel regarding their exposure to potentially hazardous procedures, substances and equipment. Understanding relationships between best practices, risk management and safety is thus imperative for achieving a successful outcome. A best practice is thus inherently dependent upon managing risks that might lead to its failure or cause harm and, it is an activity or process that is:

1. More effective at delivering an outcome than any other method.
2. The most efficient and effective way of accomplishing a task.
3. Produces an outcome with fewer problems.

Risk assessment provides a useful framework in which to develop best practices, although it is important to be aware that managing risk can become prescriptive where regulatory and statuary obligations come into force. Non-compliance with regulations pertaining to the acquisition, transfer, safe movement and conservation of germplasm in an IVGB is a serious risk. Compliance also includes risks to personnel health and safety in the workplace and conforming to regulations, these must take precedence as they dictate the route by which a technical procedure is safely undertaken. Implicitly a best practice must not put at risk any person, component, process or procedure and it is essential to be aware that levels of risk and tolerance can change during research and development. For example, as a methodology proceeds from research to validation and becomes implemented as a routine procedure. Changes in statutory, regulatory policies are often invoked in response to an unforeseen event or accident and can have ramifications for existing best practices (Pegg 1999; Fuller and Dijk 2008). This is complicated by personnel health and safety risk assessments having various levels of compliancy in different countries, regions, institutions and sectors (Tomlinson and Sakkas 2000; Tomlinson and Pacey 2003; Tomlinson 2005, 2008). These regulations take priority where extreme hazards are concerned, as would be the case for the handling of LN, pressurized gases and hazardous chemical substances. Risk assessment is not static, as new knowledge about a process, substance or pathogen can change, the level at which a risk is assessed may also necessitate changes to a best practice. For example, experience gained from a failure to identify a LN containment problem precipitated an immediate change in risk management, new regulation, and improved best practices in the medical cryopreservation community (HFEA 1998, 2007; Pegg 1999).

Risk assessment has therefore an inextricable role in developing best practices, and can be used to advantage by: (1) providing a robust framework in which best practices are developed; (2) signposting where best practices need to comply with regulatory obligations and (3) creating opportunities to refine existing best practices. Practically, this is the case when new information emerges from risk mitigation research, training and development.

5.2 Developing risk assessments for *in vitro* genebanks

Identifying type of hazard and its associated risks are the first steps of any assessment for which, there are two main categories of risk. Unavoidable risks are outside the control of the operator, as no matter what safety measures are in place they cannot offset the risk. These are usually a *force majeure* and include climatic disasters, armed conflict and terrorism. Avoidable risks are potential hazards resulting from routine operations conducted without due care and vigilance; these are identified and measured in the first stage of a risk assessment. The second stage describes actions required to prevent incidents from happening and the third informs how to deal with their consequences as effectively as possible. These measures are formalized by a risk assessment which is a systematic, recorded operation that rationally foresees and puts into place measures to protect against an adverse or damaging incident occurring. An integral part of a risk management process involves the reporting of accidents and incidents, usually formalized in a no blame culture. Incident outcomes involve the fourth risk management stage, which is learning from accidents in order to refine risk management procedures.

Various information sources are used to compile a technical risk assessment: international and national regulations, guidelines and agreements (e.g. for phytosanitary control) providers of chemicals and their Material Safety Data Sheets (MSDS), specialist cryogenic instrument and gas suppliers and international and national government health and safety bodies. The UK's Health and Safety Executive (HSE 2006) suggest five steps to start a risk assessment:

Step 1 **Identify** the hazards.

Step 2 **Decide** who or what may be harmed.

Step 3 **Evaluate** the level of risk and decide on precautions.

Step 4 **Record** the finding and implement them.

Step 5 **Review** the assessment and update regularly.

A hazard is defined as anything or a process that can potentially cause harm, a risk is the chance high, or low of harm being caused. Identified hazards can be cumulative and risk assessment involves the whole process or procedure, in its totality as well as its component steps. This includes an indication of how serious, the harm could be and its long-term effect, as well as the impact of hazards, an analysis of which can be formally structured using quality assurance systems such as the Hazard Analysis Critical Control Point (HACCP). This may be applied to plant tissue culture and IVGB operations, for example, as adapted from Leifert and Cassells (2001) a critical point assessment for microbiological contamination might include:

1. In depth analysis of all contamination sources using the identification of indicator organisms for specific contamination sources.
2. Establishing monitoring systems for all contamination sources at critical control points (CCPs) and developing improved detection methods for contaminants and their sources.

3. Improving methods for prevention of contamination at critical control points.
4. Developing deterrence methods in case preventative strategies fail.

The HACCP method is useful for developing risk assessment in IVGBs that have problems with persistent, latent (i.e. endophytic) bacterial contamination (Leifert and Waites 1990; Cassels 1991; Leifert and Cassels 2001) and assessing containment risks during complex manipulations, such as meristem excision and cryoprotection.

5.2.1 Collective action for risk assessment in CGIAR'S *in vitro* genebanks

Risk assessments undertaken within GPG2 are formalized using a generic template and assessment metrics. Training in risk management and working towards a common consensus across a community of practice is advisable.

5.2.1.1 Risk identification and mapping

Technical risk auditing for biorepositories involves several levels of complex evaluations which can be simplified using a logical risk matrix (Table 1). Possible strategies for generic risk identification can be based on personnel, sample, process, facilities, and infrastructure. Mapping out risk zones, areas and processes within genebank activities and operations aids risk identification and reduces the possibility of overlooking a potential hazard. Work flow-charts used in quality assurance exercises can also be used to plot risks in a logical order. In developing a risk map for an IVGB, the following exemplars may be included:

1. **Institutional infrastructures:** essential infrastructures and services (e.g. electricity, generators water, gas, maintenance; IT support and computers; security systems, alarms, alert devices, fire).
2. **Specialist facilities:** LN supply; special containment zones, laminar airflow cabinets, ventilation/air conditioning, culture/growth rooms, task lighting (for culture rooms); autoclave, laboratory equipment and instrumentation (calibration, validation, auditing, safety and routine maintenance); cryotanks, freezer, refrigerator; cryotank surveillance and monitoring, LN-low-level security systems alarms, low level-O_2 personnel safety monitors, alarms and alert devices.
3. **Generic methodologies and processes:** compliance with regulations; germplasm acquisition, collection, germplasm and tissue culture selection, processing, treatment; culture media quality assurance and preparation; phytosanitary treatments, quarantine, containment, monitoring and surveillance; records management, documentation, tracking and traceability; post-storage recovery procedures, regeneration, stability and field performance assessments; germplasm exchange and transfers.
4. **Specific storage methods:** germplasm selection, for slow growth (culture medium, temperature, osmotic and special treatments); germplasm selection, treatment for cryopreservation, meristem excision, risks of different cryopreservation protocols (pregrowth, cryoprotection, cryogenic protocol; cryovial, cryotank, choice of LN phase, cryotank inventories and input output procedures; rewarming, recovery, shipment in dry shippers) black box security, containment, security; regulatory safety audits of cryogenic equipment.

5.2.1.2 Risk impact, probability and acceptability assessments

Once a consensus for risk assessment has been collectively agreed upon, a logical framework is created to sequence, group and assess the risk; each step is identified with a specific level of

risk, with respect to its impact, probability, and acceptance. It is usual to prioritize risks into levels from very high to very low, with the greatest loss and greatest probability of a risk occurring being considered first. This can be difficult to rationalize, particularly for complex and multiple procedures and to assist this process Tomlinson (2005, 2008) has adopted the Australian/New Zealand model for managing risks associated with cryopreservation. The score is the product of the consequences and the chance of it occurring, i.e. risk x likelihood, it indicates the adequacy of controls and informs as to whether to accept the risk or not.

Table 1. Generic risk assessment metrics for GPG2 Activity 1.1 used to develop and implement risk management procedures in CGIAR's crop genebanks.

Description	Assessment and score metrics
Genebank objective	IVAG (MTS) IVBG (LTS)
Genebank activity Operations component	e.g. culture initiation, culture media preparation, slow growth, cryoprotection, cryopreservation, phytosanitary treatments, storage, recovery and regeneration procedures, cryogenic transit shipments, black boxes
Risk identification	Sequential identification of risk pertaining to each procedure and activity
Risk potential impact	Very Low (VL), Low (L), Medium (M), High (H), Very High (VH)
Risk probability	Very Low (VL), Low (L), Medium (M), High (H), Very High (VH)
Accepted risk	No (N), Yes (Y)
Actions and strategies to eliminate or manage risks	Sequential corrective actions

A well-audited, accurately scored technical risk assessment is desirable as it guides where resources, personnel, and funding are required to ensure successful risk mitigation. Validation of risk assessment and safety procedures is also desirable and has been undertaken in cryobanks to minimize the risks of contamination (Maertens et al. 2004). Technical risk assessments of complex IVGB processes should yield concise, accurate, and easy to understand information. This is necessary as all technical operators must understand the process and the process informs how risk management decisions are prioritized and acted upon efficiently and cost effectively (Tomlinson 2008).

5.3 Actions and strategies to eliminate or manage risks

Once a risk assessment exercise has been performed for all the components of an activity, actions and resources can be put in place to eliminate and manage the risks, for example:

1. **Compliance:** with standards and regulations specifically designed to offset and eliminate risk and adherence to manufacturers' and chemical suppliers' instructions.
2. **Risk avoidance:** not performing an activity that carries a risk.
3. **Risk elimination:** putting in place measures, controls, countermeasures to eliminate risk.

4. **Risk reduction:** putting in place measures controls and countermeasures that reduce the severity of a risk.
5. **Risk contingency and emergency:** putting in place measures controls and countermeasures that deal with the outcomes of an incident or accident should all risk countermeasures fail.

5.3.1 Examples of risk amelioration strategies for IVGBs

1. Staff safety equipment, personal protective clothing, alarms for LN or O_2 depletion, putting medical, health and safety protocols in place in case of an accident.
2. Screening for pathogenic, adventitious, endophytic, systemic and covert microbial flora and pests before allowing entry to the IVGB.
3. Physical security and containment of growth rooms, clean rooms and cryotanks.
4. A reliable LN supply and a back up or alternative supply for contingencies.
5. Back up duplication of accessions in black box collections.
6. Containment of culture and cryostorage vessels.
7. Correct choice and containment of cryovials, to reduce transmission of cross and adventitious contamination by direct exposure to LN and to prevent explosion hazards.
8. Appropriate choice of LN phase storage to avert cross contamination of samples and the destabilization of vitrified germplasm around the critical Tg zone.
9. Locating cryotanks in clean rooms to minimize cumulative adventitious contamination.
10. Alarmed cryotanks and auto-fill systems.
11. Avoiding use of equipment unsuitable for cryogenic manipulations.
12. Adherence to manufacturers' safety advice for use of cryogenic equipment.
13. Witnessing, security labelling and barcode tracking.
14. Spot-check monitoring and use of sentinels for pathogenic, adventitious and covert microbial flora, pests and mites throughout the genebank process chain.
15. Stability surveillance and assessments.

5.4 Risk management reviews and training

Continual reviewing and updating of risk management procedures is required for risk mitigation, reviews should be scheduled on a routine, recurrent basis and as acute responses to incidents and accidents. Surveillance of safety literature and manufactures' information should be ongoing in order to assimilate new information that may require immediate changes to safety procedures and policy. These actions ensure risk assessment criteria and operations remain applicable and effective and that evidential changes to processes are implemented. This is necessary if a hitherto unknown risk has been identified as hazardous; risk management is therefore an evolutionary process and it requires consensus across communities of practice. The first risk assessment exercise will not usually be optimal, requiring refinement and testing for compliance and accuracy to meet local and group needs. Training in risk management is thus essential for both new and experienced personnel as it avoids the risk of poor practices being retained and infiltrating processes.

6. General status of the *in vitro* conservation community of practice

This section overviews the development in slow growth and cryopreservation technologies based on and following the research agendas and recommendations of Normah et al. (1996), Ashmore (1997), Engelmann and Takagi (2000) and Reed et al. (2004a). A recommendation arising from the workshop on 'Cryopreservation of Tropical Plant Germplasm' proposed increased contact with institutes already using cryopreservation on a routine, large-scale, including those conserving microorganisms, and animal germplasm (Engelmann and Takagi 2000). Accordingly, this section explores developments in the wider, global biological resources communities (Caboux et al. 2007; Coecke et al. 2005; Hartung et al. 2002; ISBER 2005, 2008; NCI 2007; Parker and Hunt 2000). Many of these sectors are cooperating to pioneer quality management systems across different geographical regions and communities of practice (Kostiak 2000; Martínez-Pardo and Mariano-Magaña 2007; Smith 1998, 2001, 2003; Smith and Ryan 2001; WHO 2007). Their emphasis is on collaboration and networking for the transfer of safe storage technologies and their validation. The Organization for Economic Co-operation and Development's programme (OECD 2001, 2007) and the coordinated Global Biological Resources Centres Network (GBRCN) exemplify these activities. The microbiological sector, in particular is developing at pace, quality assured and validated management systems for securing their genetic resources in culture collections (Smith and Ryan 2008).

6.1 Utilizing quality systems for clonal crop *in vitro* genebanks

Whilst the use of biological resources is domain specific, the quest for quality systems and best practices is generic across all sectors pursuing validation (Smith and Ryan 2008), third party accreditation (von Versen et al. 2000) and risk management (Tomlinson 2008). Appraising their experiences is timely for CGIAR's crop plant genebank community, particularly as best practices are at the core of the OECD's modern 'Biological Resource Centre' concept (OECD 2001, 2007). The International Society for Biological and Environmental Repositories (ISBER) is similarly developing best practice guidelines for the collection, storage, retrieval, and distribution of biological materials (ISBER 2005, 2008). In the Biological Resource Centre (BRC) framework, conservers of genetic resources also become providers of high quality information related to their holdings. This approach concurs with De Vicente (2004) regarding the evolving role of CGIAR's genebanks, which is motivated by advances in genomics and molecular technologies. These promise to enhance genetic resources utility as an increased understanding of how a gene functions will provide powerful insights into the relationship between phenotype and genotype and can help inform how best to utilize germplasm conserved in genebanks. Consequently, genebank managers will need to offer services across different disciplines to satisfy the demands of modern molecular science. Meeting high standard *in vitro* storage and stewardship is a necessary part of this process, as stakeholders, researchers and beneficiaries require quality, authenticated and stable genetic resources (De Vicente 2004, Stacey and Doyle 1998; Stacey et al. 1999; Stacey 1999, 2004; Day and Stacey 2007; Stacey and Day 2007).

6.2 Progress in *in vitro* plant conservation strategies

The *in vitro* conservation status report commissioned by IPGRI and compiled by Ashmore (1997) involved a broad consultation process, including two international workshops.

1. International Workshop on "*In Vitro* Conservation of Plant Genetic Resources" held in Kuala Lumpur, Malaysia, 1995 (Normah et al. 1996) resulting in a consensus of Workshop recommendations (International Workshop on "*In Vitro* Conservation of Plant Genetic Resources 1996).
2. International Workshop on the Management of Field and IVGBs held at CIAT, Colombia in 1996 that informed the IPGRI Handbook for Genebanks No. 7, technical guidelines for the management of field and *in vitro* germplasm collections (Reed et al. 2004a).

Normah et al. (1996) made recommendations pertaining to training and information exchange, technique development and basic research. Reed et al. (2004a) formulated technical guidelines for initiating a collection, and operating germplasm health procedures. Because tissue culture and storage methods had been developed, but were not fully available or operational the need to standardize operational procedures for clonal plants was highlighted. Research requirements identified by Reed et al. (2004a) were:

- **Germplasm health:** virus surveys, indexing techniques, development of effective virus testing *in vitro* and whether viruses can be transmitted *in vitro*, development of indexing techniques for latent endogenous bacteria.
- **Slow growth:** research into the effects of plant growth regulators and growth retardants, light and light-temperature interactions, propagule type, size, growth stage (microtubers, bulbs, rooted plantlets, unrooted shoots), statistical rigour in experimental design, minimising the use of growth retardants.
- **Cryopreservation:** widening its applicability to more crops and genotypes, methods developed for several localities, use of cryotherapy.
- **Genetic stability:** selection pressure of *in vitro* maintenance, genetic variation in field compared to *in vitro*, field evaluations on material with known instabilities, development of markers to monitor genetic stability.

Ashmore (1997) similarly listed key issues and actions for future consideration:

Genetic stability: greater understanding of the causes and nature of SCV particularly after prolonged storage in culture and cryopreservation and establishing safe storage procedures.

Actioned by: developing improved markers and methods of characterizing SCV and monitoring genetic instability. Encouragement of research aimed at understanding causal factors of SCV and comparative assessments of genetic stability in germplasm conserved in field and IVGBs recommended.

Reproducible and wider use of slow growth and cryopreservation methods: improved, reproducible and robust protocols required for slow growth and cryopreservation optimized across genotypes held in genebanks, greater provision for species that have received limited attention, application of *in vitro* methods for safe movement of germplasm and prioritising problem species.

Actioned by: developing methods using simple facilities, with general application, optimized, and tested in IVGBs; more information on *in vitro* distribution and transportation of *in vitro* material.

Management issues: database of *in vitro* collections and their activities and operations required including routine methods and guidelines, particularly addressing what are acceptable ranges for survival and amount of material to be stored per accession for *in vitro* storage techniques. Strategies required to rationalize collections to: (1) account for issues of safety duplication and narrowing the genetic base but avoiding excessive use of resources in holding collections and (2) undertake cost analyses between IVGBs and field genebanks.

Actioned by: creating an IVBG database, encouraging use of *in vitro* techniques and development of proper guidelines for the IVGB operation including rationalization of genetic strategies and carrying out of comprehensive cost analyses to compare IVGB with field genebank operations.

Infrastructure: establishment of basic infrastructures in IVGBs for LN, supply and upgrading facilities, in many locations including back up, repairs, maintenance and servicing of equipment with a view to using simple facilities.

Actioned by: provision of adequate infrastructures, locally serviced equipment and simple methods.

Training: more information exchanges needed for knowledge sharing, collaboration and coordination with emphasis on local training for developing countries. More research carried out in developing countries and transfer of technologies between international centres and regional and national programmes.

Actioned by: better coordination of *in vitro* conservation programmes to allow greater exchange of ideas and initiating of training in developing countries.

Policy Issues: government commitment to mandate plant genetic resources conservation; sustainable funding needed as many *in vitro* projects are project-based and there is a need to emphasize the importance of *in vitro* techniques to encourage their adoption.

Actioned by: exploring sustainable funding measures, a commitment to *in vitro* conservation and promotion of positive outcomes of using *in vitro* technologies to secure and control phytosanitary status and protect plant germplasm from environmental hazards.

With an emphasis on tropical crops, Engelmann and Takagi (2000) recommended:

- More basic research needed to improve understanding of biological and physical mechanisms involved in cryopreservation, broadening its application to problem species and achieving higher survival using simplified freezing protocols.
- Systematic assessment of stability in plants regenerated from cryopreservation needed using all available detection techniques.
- More research on tropical plants especially those with recalcitrant seeds.
- Development of research emphasizing scale-up of cryopreservation.

6.2.1 Contemporary developments in *in vitro* plant conservation

Advances in cryopreservation and molecular biology have been considerable since the report by Ashmore (1997). One of the most significant advances has been in omics research, this comprises: (1) genomics, the quantitative study of genes, regulatory and non-coding sequences to yield information on DNA sequences and structure; (2) functional genomics, the dynamic, interpretive study of genes focusing on gene transcription, translation and protein interactions; (3) transcriptomics, the study of RNA, gene expression and regulation; (4) proteomics, the study of protein function and (5) metabolomics, the study of metabolites

and metabolic pathways and how they interconnect. Omics and molecular genetics technologies both support and utilize *in vitro* conservation (Carpentier et al. 2005, 2006, 2007; Ryynänen et al. 2002). Functional genomics and the use of candidate genes as tools to screen for useful traits in germplasm collections is expanding and collectively these approaches will enhance the greater utilization of banked material (de Vicente 2004).

Technical progress in cryopreservation continues mainly by empirical study involving testing cryoprotectant strategies (Sakai 2004; Sakai and Engelmann 2007; Sakai et al. 2008). This approach (Figure 5) has the advantage of simplicity as direct observations are used to develop protocols.

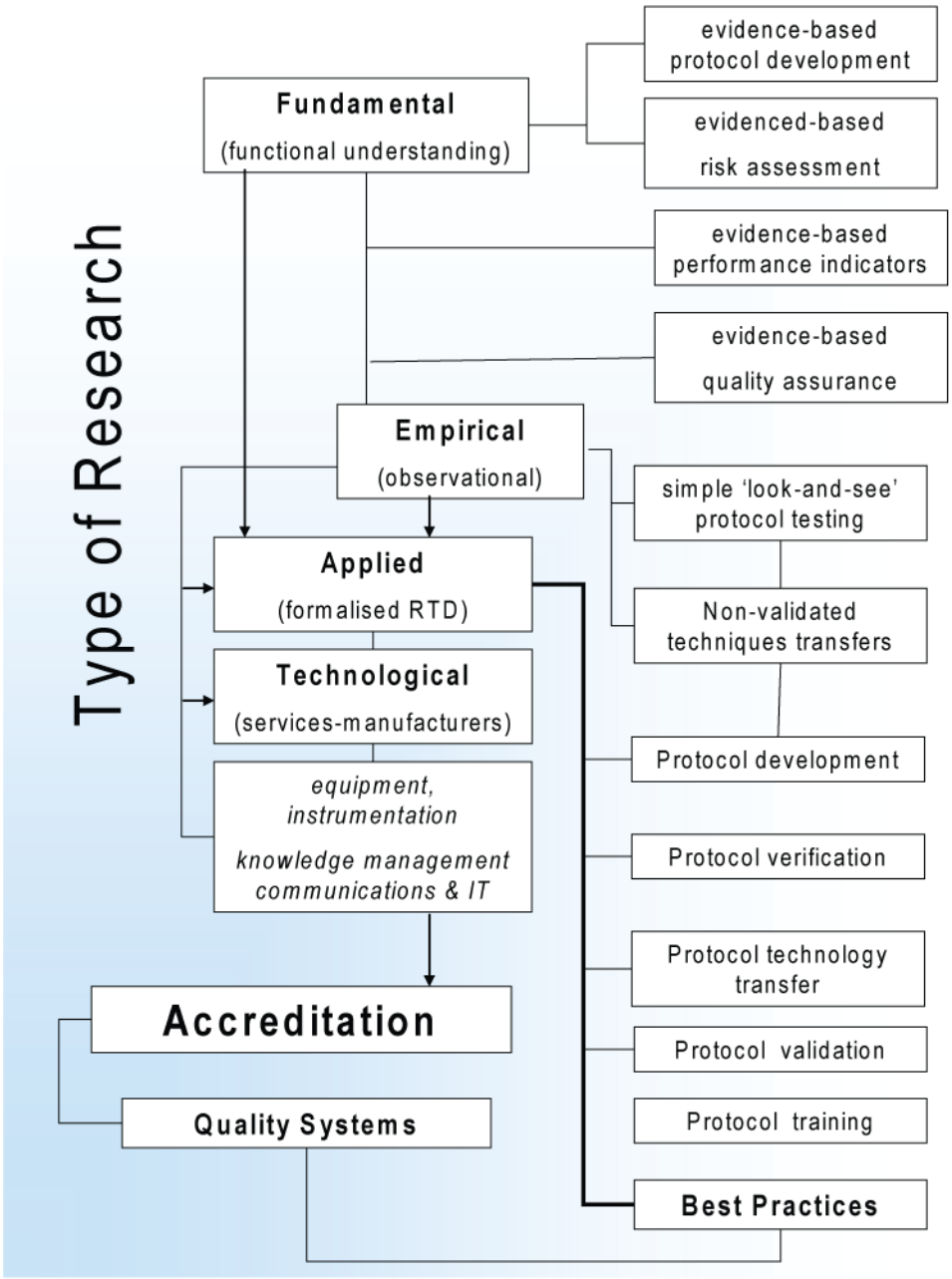

Figure 5. Road map examining the contributions of various types of research in the development of quality systems for *in vitro* conservation technologies. RTD = research, training and development.

Reed (2008) describes cryopreservation protocols for a large range of species and explants, progress has been largely vitrification-based, enabling the large-scale cryobanking of germplasm (Engelmann 2004; Gonzalez-Arnao et al. 2008, Gonzalez-Arnao and Engelmann 2006; Keller et al. 2005, 2006, 2008; Keller and Senula 2003; Kim et al. 2006, 2007; Panis et al. 2005; Sakai and Engelmann 2007). Similar approaches have facilitated the MTS of plant genetic resources (Volk and Walters 2003; Cha-um and Kirdmanee 2007). These reports have satisfied many of the recommendations made by Ashmore (1997) and Engelmann and Takagi (2000), especially for tropical species, although storage remains a problem for species recalcitrant to tissue culture and/or those that produce desiccation sensitive seeds (Ashmore et al. 2007a; Benson 2008a; Berjak 2006, Berjak et al. 1990, Berjak and Pammenter 1994, 2008; Engelmann 1999; Pritchard 2004; Withers and Williams 1980).

Advances in fundamental research (Figure 5) have enhanced the knowledge base of plant *in vitro* storage technologies. The EU CRYMCEPT project (http://www.biw.kuleuven.be/dtp/tro/crymcept/CRYMCEPT.htm) is the acronym for a European Commission Research Project (Reference: QLK5-CT-2002-01279) entitled: "Establishing CRYopreservation Methods for Conserving European PlanT Germplasm Collections", which has added substantially to fundamental plant cryopreservation research. The EU COST ACTION project "CRYOPLANET" (http://www.biw.kuleuven.be/dtp/tro/cost871/Home.htm) similarly links fundamental research to cryopreservation implementation. *In vitro* conservation technology research has been appraised by the Australian International Association of Plant Tissue Culture and Biotechnology (Bennett et al. 2005). More specific research examples include the use of omics technologies to help understand storage recalcitrance (Carpentier et al. 2005, 2006, 2007; Criel et al. 2005); thermal analyses to elucidate the mechanisms involved in creating and stabilizing the glassy state (Volk and Walters 2006; Volk et al. 2006) and oxidative stress and epigenetic markers applied to monitor cryoinjury in storage sensitive genotypes (Benson et al. 2007; Fang et al. 2008; Johnston et al. 2005, 2007). Technological developments in molecular and analytical diagnostics have facilitated more stringent phytosanitary monitoring (Mumford et al. 2006) and storage stability assessments (Ahuja et al. 2002; Dixit et al. 2003; Harding 2004; Hirai and Sakai 2000; Kaity et al. 2008; Perazzo et al. 2000; Schäfer-Menuhr et al. 1997). The needs of *in vitro* conservationists are also being met by specialist cryo-engineering and products manufacturers (Benson et al. 2005). Knowledge management (KM) and information technology (IT) sectors are designing electronic barcode inventory systems products to support *in vitro* storage technologies. Collectively, these contemporary advances support the movement towards quality systems and accreditation in crop genebanks (Figure 5).

6.3 Medium-term storage: slow growth

Tissue culture provides two possibilities for storage, growth in the active state and slow growth which is designated as medium-term storage (Benson 1999). Reducing the growth rate of cultured plants enhances efficiency and reduces the costs of resources and labour intensive subculturing cycles. Disease-free, growth-arrested cultures are the primary source material for the IVAG (Figure 3) and the *in vitro* plants and propagules held in MTS constitute a complementary conservation strategy for cryobanked germplasm maintained in the IVBG (Keller and Senula 2003; Reed et al. 1998a, b). The development and implementation of slow growth is allied to other *in vitro* plant manipulations, especially micropropagation. Types of explants used in slow growth (usually) range from rooted

plants, microtubers, and storage organs to shoot cultures. Some treatments that incur slow growth for conservation purposes are similarly applied for the selection and production of stress tolerant genotypes (Ochatt et al. 1999), mutants (Luan et al. 2007) and somaclonal variants (Jain 2001). Thus, it is useful to reciprocate knowledge from these other fields of study as it has risk management relevance for clonal genebanks that are optimising MTS treatments, such that stresses imposed, do not incur permanent debilitating symptoms, or lead to phenotypic, genetic or epigenetic instability in recovered plants (Benson et al. 1989; Harding 1991, 1994, 1999; Harding et al. 1991, 2005; Joyce and Cassels 2002; Joyce et al. 2003).

6.3.1 Principles of medium-term storage

The objective of slow growth (or minimal growth) is to reduce subculture intervals to a critical level that does not impose a long-term deleterious effect on germplasm, or put at risk the stability of regenerated/regrown plants. However, slow growth treatments incur some level of stress and it is essential to optimize MTS with respect to the timing of subculture regimes and regeneration. When this is achieved, slow growth is a successful method of securing plant germplasm in MTS (Cha-um and Kirdmanne 2007). Minimal growth storage is useful for genotypes that cannot be cryopreserved and it is a key part of *in vitro* genebanks that clonally propagate crops for distribution services. Several MTS treatments are applied, either singularly or in combination to retard growth:

- **Physical growth limitation**
  - low temperature
  - low light/restricted photoperiod
  - minimal containment
  - minimal O_2
  - osmotic (water) stress.
- **Chemical growth limitation**
  - growth regulator retardation
  - growth inhibitors.
- **Nutrient limitation**
  - low macro nutrient levels
  - low micro nutrients levels.

Choice of treatment is largely species-dependent and it is dictated by the ability to withstand the stresses incurred; the simplest and most successful slow growth strategies involve temperature and light limitation. Restrictions to MTS are deleterious effects, variable genotype responses, callus formation, hyperhydricity, and the proliferation of covert and systemic infections, including adventitious contamination entering *in vitro* vessels during extended culture (Golmirzaie and Toledo 1998). Containment is critical and Reed (1991) recommends using gas-permeable bags which have the advantage of small size, and resistance to breakages; a separate chamber for each plant reduces the risk of cross-infection and for an active collection individual bags can be easily removed for distribution. A bottle-neck in the application of slow growth is adapting a generic, growth limiting protocol to every accession of a large multi-crop genebank. This has been explored by Reed et al. (2003) who adapted a basic cold storage protocol for unrelated genera by making minor technical

modifications. Using shoot cultures of *Humulus* spp. (hops) as the test case, diverse genotypes were evaluated for MTS treatments comprising low light with cold storage (4°C) as first developed for strawberry and mint. Average storage time, without transfer for 70 *Humulus* genotypes was 14 ± 3.5 months, ranging from 6-26 months. This study confirms that a standard MTS protocol can be applied across diverse genotypes, although improvements may be required for outlying, low response performers.

6.3.1.1 Physical growth limitation

Temperature is the main limiting abiotic factor, as it is applicable across both tropical and temperate plants. Low temperature (20°C) storage of cassava reduced shoot growth by a fifth compared to those propagated under standard growth at 25-30°C (Angel et al. 1996). Minimal growth conditions comprising low light (1000 lux) and temperature (15°C) have been used to maintain several *Musa* genotypes for 13-17 months (Banarjee and de Langhe 1985). A low temperature treatment of 2°C and 10°C is used for the MTS of *in vitro Allium* cultures at IPK, extending the culture cycle to 12 months (Keller et al. 2006). Mint is generally amenable to *in vitro* culture and MTS, for which most *in vitro*-maintained clones at IPK can be stored at 2°C for up to 15-18 months (Keller et al. 2005); combinations of different growth limiting treatments are also effective for mint (Reed 1999). Reed (1992, 1993, 1999, 2002; Reed et al. 1998b; Reed and Aynalm 2005) extended and improved MTS for genotypes of several clonal crops by moderating photoperiod, light, nitrogen and temperature, thus complementing conservation in LTS. Complete light limitation in combination with 5°C storage has been applied to shoot cultures of *Trifolium repens*, extending the culture interval to 10 months (Bhojwani 1981). Son et al. (1991) report one of the most extended cold storage cycles as 5 years at 4°C for *in vitro* hybrid cultures of poplar shoots. Cha-um and Kirdmanee (2007) showed that small culture vessels minimized growth and development of plants by limiting gaseous exchange, space and nutrient supply; they comment this has cost-benefits in terms of space, media and personnel time. Adding osmotica to culture media to simulate water stress has been used for various crops (Cha-um and Kirdmanne 2007) including, mannitol for potato (Harding 1991, 1994), sorbitol for sweet potato and potato (Golmirzaie and Toledo 1998; Golmirzaie et al. 1999) and sucrose in combination with mannitol for yam (Ng et al. 1999). However, mannitol may cause epigenetic changes exacerbated by stress (Harding 1991, 1994).

6.3.1.2 Chemical growth limitation

Reducing growth regulators as a minimal growth strategy has been applied to *in vitro* coffee (Bertrand-Desbrunais et al. 1991, 1992), low concentrations of 6-benzyladenine (BA) were effective, dependent upon genotype and morphogenetic proliferation. The substituted pyrimidine, Ancymidol is a potent plant growth regulator and growth retardant and it inhibits gibberellic acid biosynthesis. Sarkar et al. (2001) have used Ancymidol in the slow growth of potato; when used in combination with sucrose and temperature limitation it extended the culture cycle to 16 months. Storage of potato microplants using acetylsalicylic acid has been used as an alternative to applying mannitol at 18°C; demonstrating that subcultures could be maintained for up to 6 months (Lopez-Delgado et al. 1998).

6.3.1.3 Nutrient limitation

Cold-sensitive genotypes can be stored under slow growth conditions by limiting sucrose and/or nitrogen, this strategy was applied to chill-sensitive *Rubus* genotypes by culturing *in vitro* plants at 25°C under conditions of reduced nitrogen (Reed 1993); for papaya, sucrose

was replaced with fructose to limit growth (Drew 1992). Nutritional factors are important for maintaining health status, as demonstrated for the *in vitro* culture of hops. Iron formulations applied during cold storage affect growth, leading to the recommendation of Reed and Aynalem (2005) to use standard, EDTA-chelated iron rather than sequestrene iron. Calcium nutrition is a factor in the minimal growth storage of *Solanum tuberosum* microplants (Sarkar et al. 2005).

6.3.1.4 Microtubers and propagules

Production of microtubers *in vitro* is an alternative storage method for potato (Golmirzaie and Toledo 1998) for which tuber dormancy can be controlled by environmental parameters (Golmirzaie et al. 1999). Slow growth maintenance of tubers generally requires a cyclic or sequential *in vitro* culture regime. In the case of the *S. tuberosum* collection held by IPK, Germany this involves: (1) the establishment of virus-free material, (2) long-day slow growth storage at 20°C for 2-3 months, (3) microtuber-induction using a short-day at 9°C for 2-4 months and (4) cold storage of microtubers at 4°C for 16-18 months (Keller et al. 2006). Cha-um and Kirdmanne (2007) highlight the preservation of storage organs and propagules of vegetatively propagated plants, including bulblets of *Allium* spp., microtubers of yam and potato and rhizomes of ginger, bamboo, orchids and turmeric.

6.3.2 Performance indicators for slow growth

Performance indicators include: (1) plant health, (2) extension of subculture interval, (3) contamination frequency and (4) capacity [viability, vigour, health status] to recover from stress treatments. Reed et al. (1998b, 2003) use descriptive scales of 0 and 1-5 to rate the performance of *in vitro Pyrus* and *Humulus* cultures maintained under minimal, low temperature growth:

0 =	all of the plantlet is brown and no visible indication of growth
1 =	very poor, questionable viability, brown, necrotic shoots, only extreme shoot visibly green, plantlet mostly brown
2 =	poor, much browning, most shoot tips necrotic, shoot tip green, leaves and stems mostly brown, base may be brown
3 =	fair, some browning, 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 and limited etiolation
5 =	excellent condition, dark green leaves and shoots, no etiolation.

Surveillance of cultures held in MTS is required and regular reviews are recommended on a 1-4 monthly basis dependent upon the crop system. It is precautionary to consider that variable genotype responses to MTS treatments can occur when using performance indicators (Van den houwe et al. 1995). This is particularly relevant when managing IVGB cultures and regeneration cycles, for which barcoded tracking and electronic documentation systems assist operations and inventory administration (Van den houwe et al. 2006).

6.3.3 Progress in medium-term storage

In a comprehensive review Cha-um and Kirdmanne (2007) report (in combination with other growth limiting factors) 12 crop and forestry species being stored at <10°C; 7 stored at 10-20°C and 10 stored at temperatures >20°C. In addition, alginate encapsulation of various explants and propagules has been combined with osmotica, low temperatures, light

limitation and chemical growth retardants for various species. Examples have been collated by Cha-um and Kirdmanne (2007) they include: *Cedrella fissilis, Chamaecyparis pisifera, Dalbergia sissoo, Fragaria x ananassa, Pinus patula, S. tuberosum, Rubus idaeus*, and *Vanilla* spp. The IPK, Germany holds >630 accessions of crop plant germplasm in slow growth storage and, as from 2006 (Keller et al. 2006) this includes 99 clones of garlic and 35 of shallot. A review of the preservation of *in vitro* active collections in the USDA's National Plant Germplasm Clonal Collections is provided by Volk and Walters (2003). Part II of the GPG2 series on clonal crop conservation summarizes surveys of the CGIAR's mandate species currently held in slow growth storage (Benson et al. 2011a); Part III (Benson et al. 2011b) provides technical guidelines pertaining to generic MTS methods and protocols.

6.3.4 Stability and slow growth storage

Conservation in tissue culture risks SCV (Rani and Raina 2000; Scowcroft 1984) during slow growth and the active growth phases preceding and following storage (Cassels and Curry, 2001). *In vitro* maintenance of cultures for long periods can potentially lead to *in vitro* ageing and neoplastic progression exacerbated by nutrient limitation and stress (Benson 2000a, b, 2008a; Gaspar et al. 2002; Häsler et al. 2003; Luan et al. 2007). Superimposed on these factors, is the possibility that minimal conditions confer an advantage on physiologically more tolerant genotypes or individuals in a clonal population, leading to the selection of resistant individuals (Ochatt et al. 1999) and/or mutations (Luan et al. 2007). Whilst clonal populations may be expected to be identical, this does not necessarily confer invulnerability to epigenetic and genetic changes occurring via the process of SCV (Harding 2004; Rani and Raini 2000; Scowcroft 1985). Moreover, individuals, in a clonal population may be expected to have different physiological attributes that confer variable propensities for tolerance which might potentially lead to selection. Thus, agents and procedures that impair growth, may cause abnormal morphogenetic responses and exacerbate epigenetic processes (e.g. DNA methylation) that have ramifications for stability and selection processes. Careful selection of donor germplasm and storage optimization was advised by Scowcroft (1984) as follows:

- Prolonged periods of tissue culture are known to increase the frequency of gross chromosomal aberrations,
    - the frequency of SCV occurring is enhanced in prolonged tissue culture.
- *In vitro* selection pressure is an effective means of generating mutants.
- Exposure to minimal (suboptimal) growth conditions over long periods can be expected to lead to genetic change.

Importantly, the problem of variation arising from clonally propagated plants is evident in both field and *in vitro* collections. Treatments can be incorporated to improve plant health and vigour during storage, whilst retaining the benefits of the minimal growth treatment. Plants maintained *in vitro* for long periods accumulate ethylene, which is detrimental to growth and exacerbates the stresses incurred during slow growth. Ethylene inhibitors such as alginate silver thiosulfate help circumvent this problem as demonstrated for potato (Sarkar et al. 1999, 2002). Reed (1992) tested the efficacy of containment on the contamination and health of *in vitro* strawberry plantlets cold-stored at 4°C, finding that a bag system was superior to boxes and tubes; as the bags are porous to gaseous exchange ethylene build-up is most likely reduced. Optimizing combinations of low temperatures, light and photoperiod improves the health of *in vitro* plants in cold stores. Studies by Reed (1993) on *Rubus* cultures

demonstrated poorer condition at warmer temperatures and that recovery from MTS can be variable and genotype dependent (Reed 1991).

Risks of SCV occurring in slow growth cultures may be minimized by reducing or omitting plant growth regulators, particularly those that have a tendency to induce callus and produce adventitious shoots (Scowcroft 1984). For some species, the addition of phytohormones is required to support minimal storage, such is the case for banana shoot cultures (Van den houwe et al. 1995) stored at 16±1°C in medium supplemented with BA and indole acetic acid (IAA). Strosse et al. (2004) found no evidence that growth regulators used in routine tissue culture directly affected the rate of SCV, but instability was increased with time in culture and number of regeneration cycles. This supports the need to develop optimal MTS methods for genotypes susceptible to genetic instability as they produce increased numbers of off-types with time in culture.

Cha-um and Kirdmanne (2007) summarize the genetic stability studies of several plant species exposed to MTS treatments, including: apple (Hao and Deng 2003); cassava (Angel et al. 1996); cedar (Renau-Morata et al. 2006); citrus and strawberry (Hao et al. 2002a, b, 2004); kangaroo paw (Turner et al. 2001); potato (Harding 1991) and silver birch (Ryynänen and Aronen 2005). Methods used to assess stability included DNA methylation, RAPD, RFLP and AFLP analyses and different outcomes concerning genetic and epigenetic stability were revealed, which in general were crop and species specific. Stability assessments of various *in vitro* shoot cultures e.g. apple (stored at 4°C) and citrus (stored at 10°C) revealed methylation sensitive amplified polymorphisms (MSAP) in some cases (Hao et al. 2002a, b, 2004; Hao and Deng 2002, 2003). This concurs with Harding (1994) who found changes in the DNA methylation status of potato plants recovered after six months of slow growth in medium supplemented by 6% (w/v) mannitol. This study showed that the majority of changes were due to preferential methylation in nuclear domains that contained EcoRII/Bst NI recognition sites, in contrast to those that contained HpaII/MSp I sites. Harding (1994) concluded that DNA methylation in slow-grown potato might be an adaptive response to osmotic stress. In contrast, Sarkar et al. (2001) did not detect any genetic variation in microplants conserved on limiting medium containing Ancymidol using RAPD analysis of genomic DNA. Angel et al. (1996) stored cassava under slow growth conditions for 10 years without evidence of genetic instability at the DNA level.

Maintaining genetic stability is a pivotal requirement for MTS and it is an aspect of quality assurance, whilst studies remain limited, findings to date suggest the changes that do occur are mainly epigenetic. Nevertheless it may be insightful to explore if they are an adaptive genomic responses to the stresses incurred *in vitro*. This approach concurs with the studies of Joyce and Cassels (2002) in which DNA methylation was used to assess variation in potato microplant morphology produced by different *in vitro* protocols. As the molecular diagnostics currently used examine a very small part of the genome (Harding 1996, 2004) an alternate or complementary approach to assess stability may be to use trueness-to-type, particularly if the use of morphological and agronomic traits as stability criteria are applied in concert with germplasm and plant authentication procedures (Perazzo et al. 2000).

6.4 Long-term storage: cryopreservation

Cryopreservation is the storage of viable cells, tissues, organs and organisms at ultra low temperatures (ca. -130 to -196°C) in the vapour or liquid phase of LN. The primary role of cryogenic storage is to secure germplasm in perpetuity in the base genebank (Figure 3). Contemporary developments benefit from data emerging on cryobank longevity, including two investigations performed on: (1) cryopreserved seed germplasm stored for >10 years, by Walters et al. (2004) and (2) microalgae stored for 20-30 years (Day et al. 1997; Müller et al. 2005). Whilst it is generally assumed that metabolic activity ceases at the temperatures of LN vapour and liquid, Walters (2004) reported molecular mobility can potentially occur at cryogenic temperatures, the extent of which being affected by water status. This is confirmed by Buitink et al. (2000) who used electron paramagnetic resonance spectroscopy to study rotational motion of a spin probe in various plant tissues as a function of moisture content and temperature, finding detrimental ageing rates to be associated with the extent of molecular mobility in the cytoplasm. The work of Walters et al. (2004) constitutes one of the most interesting longevity studies performed to date on cryopreserved plant germplasm. Measurable changes in the germination rates of dry seeds cryopreserved were observed for samples held under LN for >10 years, despite this, cryogenic seed storage was predicted to prolong shelf life of lettuce seeds for significant periods (>500 years). The benefit of low temperature storage was also found to be influenced by donor plant and germplasm physiology and pre-storage treatment before cryopreservation. Day et al. (1997) report long-term viability of eukaryotic algae cryopreserved for ca. 20 years. Müller et al. (2005) compared AFLP profiles of strains of *Chlorella vulgaris* maintained in a cryobank for almost 30 years with a population of the same strains grown in serial subculture. No significant genomic differences were found between strains derived from cryostorage compared to their actively cultured counterparts. Published reports regarding the long-term viability of preserved organisms are few; this is largely because information on extended storage timelines is only now emerging. Stacey and Day (2007) collated cryostorage longevity from diverse biological resources across timescales of 5-35 years, reporting no obvious or significant loss of viability, stability and function was found.

6.4.1 Principles of long-term storage, cryopreservation

Since Ashmore (1997), advances in cryopreservation research and storage technologies have been considerable across all disciplines; this is due to three main lines of progression:

1. The formulation of new and adapted cryopreservation protocols, particularly using vitrification (Day and Stacey 2007; Day et al. 2008; Fuller 2004; Reed 2008).
2. An explosion in fundamental cryobiological knowledge related to cryoprotection and understanding the effects of freezing on biological systems *in vitro* and *in vivo* (Fuller 2004; Fuller et al. 2004; Day et al. 2008).
3. A greater emphasis on regulatory issues and cryobank risk management (Stacey 2004; Tomlinson 2008).

Developments in cryoengineering and thermal analysis have also aided the technical and theoretical study of cryopreserved systems (Benson et al. 2005). Examples of some basic equipment required for plant cryopreservation are shown in Figure 6.

Advances have also helped to elucidate the principles of ultra low temperature storage, these initially involved the biophysical interpretation of cryoprotectant behaviour

(Mazur 2004). However, a paradigm shift is occurring (Baust and Baust 2007). This is due to the development of molecular, and omics research, which offers new and different perspectives as to what, contributes to cryopreservation success and failure. For plants, increasing advances in omics technologies are unravelling the complexities of cold and freezing stress (Xin and Browse 2000; Fowler and Thomashow 2002; Xiong et al. 2002; Kaplan et al. 2004; Gray and Heath 2005; Hannah et al. 2005; Yang et al. 2005; Chinnusamy et al. 2006; Fujita et al. 2006; Nakashima and Yamaguchi-Shinozaki 2006; Beck et al. 2007; Zhu et al. 2007; Basu 2008). Proteomics technologies in particular are now being used to study cryostorage recalcitrance (Carpentier et al. 2005, 2006, 2007).

Figure 6. Basic equipment used for the cryopreservation of plant genetic resources: Programmable freezer comprising freezing chamber, computer and pressurized liquid nitrogen coolant; solvent-cooled, passive small-scale freezing unit 'Mr Frosty™', cryovial loading into cooling chamber and small (ca 50 L) storage Dewar, LN level alarm attached to large-capacity (ca 200 L) cryotank.

Omics research promises a greater understanding of the fundamental processes that limit freeze, osmotic and dehydration tolerance. Technological innovations are also supporting the development of quality systems in modern biorepositories (Smith 1998, 2001, 2003; Benson et al. 2005; Day and Stacey 2007; Benson 2008a; Day et al. 2008) this is leading to an expansion of published literature pertaining to regulatory issues and cryobank security (Stacey 1999, 2004; Stacey and Day 2007). Developments have stimulated considerable activity in biobank risk management, especially related to microbiological containment and minimising adventitious and cross contamination in long-term cryobanks (Tedder et al. 1995; Fountain et al. 1997; Pegg 1999; Stacey 1999; SLTB 1999, 2008; Bielanski et al. 2000, 2003; Tomlinson and Sakkas 2000; Khuu et al. 2002; Kipp et al. 2004; Bielanski 2005a, b; Morris 2005; Tomlinson 2005, 2008; Mazilli

et al. 2006). Benson (2008a) reviews the possible implications of studies from other bioresources sectors for plant germplasm containment and highlights the importance of regulatory phytosanitary control before materials are cryobanked.

The following sections describe the development of the main cryoprotection and cooling techniques that constitute: (1) colligative cryoprotection and slow (controlled rate) cooling, (2) vitrification-based cryoprotection and rapid cooling and (3) droplet freezing and vitrification.

6.4.1.1 Colligative cryoprotection and controlled rate cooling

Mazur's 2-factor hypothesis of cryoinjury comprises dehydration damage caused by excessive dehydration and ice (see Mazur 2004 for a review). Colligative properties are those attributed to the behaviour of solutions that are dependent upon the number of molecules or particles in a given volume of solvent. Colligative cryoprotectants must be non-toxic at the concentrations required for their efficacy, and they must penetrate the cell (Fuller 2004), this is because their mode of action depends on reducing injuries caused by excessive cell volume changes and toxic solution effects. Colligative cryoprotectants also depress the freezing point, such that when ice nucleation does occur it is not so injurious; the most common are glycerol, and dimethyl sulphoxide (DMSO) although cryoprotectant permeability and toxicity can be germplasm and plant dependent (Fuller 2004). The level at which colligative cryoprotectants are applied is variable, e.g. they can range from 5-10% (v/v) DMSO or 0.5 to 1 M glycerol. Colligative cryoprotectants are normally used in combination with controlled rate cooling using a programmable, computerized cooling unit, that regulates stepwise rates of cooling and hold times at subzero temperatures. The process takes advantage of the supercooling properties of water; the lowest point in nature is the temperature of homogeneous ice nucleation at, or around, -40°C. During cryopreservation the point at which ice is formed is induced by 'seeding' which is the initiation of ice crystals in the extracellular matrix. When liquid water is removed from an aqueous system by ice formation, solutes become concentrated, a process that has two important effects on the cryopreserved cell. First, the temperature at which further ice is formed is lowered (super cooling); secondly, a water vapour deficit is created across the cell membrane causing movement of intracellular water to the outside. In controlled rate cooling the operator optimizes cryoprotection, seeding, cooling rates and hold times to allow just enough intra-cellular water to exit without causing colligative injury. When the germplasm is finally plunged into LN ice damage should be sufficiently limited to be non-lethal, any ice crystals formed are so small they are non-injurious. In practice, it is likely that germplasm cryopreserved in this way becomes partially vitrified (Fuller 2004; Benson 2008a, b). Non-penetrating cryoprotectants are often used in conjunction with colligative additives to remove potentially freezable water from the cell by osmosis; this approach is useful for preserving differentiated cells containing large vacuoles.

Once optimized, an advantage of controlled rate cooling is that large batches of germplasm can be cryopreserved simultaneously in a programmable freezer chamber, providing efficient, high throughput methods for large-scale cryopreservation; this is of particular benefit for genebanks holding many accessions. If the cryoprotectant strategy is robust and reliable then controlled cooling is cost effective in the longer-term as cryoprotectant addition is undertaken in the cryovial. In contrast, some vitrification protocols can be laborious (e.g. aluminium foil preparation, dispensing micro-drops, hand manipulation of each sample for cryoprotectant additions) and take up more technical preparation and handling time. Computerized processing facilitates multiple manipulations

and offers cooling programme permutations, providing the operator with more variables to aid optimization. Electronic data handling and inventories store process information and support digital sample tracking which is an added advantage for quality auditing. The main economic disadvantages of programmable freezers are the initial cost outlays for equipment which requires regular servicing and the possibility that pressurized LN appliances need to conform to regular safety audits. Programmable freezers can use large amounts of LN to cool the chamber and together all these factors add regulatory, safety and cost burdens. An alternative low budget system is the passive freezer, Mr Frosty™ (Nalgene®) however, the major limitation of controlled rate cooling remains that certain types of plant germplasm are not amenable to the approach. Cases include complex tissues comprising different types of cells and many tropical plants that produce large, recalcitrant seeds and multipart vegetative tissues for which it is difficult to optimize cooling rates to protect cells with variable water contents. For these reasons, vitrification has been used to great advantage for plant germplasm that is not generally amenable to controlled rate cooling.

6.4.1.2 Vitrification-based cryoprotection and rapid cooling

Vitrification is the solidification of a liquid without crystallization, it is termed an amorphous "glassy state" as it lacks organized structure, whilst possessing the mechanical and physical properties of a solid (Fuller 2004; Taylor et al. 2004). Cryopreservation in the absence of ice has the major advantage of reducing cryoinjury through ice formation and its associated colligative effects. Vitrification is consequently the method of choice for plant germplasm that is unresponsive to colligative and controlled cooling protocols or, where suitable equipment is not available. The glassy state is created via several mechanisms, for plants these include: osmotic, evaporative, and chemical dehydration, as well as the loading and unloading of penetrating cryoprotectants. Increasing cell solute concentration to a critical viscosity and temperature, termed the glass transition temperature (Tg) is the point at which the vitrified state is formed. The glassy state is metastable and it can revert to a liquid by devitrification, this process can involve ice formation on rewarming and it is one of the main disadvantages of vitrification; this process is sometimes called cold crystallization (Bart Panis pers comm). In addition, the material properties of unstable glasses make them susceptible to fractures or cracking on rewarming, particularly if it is undertaken rapidly. Rewarming of vitrified germplasm is often undertaken in two steps, the first is slow to allow for glass relaxation, usually at ambient room temperatures followed by more rapid rewarming at ca. 45°C to avoid ice nucleation. When utilized with care the metastable vitrified state has proved to be an effective means of cryopreserving germplasm that is not amenable to controlled rate cooling and colligative cryoprotection (Fuller 2004; Reed 2008a). Assessment of the advantages and disadvantages of the various rapid cooling and vitrification-based protocols is presented in Section 6.4.2.

6.4.1.3 Droplet freezing and vitrification

Two other cryoprotection strategies that are applied to plant germplasm on a large scale are droplet freezing and droplet-vitrification, (Schäfer-Menhur et al. 1996, 1997; Panis et al. 2005; Ashmore et al. 2007a; Sakai and Engelmann 2007; Gallard et al. 2008; Sakai et al. 2008; Sant et al. 2008). Both methods use a rapid cooling protocol, but their *modus operandi* is different due to the behaviour of water molecules contained in micro-droplets of single cryoprotectants (DMSO) or vitrification solutions (PVS2). Micro volumes (μL) of cryoprotective additives are deposited on highly efficient, heat conducting surfaces, usually aluminium foils or hypodermic

needles (Grout and Henshaw 1978; Kartha et al. 1982). If the biophysical conditions are optimal, the droplets vitrify on direct exposure to LN or, in the case where ice crystals actually do form; they are so small that they do not cause lethal damage. This process is ultra rapid or flash-freezing although for certain cryoprotectants it is perhaps more accurate to use the term flash-vitrification; the protective principle of droplet-based techniques is yet to be elucidated. It is most likely crystallization caused by the alignment of H_2O molecules forming H-bonds is impeded in micro-droplets due to insufficient numbers of H_2O molecules, surface tension-cohesion effects, cryoprotectants and cooling rates of thousands of degrees per minute (Benson 2008b, Benson et al. 2005; Orief and Schultze-Mosgau 2005). A comparative assessment of the advantages and disadvantages of droplet protocols is presented in Section 6.4.2.

6.4.2 Overview of progress in long-term storage

Controlled rate cooling was the first method developed for routine plant germplasm cryopreservation, based on the original method of Withers and King (1980) and derivative protocols (Benson 2004, 2008a, b; Reed 2008). Controlled rate cooling is effective for cryopreserving a wide range of germplasm including: shoot tip meristems from temperate woody perennials and clonal crops (Reed 2001, Reed et al. 2003); dedifferentiated plant cell cultures (Heine-Dobbernack et al. 2008) and totipotent cell lines and embryogenic cultures used by the clonal forestry industry (Park et al. 1998; Cyr 1999; Cyr and Klimaszewsak 2002; Park 2002; Gale et al. 2007, 2008). Nevertheless, it has a major limitation in the cryopreservation of recalcitrant species and physiologically complex types of germplasm. The purchase of expensive programmable freezers is also a restriction (although the Mr Frosty™ system provides a cheaper option for poorly resourced genebanks). Consequently, progress in plant cryopreservation has preferentially lead to the development of vitrification-based and droplet freezing protocols that utilize permutations of the following treatments:

1. Evaporative desiccation (air, silica gel) and osmotic dehydration (sucrose, sorbitol, mannitol).
2. Osmotic dehydration, in combination with alginate bead encapsulation and evaporative desiccation (encapsulation-dehydration) based on the original method of Fabre and Dereuddre (1990) as reviewed by Engelmann et al. (2008).
3. Vitrification using cryoprotectant cocktails of DMSO, ethylene glycol, polyethylene, glycol, sucrose and glycerol. The most common is the Plant Vitrification Solution (PVS) series, PVS2 is the most widely applied; PVS3 is an alternative method (Sakai et al. 2008).
4. Encapsulation-vitrification, chemical additives combined with alginate bead encapsulation (Sakai et al. 2008).
5. Droplet freezing and droplet-vitrification, in which micro-droplets of respectively DMSO or vitrification solutions are cooled at ultra rapid rates by direct exposure to LN; based on the methods of Kartha et al. (1982) Schäfer-Menuhr et al. (1996, 1997) and Panis et al. (2005).

6.4.2.1 Evaporative and osmotic dehydration

This is a simple, cost-effective method involving dehydration and desiccation treatments using osmotica, heat-activated silica gel or air, followed by direct plunging into LN and, on retrieval from the cryobank, rewarming at ambient temperatures. Cryoprotective dehydration has been applied with success for the cryopreservation of recalcitrant and orthodox seed (Hamilton et

al. 2008; Normah and Makeen 2008; Pritchard and Nadarajan 2008; Walters et al 2008). The main disadvantage of this approach is its restriction to desiccation tolerant germplasm.

6.4.2.2 Vitrification using cryoprotective additives

Chemical cryoprotectants used for vitrification are applied at higher levels than is the case for colligative cryoprotection. Solutions formulated by Sakai and colleagues (reviewed by Sakai 2004; Sakai and Engelmann 2007; Sakai et al. 2008) are the main protocols applied to plant germplasm, of which the PVS series (PVS2 and 3) are the most popular. Because vitrification solutions are highly concentrated, protocol permutations often incorporate pregrowth and dehydration treatments to enhance recovery in osmotically sensitive genotypes. This involves pre-loading germplasm with 2 M glycerol and 0.4 M sucrose, followed by sequentially increasing the PVS to a final cryoprotective concentration. For PVS2 this is 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose. For PVS3 the composition is 50% (w/v) each of sucrose and glycerol, prepared in standard liquid culture medium (Sakai et al. 1990; Nishizawa et al. 1993; Sakai et al. 2008). Vitrification solutions are dispensed at ambient temperature or on ice to reduce the effects of osmotic shock and PVS-cryoprotected germplasm is directly plunged into LN. Vitrified germplasm is rewarmed rapidly in a water bath at 45°C; alternatively the process involves two stages to prevent glass fracturing due to glass relaxation which risks devitrification and ice formation occurring as the system progresses through temperatures at and close to the Tg. This warming procedure can entail holding for 1 min in LN vapour or, at ambient temperatures (to allow for glass relaxation) followed by rapid warming for 2-3 min in a water bath at 45°C. PVS mixtures are usually removed by rinsing in an unloading solution of 1.2 M sucrose.

A technical and practical disadvantage of vitrification is the need to stabilize glasses around the critical Tg, during LTS, vapour phase transit storage and on sample input and retrieval from a cryobank. Volk and Walters, (2006) recommend germplasm cryopreserved using PVS2 should be maintained in the liquid phase of LN and it is prudent to check the stability of glasses and germplasm viability after transit and transfers between different cryotanks. Dry shippers have to operate in the vapour phase of LN for safety reasons, and the materials contained within them may reach temperatures outside the critical Tg zone. It is also essential to adopt stringent removal and addition strategies for vitrified germplasm held in cryobanks, particularly for samples at the top of inventory systems. Devitrification becomes more problematic if the cryoprotectant treatment duration and loading has not been fully optimized (Benson et al. 1996b). It is advisable to be aware of the thermal behaviour of glasses during the optimization of PVS cryoprotectant loading, cooling *and* rewarming. Sakai et al. (2008) and Sakai and Engelmann (2007) report the following thermal characteristics of PVS2: (1) solidification of a metastable glass at ca. -115°C; (2) a slow rewarming Tg at ca.-115°C; (3) an exothermic Td devitrification (ice crystallization) event at ca. -75°C and (4) an endothermic melt Tm, at ca -36°C.

The technical advantage of chemical-additive vitrification is that the need for controlled freezing equipment is circumvented; the main methodological disadvantage is that processing many samples is labour intensive as compared to programmable controlled rate cooling. The PVS 'cocktails' can be toxic to some types of plants and germplasm although pretreatment and careful addition of the cryoprotectant reduces their toxicity (Sakai et al. 2008). The main advantage of PVS-based cryoprotection is its efficacy and successful application for the cryopreservation of germplasm from many different tropical and temperate species (Sakai et al. 2008). It has been used as the method of choice for tropical

plants and germplasm that are not amenable to cryopreservation using desiccation and controlled rate cooling methods (Sakai and Engelmann 2007).

From the reports of Sakai et al. (2008) and others, PVS-based cryoprotection strategies (i.e. PVS2 and PVS3) have been applied to germplasm from: *Allium porrum, Allium sativum, Allium wakegi, Ananas comosus, Arachis* sp. *Armoracia rusticana, Artocarpus heterophyllus, Asparagus, Atropa beladona, Beta vulgaris, Bletilla striata, Brassica campestris, Camellia sinensis, Carica papaya, Castanea sativa, Chrysanthemum, Citrus* spp., *Colocasia esculenta, Cymbidium* spp., *Cymbopogon, Daucus carota, Dendranthema, Dianthus caryophyllus, Dioscorea* spp. *Diospyros kaki, Doriteanopsis, Dortis pulcherrima, Fragaria* x *ananssa, Gentiana* spp., *Grevillaria scapigeram, Hyosciamus niger, Ipomea batatas, Lilium japonicum, Limonium* spp. *Macropidia fuliginosa, Malus* spp., *Manihot esculenta, Mentha* spp., *Musa* spp., *Nicotiana tabacum, Oryza sativa, Picorhiza kurroa, Populus alba, Prunus* spp., *Pyrus* spp., *Quercus robur, Quercus suber, Ribes* spp. *Secale, cereale, Solanum* spp., *Solemostemon rotundifolius, Trifolium repens, Vitis vinifera, Wasabia japonica*, and *Xanthosoma* sp.

6.4.2.3 Encapsulation-dehydration

This vitrification-based strategy was first developed for potato by Fabre and Dereuddre (1990), the procedure involves encapsulating germplasm in a calcium-alginate matrix, osmotic dehydration in sucrose, and evaporative desiccation in an air flow, or over silica gel, followed by direct plunging into LN. The glasses formed in the alginate beads are usually stable once desiccation conditions are optimized (Benson et al. 1996b, Gonzalez-Arnao and Engelmann 2006). Therefore, rewarming can be undertaken at ambient temperatures, usually for 20-30 min in a laminar airflow cabinet, followed by rehydration of the beads in liquid medium for 20 min to remove sucrose, before transfer to recovery medium. This method has the advantage of being a low technology protocol and like other vitrification protocols it is available to laboratories without access to controlled rate cooling equipment. An advantage of encapsulation-dehydration is its proven efficacy for germplasm unresponsive to controlled rate cooling (Gonzalez-Arnao and Engelmann 2006; Engelmann et al. 2008). One practical benefit is its application in artificial seed production, which is an advantage in clonal forestry (Gale et al. 2007, 2008). The main disadvantage of encapsulation-dehydration is that desiccation-sensitive germplasm remains problematic and care must be taken to achieve non-lethal desiccation (Benson et al. 1996b). Reproducibility of air desiccation may be a problem in laboratories operating in an unregulated ambient environment and in these situations, silica gel evaporation is advised (Sherlock et al. 2005). Encapsulation-dehydration is a labour intensive protocol and requires several steps, sometimes undertaken over 1-2 days; therefore it may not be cost effective for processing large numbers of samples.

Encapsulation-dehydration appears to be preferentially used for the cryopreservation of shoot tips (Engelmann et al. 2008; Reed 2008) and has been applied to germplasm from over 70 plant species. Including, as reported by González-Arnao and Engelmann (2006) the following: *Actinidia* spp., *Anthirrnium macrophyllum, Armoracia rusticana, Auricularia, Beta vulgaris, Brassica napus, Cychorium intybus, Chrysanthemum morifolium, Citrus* spp., *Cocos nucifera, Coffea racemosa, Cosmos atrosanguineus, Daucus carota, Dioscorea* spp., *Fragaria x ananassa*, *Holostemma annulare, Ipomea batatas, Iris nigricans, Lilium* spp., *Malus* spp., *Mentha* spp., *Musa* spp., *Morus bombysis, Olea europea, Polygonium aviculare, Prunus* spp., *Pyrus* spp., *Ribes* spp., *Solanum* spp., *Vitis vinifera, Wasabia japonica* and *Xanthosoma* sp.

6.4.2.4 Encapsulation-vitrification

Sakai and colleagues (Sakai 2004; Sakai and Engelmann 2007; Sakai et al. 2008) developed the encapsulation-vitrification protocol which combines alginate encapsulation with PVS2 vitrification. As applied to shoot meristems, shoots are first pregrown with sucrose, optimized at levels of ca. 0.3 M, followed by alginate encapsulation, loading with 0.4 M sucrose, or a mixture of 2 M glycerol and 0.4 M sucrose for 30-60 mins, followed by treatment with PVS2 for 1 h at 25°C; beads are removed and placed in cryovials to which 1 ml of PVS2 solution is added. For storage, samples are directly immersed into LN; on retrieval the beads are rapidly rewarmed in a water bath at 35-45°C for ca. 1 min, the PVS2 is drained away and replaced with 1.2 M sucrose unloading solution for 10 min. The encapsulation-vitrification protocol was designed by Sakai (see Sakai et al. 2008) to process large numbers of meristems by eliminating the lengthy desiccation treatments required for encapsulation-dehydration. An advantage is the substitution of evaporative desiccation with osmotic dehydration making cryopreservation more accessible to desiccation-intolerant tropical plant germplasm. Encapsulation-vitrification has been successfully applied to different species as reviewed by Sakai and Engelmann (2007) and Sakai et al. (2008); they include: *Ananas comosus, Citrus aurantium, Daucus carota, Dianthus caryophyllus, Dioscorea* spp., *Diospyros kaki, Fragaria x ananassa Duch., Gentiana* spp., *Ipomea batatas, Lilium* spp., *Malus domestica, Manihot esculenta, Mentha spicata, Olea europea, Poncirus trifoliate x Citrus sinensis*, *Prunus domestica, Pyrus* spp., *Rubus idaeus, Saintpaulia ioantha, Solanum tuberosum*, and *Wasabia japonica*.

6.4.2.5 Droplet freezing and vitrification

These methods are permutations of ultra rapid freezing and vitrification protocols; Kartha et al. (1982) originally developed the droplet freezing protocol for cassava and this was adapted for potato shoot tip cryopreservation by Schäfer-Menuhr et al. (1996, 1997). DMSO is the cryoprotectant used in droplet freezing which, when applied with PVS2 (Benson et al. 2007, Panis et al. 2005; Sakai and Engelmann 2007) is referred to as droplet-vitrification. Both protocols involve the ultra rapid cooling of the sample by direct exposure of the germplasm to LN. Pretreatments are often applied before the cryoprotectant stage, which in the case of droplet freezing involves dispensing 2.5-20 μL droplets of 10% (v/v) DMSO onto sterile aluminium foil strips of 2-3 x 0.5-1.0 X 0.003 to 0.005 cm^3 dimensions. After loading of 5-10 droplets/strip with shoot meristems the foils are directly exposed to liquid phase LN and transferred to cryovials containing LN (2 foils per vial) after which they are stored in the liquid phase of LN. On rewarming, the foils are removed and placed directly into liquid medium at ambient room temperatures (ca. 25°C); the shoots dislodge and are plated onto recovery medium. In the case of droplet-vitrification, one method described by Panis et al. (2005) for *Musa* is as follows: shoot tip meristems are pretreated with an osmotic additive, followed by a preparative loading treatment with pre-chilled PVS2. Meristems are then transferred to 15 μl droplets of chilled PVS2 solution, dispensed onto strips of aluminium foil, plunged directly into liquid phase LN and transferred for permanent cryostorage to 2 ml cryovials pre-filled with LN. On rewarming, foils are placed in rinsing solution at ambient temperatures and after blotting on filter papers they are transferred to culture medium.

The major advantage of droplet-vitrification is its broad-ranging success in cryo-preserving shoot tip meristems of different types of crop plant germplasm (Panis et al. 2005; Halmagyi and Pinker 2006; Ashmore et al. 2007a; Kim et al. 2007; Sant et al. 2007; Gallard et al. 2008). In this capacity, it is helping to alleviate the bottleneck of differential genotype response which is a major limiting factor in the large-scale cryobanking of crop plant genetic

resources. Droplet-vitrification (PVS2) and droplet freezing (DMSO) protocols are critically dependent upon using ultra rapid cooling and rewarming requiring direct exposure to LN, making non-containment the main disadvantage of both methods. Direct exposure to LN is essential to achieve the rapid rates of cooling required for survival, both droplet-vitrification (PVS2) and droplet freezing (DMSO) often involve purposeful filling of cryovials with LN which may have consequences for cross-contamination by microbial agents and pathogens. Stringent adherence to phytosanitary and indexing procedures and double-testing for endophytic contaminants before germplasm is cryopreserved using these protocols is therefore recommended. In some countries and institutions non-adherence to manufacturers' safety recommendations, including the purposeful infiltration of cryovials with LN may not comply with health and safety regulations. Droplet freezing has the advantage of proven applicability across a wide crop-genotype range, as first applied to potato shoot meristems and afterwards for the cryobanking of plant germplasm in genebanks (Kryszczuk et al. 2006; Schäfer-Menuhr et al. 1997). Droplet-PVS2 vitrification is being increasingly applied to major crop plant species and has proved very successful in its application to diverse crops and different genotypes (Leunufna and Keller 2003, 2005; Kim et al. 2006, 2007; Ashmore et al. 2007a; Sakai and Engelmann 2007; Sant et al. 2007; Sakai et al. 2008). Examples of which include: *Allium sativum, Carica papaya, Colocasia esculenta, Cychorium intybus, Dendrathema grandiflora, Dioscorea* spp., *Fragaria x ananassa., Ipomea batatas, Lilium* spp., *Musa* spp., *Pelagonium* spp., *Phoenix dactylifera, Saccharum officinarum, Solanum tuberosum, Solanum stenotomum, Solanum* spp. and *Thymus*.

Part II of the GPG2 series on clonal crop conservation collates and summarizes surveys of the mandate species currently held in CGIAR's cryobanks (Benson et al. 2011a). Part III (Benson et al. 2011b) of the series provides technical guidelines pertaining to generic LTS methods and protocols.

6.4.3 Large scale deployment of cryopreservation

The development of a wide range of protocols (Reed 2008) has resulted in the large-scale deployment of cryopreservation in international genebanks and biorepositories. Keller et al. (2008) report the status for major cryobanking initiatives at a number of international repositories, including, at the IPK, Gatersleben regarding 1,017 accessions of potato, 38 of garlic, 19 of mint, 1 for yam and 4 for other medicinal and aromatic plants; at the Northwest German Forest Research Institute, 450 elm accessions; in the Czech Republic's crop plant genebank, 15 accessions of hop and potato; in the USDA's germplasm repository, 27 accessions of garlic, 17 of *Fragaria*, 3 of *Humulus*, 20 of *Ipomea*, 2,201 of cold-hardened *Malus* buds, 44 of *Mentha*, 57 of *Prunus*, 100 of cold-hardened *Pyrus* buds, 27 of *Ribes* and 30 of *Rubus*, as well as 37,654 cryopreserved seed accessions held in the Fort Collins cryobank and in the South Korean Genebank, 300 accessions of garlic. Gonzales-Arnao et al. (2008) review the large-scale application of cryopreservation for shoot and embryo cultures represented by citrus, various tropical crops, cassava and potato. Volk and Walters (2003) report on the use of cryopreservation and the status of *in vitro* active collections held in the USDA's National Plant Germplasm Clonal Collections.

6.4.4 Cryotherapy

Virus elimination by cryogenic treatment is now used to eradicate pathogenic organisms from some cultures. The procedure is useful in that it circumvents the laborious dissection of small meristems and is applicable across different meristems sizes. First tested by Brison et al. (1997)

for plum, cryotherapy has been demonstrated by Wang et al. (2003) to eradicate grapevine virus A and by Kim et al. (2007) to eradicate *Allium* viruses. Wang and Valkonen (2008) also found cryotherapy effective for eliminating sweet potato little leaf phytoplasma. Helliot et al. (2002b) describe cryotherapy as a micro-scalpel, although cautioning not all viruses and plants responded effectively regarding complete pathogen eradication.

6.4.5 Stability and cryopreservation

Cryogenic stability pertains to the biophysical and molecular mobility of the vitrified state and the genetic stability of plants regenerated from cryobanked germplasm.

6.4.5.1 Biophysical stability

Thermal analysis elucidates critical factors in cryopreservation protocols (Angell 2002; Block 2003; Benson et al. 2005; Hor et al. 2005; Šesták and Zámečnik 2007) and provides information concerning the stabilization of the glassy state (Volk and Walters 2006). The International Society for Biological and Environmental Repositories (ISBER 2008) define Tg as: "the temperature at which a cell is dehydrated to the degree that the remaining liquid within it is so viscous that molecules have insufficient energy to order into a crystalline state. Below this temperature (generally regarded as -132°C), no diffusion can take place within the cells and its surroundings. Without this diffusion the 'biological clock' stops."

Thermal analyses are useful, not only in optimising cryoprotective dehydration and desiccation, but also in risk management as they can confirm the principal storage temperatures required to stabilize vitrified germplasm (Benson et al. 1996b, 2005; Martínez et al. 1998; Dumet et al. 2000; Angell 2002; Block 2003; Šesták and Zámečnik 2007). Manufacturers of cryogenic storage equipment recommend cell storage to be performed below the water recrystallization temperature of -130°C (Nunc™ 2005). Consequently, knowledge of cryoprotectant Tgs, as well as their ice nucleation and re-crystallization temperatures is a quality assurance parameter for storage stability. Walters (2004) cautions timescales for changes in viability of cryopreserved germplasm have yet to be calculated, noting that molecular mobility studies can provide approximations of deteriorative reactions that might affect longevity. For long-term cryobanks, knowledge of water status and Tgs provides evidence for creating standard operating procedures (e.g. for cryovial input and withdrawal) that do not compromise stability. Volk and Walters (2006) comment that the mechanism by which PVS2 exerts its protective properties has ramifications for the longevity of vitrified germplasm and recommend storing cryopreserved materials well below their Tgs as reduced cellular viscosity increases long-term survival. Knowledge of thermal behaviour in different glasses informs optimal choice of LN phase storage and the use of vapour phase-only transit shippers. Taking into account the stability of Tgs for germplasm held at different storage temperatures concurs with the best practice recommendations of ISBER (2008).

6.4.5.2 Phenotypic and genotypic stability

In vitro stability is a cross-cutting issue concerning MTS and LTS and cryopreservation is dependent upon culture manipulation before and after LN treatment. Harding (2004) summarizes the methods used to assess stability in cryopreserved germplasm as:

- **Phenotype variation:** morphological assessment (*in vitro* and field-tested, preferably using crop plant descriptors) at sequential stages of short and long-term recovery.

- **Morphological assessments:** *in vitro* characteristics (differentiation, dedifferentiation, adventitious shoot development) meristematic chimeras, vegetative and reproductive development in regenerated plants.
- **Biometric studies:** comparative studies of development using principle component analyses, for different stages of plant development.
- **Histological and cytological analysis:** tissue integrity, chromosome stability, ploidy status.
- **Biochemical and metabolite analysis:** production of secondary metabolites.
- **Protein, enzyme analysis:** isoenzyme profiles, proteomics technologies, 2D electrophoresis.
- **Molecular genetic analysis:** genome structure, DNA-DNA hybridization, PCR-based techniques: RAPD, SSR, AFLP analysis, and the stability of transgenes.
- **Epigenetic**: (non-Mendelian inheritance) chromatin analysis, DNA-methylation, methylation-specific PCR.

Scowcroft (1984) suggested cryostorage could reduce the constraints of slow growth as the biological processes causing instability are arrested at ultra low temperatures. Some factors (osmotica, cold) are however, similar to those used for slow growth, albeit for significantly shorted durations. Using cryogenic storage to confer stability on cultures is a common principle that has been widely applied to microorganisms, human and animal cells. In these cases, both base and active collections are maintained in cryobanks (Stacey and Day 2007).

Stability consensus

To date, the consensus for stability assessments of cryopreserved germplasm and plants regenerated from cryobanks is positive and there exist various reports which confirm stability using different approaches applied to diverse species. Examples include: stable RAPD profiles of *Melia azedarach,* cryopreserved using encapsulation-dehydration (Scocchi et al. 2004) and genetic fidelity demonstrated by RAPD analysis of *Populus tremula* x *Populus tremuloides*, which was cryopreserved using controlled, slow cooling and PVS2 (Jokipii et al. 2004). Phenotype and karyotype analysis also indicated stability in *Cyrtopodium hatschbachii*, cryopreserved using encapsulation-dehydration (Surenciski et al. 2007). Ploidy status was retained in *Solanum* spp. cryopreserved using ultra rapid freezing or encapsulation-dehydration (Ward et al. 1993; Benson et al. 1996a). Various molecular studies have been performed on potato germplasm, including microsatellite analysis and DNA-DNA hybridization of nuclear and chloroplast DNA which demonstrated stability in *S. tuberosum* cryopreserved using encapsulation-dehydration (Harding and Benson 2000; Harding and Benson 2001). Schäffer-Menuhr et al. (1997) confirmed stability using RFLP, cytological and phenotypic analyses for a large-collection of potato germplasm cryopreserved using droplet freezing. Stable RFLP profiles were also observed in DNA extracted from mahogany trees recovered from germplasm cryopreserved by controlled rate cooling (Harding et al. 2000). For *Arachis* germplasm cryopreserved using PVS2, RAPD marker analysis confirmed stability (Gagliardi et al. 2003). Stable Inter Simple Sequence Repeats (ISSR) and RAPD profiles were respectively observed in apple (Liu et al. 2008) and citrus (Hao et al. 2002b) following recovery after vitrification.

For some studies, multiple approaches have been applied to monitor stability, this is exemplified by the use of phenotypic, cytological, RAPD and AFLP analyses to confirm and corroborate stability in plants derived from of *Prunus* germplasm that had been cryopreserved using PVS2, encapsulation-dehydration or controlled rate cooling (Helliot

et al. 2002a). Similarly, stable morphologies, biosynthetic capabilities and RAPD profiles were found in *Dioscorea bulbifera* plants recovered after encapsulation-dehydration (Dixit et al. 2003). No detectable differences were found in protein, isozyme, RAPD and AFLP profiles of apple, plants recovered from cryopreserved germplasm using vitrification (Liu et al. 2004). Taking a different approach, the stability of integrated transgenes was confirmed in transformed embryogenic lines of European chestnut that had been cryopreserved using a PVS2 vitrification protocol (Corredoira et al. 2007).

There are also reports of instability and/or changes in development competency found in plants regenerated from cryopreserved germplasm, it is precautionary to use this information to guide best practices and risk management in quality assurance strategies for LTS. Risk assessment for cryopreservation may be advised to include associated tissue culture manipulations as different components of a cryopreservation protocol may incur various risks to stability and in practice protocol success depends upon both cryogenic and tissue culture procedures. Examples of cryopreservation treatments incurring instability and/or changed phenotypic and genotypic characters include: ribosomal RNA gene DNA methylation in *S. tuberosum* cryopreserved using ultra rapid freezing (Harding 1997). Variation in RAPD profiles in DMSO-treated (non-cryopreserved) embryogenic cultures of *Abies cephalonica* were observed by Aronen et al. (1999). DNA-methylation changes have been found in citrus cryopreserved using PVS2 (Hao et al. 2002b); in strawberry, cryopreserved using encapsulation-dehydration (Hao et al. 2002a) and other epigenetic chromatin changes in mahogany, cryopreserved using controlled rate cooling (Harding et al. 2000). This suggests the propensity for epigenetic processes occurring during cryopreservation is genotype/species and protocol independent. A changed RAPD profile was observed in one regenerant of *Dendranthema grandiflora* cryopreserved using encapsulation-dehydration (Martín and González-Benito 2005). Possible SCV was observed in white spruce, cryopreserved using controlled rate cooling (DeVerno et al. 1999). Genotype-dependent genetic variability based on RAF (Randomly amplified DNA fingerprinting) and AMP (Amplified DNA methylation polymorphism) markers were noted in papaya plants recovered from droplet-vitrification (Kaity et al. 2008).

Which is the best method to use?

The issues of how to: (1) assess stability in cryopreserved germplasm and (2) minimize and manage the risk of instability are critically important in developing best practices, high standards of stewardship and quality assurance criteria for all types of biorepository (Stacey 2004). For plant germplasm, these challenges are confounded by the tendency of plants to manifest SCV in tissue culture; this is also exacerbated by epigenetic and genetic changes in response to conditions of stress. Consequentially, Harding (2004) highlighted the need to develop more robust, practical approaches to assess stability in regenerated cryopreserved germplasm, giving rise to the concept of cryobionomics. This focuses on understanding the linkages between genetic stability and its possible causes (e.g. cryoinjury and somaclonal variation), and elucidating how they affect stability and quality (trueness-to-type). Harding (2004) reviewed the applicability of the different molecular analyses currently used to assess genetic stability, concluding that most analyse a very small fraction of the genome, which may not provide a sufficiently robust measure to detect variability in cryopreserved clonal crop germplasm. Using different levels of evaluation is prudent; assessments of trueness-to-type and field performance are more rigorous than only using molecular analyses.

Smith and Ryan (2008) recommend using multiple physiological, metabolic and genetic tests to assess stability of conserved strains of microorganisms held in cryobanks and advise tests should be validated and implemented on a periodic, rather than a regular basis. Routine checks will ensure standard procedures are being adhered to and well documented. AFLP analysis is the method of choice suggested by Smith and Ryan (2008) as it is both stringent and relatively reproducible, however AFLP analysis requires expensive, specialist equipment and alternative suggestions are the use of RAPDs, minisatellites or Variable Number Tandem Repeats (VNTR), Simple Sequence Repeats (SSR) and Inter Simple Sequence Repeats (ISSR). Smith and Ryan (2008) cautioned that PCR-based techniques are prone to interference and this can result in different banding patterns, this problem may be complicated by contaminated, non-axenic cultures (Stacey 1999, 2004). Long-term biometric, phenotype, and field assessments of plants recovered from cryopreserved germplasm remain valuable performance indicators for assessing stability (Harding and Benson 1994; DeVerno et al. 1999; Côte et al. 2000; Harding and Staines 2001; Mártinez-Montero et al. 2002; Konan et al. 2007; Medina et al. 2007). Ideally, these are best used to complement molecular-based tests; for the sake of efficiency their application may be rationalized by first assessing the propensity of genotypes to produce off-types.

Long-term stability management

Managing the risks of instability in IVBGs is critical, though it is wise to take a pragmatic approach as overall; the risks of not securing germplasm in *in vitro* genebanks might be greater than the risk of loss (or instability) being generated by other types of storage. Moreover, a number of strategies and practices can be applied to reduce and offset the potential problem of instability. These have been explored in the detailed study of *Prunus*, by Helliot et al. (2002a) which reiterates the potential problems of SCV first highlighted by Scowcroft (1984, 1985). It is advisable not to use, as source material *in vitro* plants that have been maintained in long-term culture, if this is not practicable it is precautionary to compare genetic stability at each stage of a protocol. Where it is necessary to cryopreserve germplasm that has been maintained in culture for longer periods, it is cautious to be aware of the increased risks of generating SCV over extended culture times; these risks highlight the practical importance of rejuvenation, regeneration and verification (see Figure 7). This is exemplified by the study of DeVerno et al. (1999) in which somaclones from cryopreserved cells of white spruce were attributed to tissue culture factors rather than cryopreservation *per se.* Medina et al. (2007) also observed undesirable agronomic characters in strawberry plants regenerated from cryopreserved shoot meristems. These variants were assigned to *in vitro* manipulations rather than to cryogenic factors. Nonetheless, as tissue culture is a necessary component of most cryopreservation protocols measures are required to reduce the effects of both cryogenic and non-cryogenic factors on long-term stability.

Integrated processes in the medium and long-term storage of clonal crops

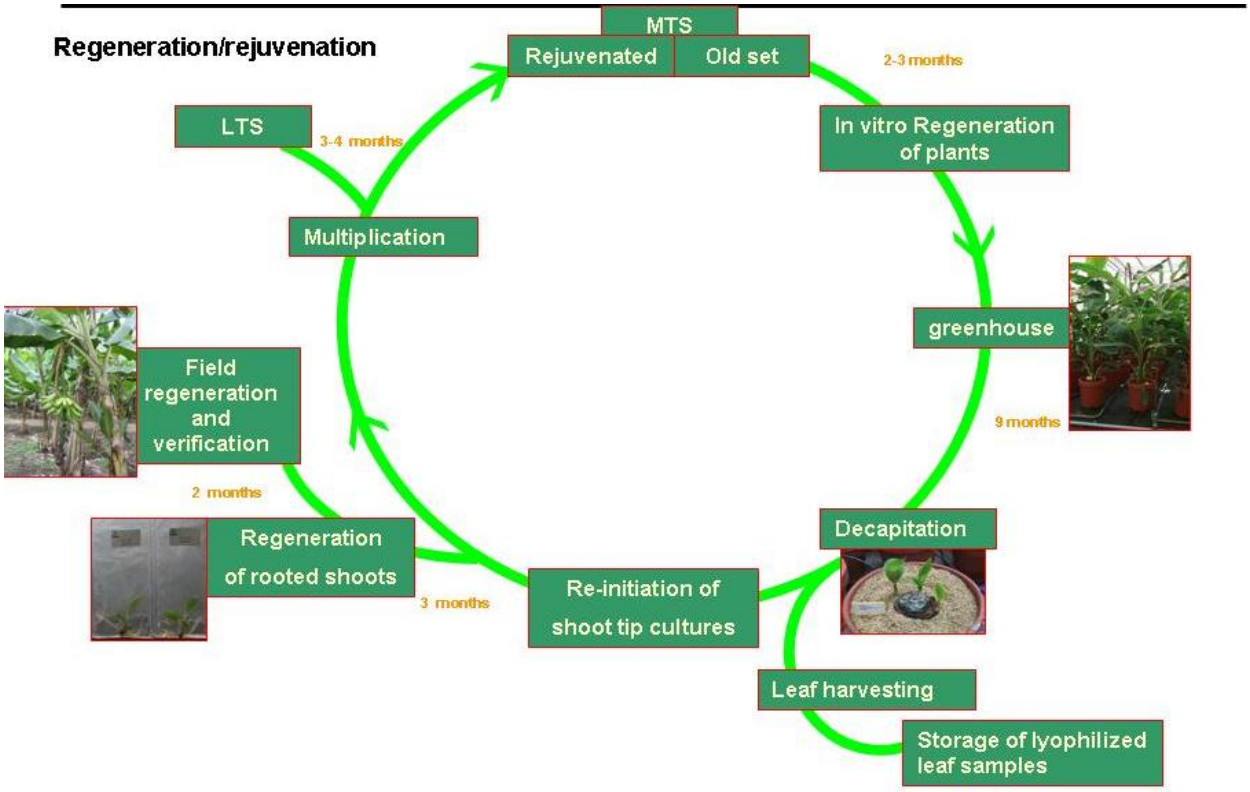

Figure 7. Schematic showing the integration of regeneration and rejuvenation cycles in the *in vitro* conservation of *Musa* germplasm, highlighting the role of field regeneration and verification as quality measures in slow growth (MTS) and cryopreservation (LTS).

The image is kindly supplied by Ines Van den houwe, Bioversity International.

7. Research, validation, and best practices in *in vitro* genebanks

Fundamental knowledge and practical outcomes of empirical research (Figure 5) are the building blocks of storage protocol development; however, progress towards quality management systems and best practices requires a more formal approach. Whilst contemporary advances in slow growth and cryopreservation have been achieved through several modes of research (Figure 5), it is critical that best practice development and validations in IVGBs are considered more holistically as shown in Figure 7.

Validation is the mechanism by which a process is assessed against a specific criterion as fit-for-purpose; this could pertain to expected levels of survival and regrowth after cryopreservation and slow growth treatments. In a quality management system, validation provides confirmation that the needs of a client or end user are met, it necessitates documented evidence that a process or a system used within specific parameters can perform: (1) effectively and (2) reproducibly, with respect to predetermined specifications. A validated storage protocol also confers a level of confidence that is critically dependent upon carefully selecting and corroborating validation criteria which are linked to fitness-for-purpose. In the case of validating a cryopreservation protocol for shoot meristems, the criterion should not be survival (viability) but shoot and preferably plant regeneration, this is because survival is not a sufficiently robust indicator that recovered germplasm is fit-for purpose. Albeit, in working up a protocol, viability is useful as an early indicator, it is not an appropriate measure of competency when implementing the protocol as a best practice and routine method. Different performance criteria (e.g. phenotype, genotype assessments) are also used to evaluate plants recovered from IVGBs and their methodologies can also be validated (Day et al. 2007). Various criteria may be required for different protocols and types of germplasm, noting that validation will remain an aspiration for methods under development. In progressing towards a quality assured management system, it is cautionary to build in a formal validation process for a method before it is disseminated for routine use as a best practice or standard operating procedure across collaborating consortia.

7.1 Fundamental research in protocol development

Fundamental research supports evidence-based decisions for storage protocol development, validation and risk assessment and it helps to calibrate performance indicators for best practices formalized in RTD programmes (Figure 5). One of the most insightful recent applications of fundamental knowledge is the biophysical study of cryopreserved germplasm longevity as this has implications for long-term risk management. Walters et al. (2004) found that cryogenic storage temperatures progressively increased seed longevity, but were not sufficient to stop deterioration as the benefits of low temperatures (at -18 or -135°C) on longevity were progressively lost if seeds were first stored at 5°C. This suggests that physiological status and pre-storage treatments applied before cryopreservation may have consequences for longevity. Walters et al. (2004) also found variability in seed ageing kinetics could not be accounted for by water content and temperature alone, demonstrating differences in germination across accessions, provenances and between collecting years. This implies that variability is not solely due to genetic factors, but can be affected by other parameters and cryobank operators should not always assume a particular accession will exhibit average

deterioration kinetics. Thus, basic knowledge assists storage protocol risk assessments, for example, where germplasm is exposed to phase-fluctuations in LN temperatures, during excursions in, and out of cryobanks and in dry shipper transport. Knowledge of Tgs in germplasm held at different storage temperatures concurs with the best practice recommendations of ISBER (2008) and thermal profiling helps optimize cryoprotection (Benson et al. 1996b, Dumet et al. 2000; Šesták and Zámečník 2007; Nadarajan et al. 2008) particularly in desiccation sensitive germplasm. Knowledge of stress physiology can be used to help develop risk management strategies and to ensure germplasm is in an appropriate state before it is cryobanked. For example, stress markers can help to refine cryoprotection strategies (Johnston et al. 2007) and proteomics research can be potentially used to elucidate the basis of storage recalcitrance (Carpentier et al. 2006, 2007) and inform corrective actions.

7.2 Storage protocol validation and critical point assessment

Empirical approaches to technology transfer usually involve non-validated skills training and protocol transfers (Figure 5), but achieving a validated *in vitro* storage protocol, sufficiently robust to become a collective best practice across dispersed genebanks requires formal procedures. This is important for validating storage protocols across communities of practice undertaking shared research, training and technology development activities. Validation provides added confidence that all parties are performing their tasks effectively to specific criteria and performance indicators. Similarly, validation of *in vitro* conservation practices is desirable for genebank personnel involved in the reciprocated safe duplication of their holdings in black boxes. For example, in the event black boxes succumb to catastrophic events (e.g. earthquakes) validated rescue protocols may be required as recovery procedures.

Initially steps towards validation might involve internal (in house) verification of a protocol to meet specific performance criteria. This would be followed by technology transfers to ensure, through critical point analysis, all requirements are met for successful technology implementation in a recipient institute (Reed et al. 2001, 2004b). The final deployment of the protocol often needs adaptation to local circumstances to accommodate different facilities and operational conditions (Reed et al. 2001; Elster et al. 2008; Harding et al. 2008; Lukešová et al. 2008). Optimally, external validation (Reed et al. 2004b; Day et al. 2007; Dyer et al. 2007) is calibrated and corroborated by validators with different levels of expertise and across different institutions. This more robust approach tests quality of supporting information and experimental designs, ensuring a best practice is the successful outcome (Figure 8). Currently there is no formal validation system in place for cryopreservation in BRCs (Smith and Ryan 2008) this is mainly due to variation in and between storage protocols applied to different germplasm types. Methods often require technical adaptations for local institutes and different bioresources, although this need not be a constraint as long as the validation is undertaken systematically. Few detailed cryopreservation validation exercises have been reported in the literature, although three from very different sectors have been successful: (1) shoot meristems of clonally propagated culture of *Ribes nigrum* cv 'Ojebyn' (Reed et al. 2001, 2004b); (2) *Chlorella vulgaris*, SAG 211-11b, (Day et al. 2007) and (3) peripheral blood mononuclear cells (Dyer et al. 2007). Despite different biomaterials being cryopreserved these exercises had similar outcomes, comparable management criteria and modes of intervention. This provides confidence that validations based on basic principles can be undertaken across dispersed and diverse biobanks that service different communities of practice.

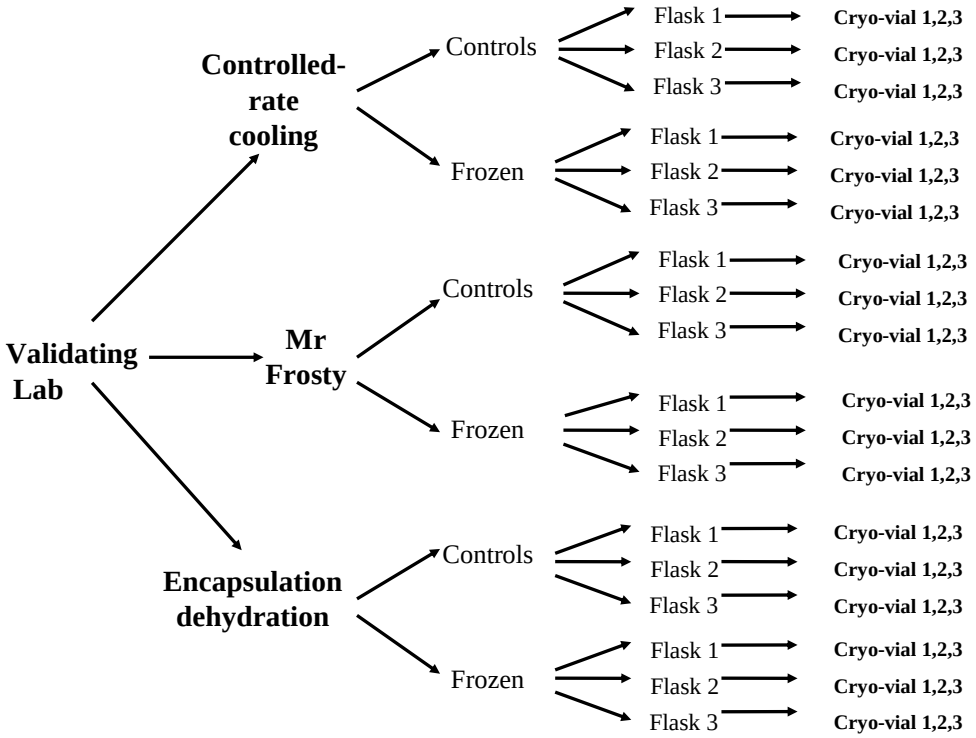

Figure 8. Experimental design for validating three cryopreservation protocols using encapsulation-dehydration and controlled rate cooling with a programmable freezer and a Mr Frosty™ unit. From the algal cryopreservation validation exercise of Day et al. (2007) which comprised seven validating laboratories.

Based on the experience of European Union's 5th Framework Programme, Quality of Life and Management of Living Resources, Research Infrastructures Biological Collections, COBRA Project QLRT-2000-01645.

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7.3 Designing and evaluating a storage protocol validation exercise

Ideally storage protocol validation involves a common experimental design applied in exactly the same way, concomitantly across several institutes, using an identical test culture, organism or cell line provided from the same source (Figures 8 and 9). A risk assessment should be undertaken prior to the validation exercise to ensure that the potential hazards of transmitting pathogens are offset. Validation requires detailed planning; arriving at consensus as to how the process is undertaken and engendering collective ownership of validation data that may be used for quality assurance. Some lead in time is necessary to dispatch test cultures to different validators, which on their receipt should be processed for health and competency. It is essential test cultures are cultivated similarly using the same growth media across participating laboratories, this requires crosschecks to ensure cultures are performing within expected criteria before they are used to test storage protocols. It is

preferable the test culture has a known and well-proven amenability to storage (Reed et al. 2004b; Day et al. 2007) as this can be used as a performance indicator (viability, shoot regrowth or colony forming units). The protocol should have been developed, tested and verified by at least one participating partner who usually takes the lead in the wider validation exercise. Once a protocol has been validated for test material, it can be extended to other organisms and cultures. It is critical that identical handbooks and technical instructions are provided so that technical operators are guided by the same information, which has been checked for understanding, eligibility, and accuracy, particularly if it is translated into different languages. Validation by personnel unfamiliar with *in vitro* storage procedures requires adjunct training, workshops and phone-conferences (Figure 9) instigated at each stage of the process (Reed et al. 2004b; Dyer et al. 2007). Training and knowledge transfers may not be necessary to achieve a successful outcome for validators with prior experience.

Good communications throughout validation programmes are required and this is best achieved using face-to-face visits, conference calls and a virtual hub such as a project–dedicated website. In the case of the European Union's 5th Framework Programme for Research Infrastructures in Biological Collections, the 'COBRA Project' used both virtual and physical infrastructures enabling the team to validate cryopreservation protocols using a test strain of the single-celled green algae *Chlorella vulgaris* SAG 211-11b (Day et al. 2007). This reduced the risks of non-compliancy (Table 2) by the seven validators and problems could be largely rectified early in the process; changes to a validation exercise should be meticulously recorded using a commonly agreed template. This precautionary measure can be used as a diagnostic to explain why an expected performance indicator has not been satisfied and it assists in a successful outcome becoming a sustainable best practice, thereby supporting external accreditation at a later stage (Figures 5 and 9).

7.3.1 Performance indicators

Robust performance indicators are essential for storage validation, for the study concerning *C. vulgaris* SAG 211-11b (Figure 8) the exercise used prior experience of taxa with good levels of post-thaw survival (>50%) and a ~15% variation in batch-to-batch viability which was known to have no discernable effect on recovery or customer acceptability regarding sample quality. These parameters were already adopted as the CCAPs' 'in-house' quality standard and, as such they set the performance target for validation within an acceptable level of variability of 15% of the mean or median.

Day et al. (2007) also validated the assays used to assess survival (viability) and recovery as colony forming units (CFUs). Vital staining indicated significantly ($p<0.0001$) higher levels of viability following controlled rate cooling compared to measuring recovery as regrowth. Vital staining over-estimated survival compared to methods that use CFU and cell growth as the performance indicator, statistical analysis showed a significant ($p<0.01$) interaction between cooling technique and viability assessment method. In contrast, meristem survival is not a reliable assessment of recovery in the validation of *Ribes* cryopreservation protocols and Reed et al. (2001, 2004b) chose regrowth at six weeks as the performance indicator. It is therefore necessary that recovery times extending to several weeks are included in validation experiments.

7.3.2 Critical point assessment: creating compliancy across consortia

Validation exercises should identify the critical points that may affect successful outcomes, including non-compliancy and factors outside the validators' control. These are usually constraints in local facilities and operational practices but they still need to be part of the

analysis. As an exemplar (Figure 8), *C. vulgaris* SAG 211-11B was successfully cryopreserved using two controlled rate cooling methods and an encapsulation-dehydration protocol (Harding et al. 2008). Seven validating laboratories and two parameters were tested: (1) the cryopreservation protocol and (2) the performance criteria for viability. Validators comprised two groups, those internal to the project consortium were the UK's Natural Environment Research Council's Culture Collection of Algae and Protozoa (NERC-CCAP), the Universität Göttingen, Sammlung von Algenkulturen (SAG), Germany; Institute of Botany, Culture Collection of Algal Laboratory (CCALA), Czech Republic; Institute of Soil Biology, ISB Collection (ISB), Czech Republic; Universidade de Coimbra, Culture Collection of Algae, University of Coimbra (ACOI), Portugal; two validators external to the consortium: University of Caen, Algobank, France; CABI Europe-UK. These were selected on the basis of technical skills and knowledge, but had no direct experience of certain aspects of the procedure. Algobank had no previous practical experience of cryopreservation and CABI had limited phycological experience.

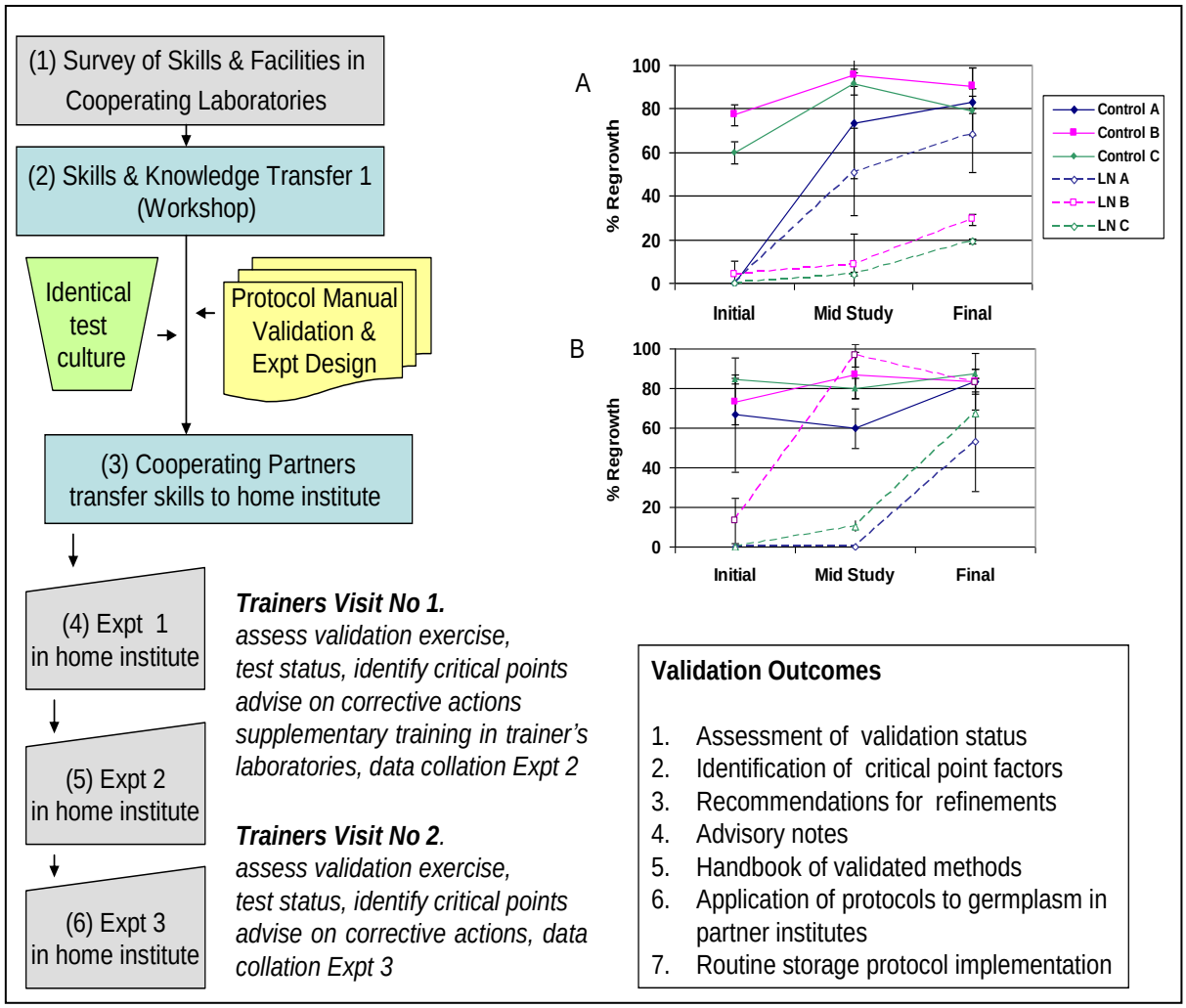

Figure 9. A programme for critical point analysis of cryopreservation protocol technology transfer and validation for shoot meristems of *Ribes* cryopreserved by: (A) PVS2 vitrification and (B) encapsulation-dehydration. The process was undertaken in three independent laboratories in Kazakhstan, Poland and Germany supported by the USDA Foreign Agricultural Service, Scientific Cooperation Programme. Schematic based on Reed et al. (2004b), reproduced with the permission of CryoLetters.

In validating a controlled rate cooling protocol for *Ribes* shoot meristems, Reed et al. (2001) found that the type of programmable freezer was a critical point factor due to different seeding (ice nucleation) mechanisms. These influenced germplasm survival and shoot growth, although local adaptations of the protocol resulted in acceptable levels of survival and shoot recovery. In the case of *C. vulgaris*, all participating laboratories successfully validated three different cryopreservation protocols (Day et al. 2007). A significant difference ($p<0.010$) was observed between data generated by individual partners, personnel experience was identified a critical factor when staffing changes at one institution affected control and post-cryo CFU counts. This was the most likely cause of inter-individual variability, whereas overall variability between validating labs demonstrated a well controlled/regulated validation experiment even though significant differences ($p<0.010$) were sometimes observed (Day et al. 2007). The outcome of this study was that all partners achieved viability levels within 15% of the median value and they were considered to have achieved the predetermined specification. The cryopreservation validation exercise using *C. vulgaris* demonstrates the technical rigour of the validators and measures protocol reliability. Both controlled cooling devices tested positive with high levels of viability, even with minor deviations from the code of compliance (Table 3).

Table 2 demonstrates a robust validation exercise employing both internal and external validators with different competencies from different institutions. Adherence to protocol instructions was tracked using an interactive website, enabling real-time monitoring of cooperative activities. High levels of compliance were achieved with the exception of factors outside the control of the project team (changes in personnel, variable suppliers, refrigerators). It was possible to ascertain critical point factors in the validation exercise (Tables 2 and 3) and critical point assessments of technology transfers and validation procedures provide a means of pinpointing those factors that affect deployment of protocols across different institutions. In the study conducted by Reed et al. (2004b) shoot tips derived from cultures of *Ribes nigrum* cv 'Ojebyn' were used to test the success of technology transfers across clonal fruit crop institutes in Poland, Germany and Kazakhstan. Several critical point factors were identified in the process:

- **General facilities:** growth room, laminar airflow benches, water bath, growth room for cold acclimation, general laboratory facilities, air-conditioning and ambient temperature control.
- **Personnel:** basic laboratory skills, competency and aptitude for meristem dissection, stringency of adherence to protocol procedures.
- **Instructions:** clarification of written and oral communication of methods and technical points related to cryoprotectant preparation, meristems excision, controls and handling of LN, rewarming and rehydration.
- **Source plant status:** origin, time in culture, subculture transfer interval, genotype (identical genotype/source required for validation).
- **Culture conditions:** optimized standard culture regimes, growth room parameters (light, temperature, and diurnal regime), culture medium preparation (autoclave procedure), growth regulators (quality, sterilization procedure), and subculture interval.
- **Pregrowth and recovery:** acclimation conditions, pregrowth treatment, medium for growth and recovery growth room parameters and subculture intervals.
- **Cryogenic facilities:** Dewar type, vials, inventories, labelling, LN availability.

Table 2. Compliance[1] of external/internal validators to an experimental design and protocols to validate controlled cooling and encapsulation-dehydration protocols for *Chlorella vulgaris* strain SAG 211-11B. [1]Compliance - dark grey; minor deviation – hatched; significant deviation – white; nd – not determined, [2]Colligative cryoprotection and controlled cooling using a programmable controlled rate cooler; [3]Colligative cryoprotection and controlled passive cooling using Mr Frosty®; [4]Cryoprotection by encapsulation-dehydration. Based on experience of the European Union's 5th Framework Programme, Quality of Life and Management of Living Resources, Research Infrastructures Biological Collections, COBRA Project QLRT-2000-01645.

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				Internal validators					External validators	
[1]Parameters for compliance				CCAP	SAG	AC OI	CCALA	ISB	Algo-bank	CABI
Test-strain										
Pre-cryoculture conditions		Medium								
		Environment								
CRF[2]	Experimental design	Number of flasks								
		Number of cryovials								
		Number of measure-ments	FDA							
			CFU							
	Methodology	Cryoprotection								
		Cooling regime								
		Thawing regime								
		FDA method								
		CFU method								
Mr Frosty[3]	Experimental design	Number of flasks								
		Number of cryovials								
		Number of measure-ments	FDA							
			CFU							
	Methodology	Cryoprotection								
		Cooling regime								
		Thawing regime								
		FDA method								
		CFU method								
Encap-dehyd[4]	Experimental design	Number of flasks								
		Beads/ cryovials								
		Number of measure-ments	Colour							
			Index							
			CFU							nd
	Methodology	Encapsulation								
		Sucrose treatment								
		Air desiccation								
		LN2 plunge								
		Rewarming/ rehydration								
		Colour								
		Growth index								
		CFU method								nd

Table 3. Viability analysis as a function of validating laboratory, for controlled rate cooling using two different cooling devices, CRF = controlled rate and Mr Frosty™ freezing unit.[1]Number of replicates; [2]Inter-Quartile Range (IQR). Using *Chlorella vulgaris* strain SAG 211-11B as the test organism. CFU = colony forming unit; FDA = fluroscein diacetate. Based on the experience of European Union's 5th Framework Programme, Quality of Life and Management of Living Resources, Research Infrastructures Biological Collections, COBRA Project QLRT-2000-01645.

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Lab	Cooling device	Viability assay	N[1]	IQR (%)[2]	Median (%)
CCAP	CRF	CFU	27	9.0	94.0
		FDA	27	6.0	100.0
	Frosty	CFU	27	20.0	57.0
		FDA	27	0.0	100.0
SAG	CRF	CFU	24	24.0	61.5
		FDA	24	14.5	87.5
	Frosty	CFU	26	20.0	62.5
		FDA	26	21.0	91.0
ACOI	CRF	CFU	27	6.0	103.0
		FDA	27	6.0	97.0
	Frosty	CFU	27	51.0	91.0
		FDA	27	2.0	95.0
ISB	CRF	CFU	27	6.0	89.0
		FDA	27	5.0	92.0
	Frosty	CFU	27	5.0	94.0
		FDA	27	3.0	98.0
ALGO	CRF	CFU	27	11.0	91.0
		FDA	27	3.0	100.0
	Frosty	CFU	27	15.0	89.0
		FDA	27	5.0	93.0
CABI	CRF	FDA	20	37.0	83.5
Total			**544**		

Final experiments achieved successful levels of post-storage recovery and training in the use of performance indicators improved experimental outcomes (Figure 8). Critical factors across all laboratories were: plant health, operator skills, prior experience and clarification of technical details. It is recommended instructions and information is tested for clarity before embarking on a validation process.

7.3.3 Learning from validation experiences

It is important not to underestimate the costs, time and resources required to plan, document and execute validation exercises undertaken across international consortia, this is especially relevant for quality assurance programmes (Dyer et al. 2007; Smith and Ryan 2008). For practitioners new to *in vitro* storage meticulous identification of: (1) critical point factors that determine successful outcomes and (2) assessment criteria are required. Mode of cryoprotection and cooling are key parameters and regrowth must use definitive assessment criteria, preferably followed by quantifiable phenotypic and genotypic assessments that confirm plants recovered from stored germplasm are fit-for-purpose (Day et al. 2007; Harding 2004; Reed et al. 2004b). Commonly identified general principals are practical "hands on" training, reciprocal visits, workshops, and pilot studies of the protocol before embarking on its validation; in the case of inexperienced personnel these activities are an essential preliminary.

Validations must be appropriately costed, resourced, and scheduled, preferably by involving the participation of internal and external validating laboratories with different levels of expertise. It is vital that simple, clear, and detailed instructions are provided and where necessary these should be translated into a first language with stringent accuracy. Participants should check and refine draft documentation and progress a mutually agreed final version of the validation documents. Engaging as many participants as possible during the development of the process increases "ownership" of the exercise. A virtual collaborating environment and project-dedicated website is invaluable in validation management, technical communications, data compilation, transfer and interpretation. Standard reporting templates, online questionnaires (to assess conformity) and equipment calibration avoids the unnecessary bureaucracy that can be associated with validation exercises.

Recommendations arising from the European Union's 5th Framework Programme, Quality of Life and Management of Living Resources, Research Infrastructures Biological Collections, COBRA Project QLRT-2000-01645 are shown in Table 4. Smith and Ryan (2008) provide a summary of parameters that may be used to standardize and validate fungal cryopreservation protocols. It includes criteria of generic importance to all types of cryopreserved materials:

- **Performance of blind tests:** a central laboratory sends a test culture with limited information to be tested by a validating laboratory.
- **Reproducibility check:** comparing results of a validation performed at different times, comparisons of results obtained by different storage methods, comparisons of results obtained by different personnel and operators.
- **Equipment calibration:** all equipment and facilities used in the validation are critical point parameters and should be regularly maintained and checked for performance.
- **Recording:** daily recoding of temperatures and critical performance attributes of growth rooms, cryogenic and refrigerated freezers.

Table 4. Recommendations for planning international validation exercises for cryopreservation protocols, based on the experience of European Union's 5th Framework Programme, Quality of Life and Management of Living Resources, Research Infrastructures Biological Collections, COBRA Project QLRT-2000-01645.

Revised from Day et al. (2007) with permission of CryoLetters.

Compliance area	Action
Training	Ensure those performing "hands on" work participate in pre-validation workshops and training courses.
Experimental design	Ensure all participants have the equipment, logistics and staff available to undertake the validation exercise. Where appropriate translate handbooks and manuals into the first language of the validating laboratories and check for accuracy and clarity of all instructions.
Methodology	Undertake a pilot study in all participating labs and not just a sub-set of the validating laboratories. Make sure there is adequate technical support to deal with all requests immediately. Wherever possible ensure all equipment and consumables are identical, where this is not possible make detailed and accurate records as to where deviations from protocols and materials occur.
Reporting	Where possible use a central, web-based hub to manage the validation exercise and train staff in completing any on-line reporting and data input.
Data management	Engage in dialogue at the planning stage with experts in validation and an appropriately experienced bio-statistician.
Data exploitation	Ensure the design of the experimentation is compliant with publishable results allowing widespread dissemination of the key findings of the study.

7.4 Working towards accreditation: a multidisciplinary experience

Quality Assurance (QA) is evidence of action needed to provide confirmation work is done effectively and involves a systematic accounting of activities, giving sufficient confidence a process or product meets given requirements for quality. Galsworthy (2007) explains a quality management system: (1) directs and controls an organization with respect to quality and (2) establishes policies and procedures required to achieve quality objectives. These should normally include all activities in the process including, documentation, calibration and maintenance. Implicit in QA is failure testing as this anticipates a weakness or critical point in a process, in the manufacturing industries Taguchi statistics are used to assess compliance and the approach is now applied to plant cryopreservation to determine critical parameters (Nadarajan 2005; Muthusamy et al 2005; Nadarajan et al. 2006; 2007). Statistical analysis also helps to quantify compliancy for variance around critical levels of tolerance (Day et al. 2007) and when routinely used it ensures a process is correct within defined levels and limits. Quality control involves QA across the whole process ensuring that it is reliable (robust) and safe with an overarching awareness a process is fit-for-purpose.

Medical practitioners are pioneers in developing quality assured and accredited culture collections in both active culture and cryopreserved states (Day and Stacey 2007; Day et al. 2008). Von Versen et al. (2000) comment that reaching consensus within a community of

practice, for best standards helps to confirm to authorities compliance with regulatory requirements. Quality systems have also driven the manufacture of high specification storage products, security systems and risk management logistics supporting the modern service biorepository and biobank and quality management has been a benchmark for service culture collections since their instigation. Smith and Ryan (2008) and Galsworthy (2007) similarly justify the importance of quality systems as follows:

- managing risk effectively,
- improving performance,
- assuring the quality of the service or research provided,
- formalizing systems for staff competency and training,
- gaining recognition via accreditation and certification.

Accreditation is the process by which a certificate of competency is awarded to an organization by an external, third party, auditor, which is itself accredited by formal standards and procedures. The auditor is thus assured competent to test the quality assurance. Certification is a third party attestation related to products, processes, systems or personnel and accreditation is undertaken in compliance with a body recognized to assess against internationally recognized standards. The international standards body for quality management systems is the International Organization for Standardization, represented by the logo and designation of ISO. The ISO body is a global organization comprising representatives from a wide range of national standard organizations that set standards of practice. There are three main international quality systems standards (Galsworthy 2007) relevant to accreditation in BRCs and IVGBs:

1. **ISO 9001:** a quality system with limited or no technical input.
2. **ISO 17025:** a quality system for the competency of a laboratory or organization with a high level of technical input.
3. **Good laboratory practice (GLP):** covers the production of registration data for chemical submissions to registration bodies.

The ISO 9000-9001 series is now being applied to tissue and cell banking in many disciplines, it is therefore advisable to explore how different biological resources sectors have developed their best practices and accredited quality systems. Smith and Ryan (2008) report the impacts of the OECD's best practices (OECD 2007) on validating cryopreservation techniques for microorganisms held in BRCs. These involve the activities of the Global Biological Resource Centre Network (GBRNC) a worldwide federation of culture collections. Their experiences provide useful insights into how a large-scale collective proceeds towards validating best practices and developing quality criteria for active and cryopreserved cultures. The OECD best practices (OECD 2007) consolidate the earlier efforts of the UK's National Culture Collections (UKNCC, http://www.ukncc.co.uk), the European Common Access to Biological Resources and Information (CABRI) and the European BRC Network. Guidance offered by the OECD (2007) is designed to ensure biological materials held in all its institutions supply them in compliance to a common set of four quality standards related to:

- laboratory design and procedural requirements,
- handling, authenticity, preservation, and distribution procedures,

- compliance with national and international rules and regulations,
- auditing and accreditations procedures.

The OECD and UKNCC best practice guidelines are reiterated at two levels, the first is applicable to all biological resources, whilst the second is domain specific, the most rigorous specifications are in place for the microbial faculty, followed by human-derived materials. The principles on which the best practices are based are generic and as such are applicable to plant germplasm, they are listed as:

- Competent persons carry out procedures.
- Performance of stored germplasm authentication tests.
- Number of subcultures, transfers, or generations of original material before storage are kept to a minimum.
- Master stock is created from the original material.
- Master stocks are cryopreserved from which further stocks for distribution are sourced.
- Sufficient distribution stocks are made to minimize need to go back to master stocks for replenishment.
- Materials are stored under environmental parameters that assure stability of properties.
- Detail of inventory control, lead times and re-stocking practices are documented.
- Validation of methods and recording of details of the validation process.
- Details of protocols are laid down using domain-specific criteria.
- Biological materials are preserved using at least two distinct methods.
- Cryopreserved stocks are maintained in separate locations.
- Disaster measures are in place in the event collections are placed at risk.
- Establishment of baseline information on storage maintenance checks.
- Maintenance plan for periodic control of each organism preserved.
- Validation of materials after preservation.
- Any deviation from standard operations requires accounting for discrepancies and recording.
- Quality audits and review procedures are in place.

The goals of quality management systems are to ensure the availability of known, validated and precisely identified biological resources that are conserved using validated and reproducible preservation protocols (Smith and Ryan 2008). To this end the OECD continues to examine mechanisms for adopting best practices, based in part, on the UKNCC quality management system (UKNCC 1998). Adopted by many collections is the ISO 9000 (9001):2000 series certification, of which sixteen 'collection' organizations have been third party accredited (Smith and Ryan 2008). The German Collection of Microorganisms and Cell Cultures (DSMZ) holds ca. 750 plant lines certified by DIN EN ISO 9001-2000. The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany has realized ISO certification (DIN EN ISO 9001-2000). The International Potato Center (CIP), Peru, was awarded 'ISO/IEC 17025:2005 General Requirements' for the competence of testing and calibration laboratories in February 2008, by the United Kingdom Accreditation Service. This accreditation is a considerable achievement for addressing quality systems in CGIAR's clonal crop genebanks and a major milestone for the *in vitro* plant conservation community.

7.5 Future perspectives: translational *in vitro* conservation technology

The characteristics of translational research (TR) are complexity, trans-disciplinarity, and undertaking collective actions across multiple sectors and institutional types (Clackson 2006). First developed for medical disciplines, TR is described as the 'Bench-to-Bedside' approach to drug development (Littman et al. 2007) although it is now engaged by other sectors because of its broader definition of translating academic research into practical benefits. Realising best practices, managing risks, and validating protocols in the IVGB involves translational principles:

1. Consolidating practices for connecting pure and applied research.
2. Creating frameworks for delivering to practitioners and beneficiaries.
3. Charting paths from scientific discovery to application.
4. Utilizing roadmaps and technology implementation plans.
5. Developing strategies to:
    a. capture and accelerate research benefits,
    b. close gaps between discovery and knowledge use by practitioners,
    c. yield high specification "fit-for-purpose" knowledge transfers,
    d. meet professional standards and safety criteria,
    e. make more cost effective use of research and development funds.

Translational research is not a substitute for conventional research, rather it is a catalyst to achieving a desired outcome that satisfies the needs of end users. Operations within IVGBs are under regulatory control and are increasingly influenced by the changing expectations of stakeholders and beneficiaries, particularly in molecular and omics research which demand stringency in the quality and containment of genetic resources. The expectation is that IVGBs meet these demands through best practices and quality assured operations; a translational approach may help to ensure that research outcomes are acceptable within these contexts (Benson 2008a; Littman et al. 2007).

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