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 II. Status of *in vitro* conservation technologies for: Andean root and tuber crops, cassava, *Musa*, potato, sweetpotato and yam

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"; It concomitantly assists 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." The information collated on *in vitro* techniques supports the production of a training manual for the use of MTS and LTS by other partners, including NARS. Interdependent Actions: The methods in this document support the interdependent Milestone Activity 2.1 and its sub-activities leading to the compilation of best practices for clonal crops.

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Abbreviations and acronyms

AFG	Associated field genebank
AFLP	Amplified fragment length polymorphism
ARTC	Andean root and tuber crop(s)
BA	benzyl adenine (also see BAP)
BAP	6-benzylamino purine (also see BA)
CBD	Convention on Biological Diversity
CCTF	Clonal Crop Task Force
CGIAR	Consultative Group on International Agricultural Research
CIAT	Centro Internacional de Agricultura Tropical
CIP	Centro International de la Papa
DMSO	Dimethyl sulphoxide
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FDA	Fluorescein diacetate
GA_3	Gibberellic acid (isomer 3)
GBRCN	Global Biological Resources Centres Network
GPG2	Global Public Goods Phase 2
IAA	Indole acetic acid
IARC(s)	International Agricultural Research Centre(s)
IBPGR	International Board for Plant Genetic Resources
IFPRI	International Food Policy Research Institute
IITA	International Institute of Tropical Agriculture
INIBAP	International Network for the Improvement of Banana and Plantain
INIFAP	Instituto Nacional de Investigaciones Forestales y Agropecuarias
IPGRI	International Plant Genetic Resources Institute
IPK	Institut Für Pflanzengenetik und Kulturpflanzenforschung, Germany
ISBER	International Society for Biological and Environmental Repositories
ISO	International Standard Organization
ISSR	Inter simple sequence repeats
ITC	(INIBAP-Bioversity) International Transit Centre
ITPGRFA	International Treaty on Plant Genetic Resources for Food and Agriculture
IVAG	*In vitro* active genebank
IVBG	*In vitro* base genebank
IVGB	*In vitro* genebank
KULeuven	Katholieke Universiteit Leuven
LN	Liquid nitrogen
LTS	Long-term storage
MTA	Material transfer agreement (Standard-MTA)
MTS	Medium-term storage
NBPGR	National Bureau for Plant Genetic Resources (India)
NARS	National Agricultural Research System

OECD	Organization for Economic Co-operation and Development
PCR	Polymerase chain reaction
PVS	Plant vitrification solution
PVS2	Plant vitrification solution number 2
PVS3	Plant vitrification solution number 3
RAPD	Randomly amplified polymorphic DNA
RAF	Randomly amplified polymorphic DNA fingerprinting
RFLP	Restriction fragment length polymorphism
SCV	Somaclonal variation
SGRP	Systemwide Genetic Resources Programme
SINGER	Systemwide Information Network for Genetic Resources
SSR	Simple sequence repeats (microsatellites)
Tg	Glass transition temperature
TPS	True potato seed
USDA	United States Department of Agriculture
VNTR	Variable number tandem repeat

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 system", Phase 2 (GPG2) aimed at enhancing the security and stewardship of the genetic resources held in trust in CGIAR genebanks. This project is the central topic of this publication which presents the outcomes, lessons learned, and points out key challenges involved in furthering the GPG2 activity "Refinement and standardization of storage procedures for clonal crops", sub-activity "Review of *in vitro* protocols applied to clonal crops".

The GPG2 Project (2007-09) successfully promoted collective actions for the conservation of clonal genetic resources, specifically to increase their security, to use best practices across genebank processes needing validation, third party accreditation and risk management. In this context, the evolving role for germplasm curators was envisioned to satisfy stakeholders' demands in meeting high standards in storage procedures (including *in vitro*

slow growth and cryopreservation), to provide access to taxonomic and trait-related information, to develop modern genebank inventory systems for storage and delivery of accession data, and to develop high throughput screening techniques for new traits (such as abiotic stress, micronutrient and health-related phytochemical content).

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

To successfully move ahead in implementing the GPG2 objectives, key challenges requiring collective attention still need to be tackled. These include: a) establishing practical risk-amelioration strategies for *in vitro* genebanks, especially in disaster-prone areas; b) developing simple, low cost, conservation protocols to expand the *in vitro* genebanks in developing countries; c) linking fundamental and applied research in *in vitro* conservation, for expanding the range of cryoresponse in the germplasm, for increasing the subculture interval of slow growth, and improving the efficiency of disease-indexing techniques at *in vitro* level, and d) developing high throughput screening techniques for relevant new traits, such as abiotic stress, micronutrient and health-related phytochemical content.

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1 .Introduction

The System-wide Genetic Resources Programme (SGRP) of the Consultative Group on International Agricultural Research (CGIAR) unifies the collective efforts of its genebanks. In support of CGIAR's mission, SGRP created the Global Public Goods Project to upgrade the management of its in-trust collections. The Collective Action for the Rehabilitation of Global Public Goods Phase 2 (GPG2) has the overarching objective to enhance the security and stewardship of >650,000 samples of plant genetic resources held in-trust by CGIAR's genebanks. The GPG2 Project and its associated Knowledge base (see http://sgrp.cgiar.org/) were implemented by SGRP to provide a comprehensive, system-wide, work programme and information resource to enable CGIAR's in-trust commitments and facilitate collection management. The Global Public Goods Project Phase 2 is also mandated to build upon the existing competencies of CGIAR's centres, especially by developing new modes of collaboration that maximize the integration and sharing of best practices, standards and risk management. GPG2 Activity 1.2 concerns the "Refinement and standardization of storage procedures for clonal crops" and sub-activity 1.2.1 instructs to "Review *in vitro* protocols applied to clonal crops".

1.1 Aims

An overarching aim of GPG2 Activity 1.2 is to collate information for the collective validation of best practices and to develop multi-crop best practice guidelines. To aid this process, external experts, reviewers and the CGIAR's Clonal Crop Task Force have compiled three outputs:

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

This document comprises Part II, the purpose of which is to provide a status update on the GPG2 Project's mandated crops, with a view to help formulate multi-crop guidelines for CGIAR's clonal crop genebanks (Benson et al. 2011b). The process for formulating Part II involved: (1) a literature review of the wider community of practice conserving the mandated crops and (2) an appraisal of CGIAR's clonal crop *in vitro* genebanks (IVGBs). These are the Centro Internacional de Agricultural Tropical (CIAT), Centro Internacional de la Papa (CIP), International Institute of Tropical Agriculture (IITA) and Bioversity International-International Transit Centre (ITC) for *Musa*. Part II specifically reviews information concerning the Medium-Term Storage (MTS) and Long-Term Storage (LTS) of potato, cassava and *Musa*, from which the knowledge gained will help to conserve other mandated clonal crops. Data has been collected from the returns of a CGIAR clonal crop survey conducted during 2007-2008 and assessed at a GPG2 Workshop, hosted by CIP in November 2007. This document (Part II) collates the survey information (Tables 1-11) and provides a critical point analysis of its results regarding the infrastructure and conservation status of CGIAR's clonal crop genebanks. This approach is compliant with the GPG2 Project milestone for Activity 1.2 which is to: (1) compile and analyse the *in vitro* protocols in use for the medium-term, slow growth and long-term,

cryopreservation of clonal crops and (2) draw on the techniques and experience available for banana, potato and cassava and analyse the lessons learnt. This exercise will help to overcome the storage constraints for sweetpotato, yam and ARTCs and assist the development of multi-crop guidelines which are presented in Part III (Benson et al. 2011b).

1.2 Global importance of conserving CGIAR's clonal crops *in vitro*

The vision statement of Scott et al. (2000) forecasted the value of the global root and tuber food system: "By 2020 roots and tubers will be integrated into emerging markets through the efficient and environmentally sound production of a diversified range of high-quality, competitive products for food, feed and industry. These crops' adaptation to marginal environments, their contribution to household food security, and their great flexibility in mixed farming systems make them an important component of a targeted strategy that seeks to improve the welfare of the rural poor and to link smallholder farmers with the emerging growth markets".

The consortium comprising: CIP, CIAT, the International Food Policy Research Institute (IFPRI), IITA and Bioversity International recommended a systems approach for root and tuber crop production and utilization. The GPG2 Project reinforces this objective by supporting collective action across the CGIAR centres that hold in trust the world's largest collections of cassava, potato, sweetpotato, yam and Andean root and tuber crop (ARTC) species (Hermann and Heller 1997). Crop-specific *ex situ* conservation strategies have also been developed under the auspices of the Global Crop Diversity Trust for potato (Van Soest 2006) and sweetpotato (Roca 2007); similarly, a review was commissioned on the *ex situ* conservation of banana and plantain (Lusty et al. 2006). Banana and plantain crops are mainly grown by small-scale farmers in developing countries and they are one of the world's most important food staples for more than 400 million people (Panis and Thinh 2001). *Musa* cultivars are usually seedless making conservation *in vitro* the only long-term option for their germplasm security.

2. General status of mandate clonal crop *in vitro* conservation

Considerable progress has been made in the development of *in vitro* conservation methods for clonal crops (Ashmore 1997; Engelmann 2004; Engelmann and Takagi 2000; Engelmann et al. 2008; Reed 2008a, b; Sakai et al. 2008; Volk and Walters 2003). This section overviews the general status (i.e. across the non-CGIAR sector) of *in vitro* conservation for the mandated clonal crops; their conservation in CGIAR's genebanks will be reviewed in Section 3 using the survey returns of the GPG2 clonal crop task force as the primary information source. This section starts with a case study of potato, a crop with a long history of *in vitro* conservation (Bajaj 1987; Benson 2004; Espinoza et al. 1986, 1992; Gonzalez-Arnao et al. 2008).

2.1 Potato *in vitro* conservation: a case study

Storage methods for potato germplasm are varied, wild species and some crop relatives can be stored as true (botanical) seeds produced from potato berries (Towill 1982). However, native cultivars and germplasm comprising eight *Solanum* species are usually conserved vegetatively as seed (tuber) potato or *in vitro* as microtubers, or as shoot cultures and meristems in MTS and LTS respectively. A survey performed by van Soest (2006) revealed that 17 genebanks have *in vitro* conservation facilities for cultivated potato germplasm; some accessions of wild species are also included. Medium-term storage is applied across repositories using *in vitro* techniques, although the report conveys that cryopreservation is not a common practice. Current reviews (Keller et al. 2008a; Gonzalez-Arnao et al. 2008) describe the development of cryobanks for potato germplasm in several international genebanks (see *Section 2.1.2*) and their experiences provide a substantial knowledge base for developing generic best practice guidelines for mandate and other clonal crops (Benson et al. 2011b).

2.1.1 Medium-term storage of potato

Balancing the deleterious effects of stresses incurred by growth limiting treatments with the advantageous extension of subculture interval is critical for the MTS of potato which is susceptible to Somaclonal Variation (SCV), ploidy instability and epigenetic change mediated via DNA methylation (Joyce and Cassels 2002; Joyce et al. 2003; Sharma et al. 2007). Optimization of stress treatments used to limit growth and the careful monitoring of potato plants maintained in MTS are thus advisable. Minimal growth storage of potato has been pioneered by testing retardants including, mannitol, abscisic acid, chlorophonium chloride (Phosphon D) and Diaminazide; low temperatures have also been used (Westcott et al. 1977; Westcott 1981a, b).

Cha-um and Kirdmanee (2007) have collated information related to potato minimum growth regimes at various institutes. The Central Potato Research Institute, Pradesh, India, initially undertook MTS studies using treatments of 40 g/l sucrose and 20 g/l mannitol applied with a 16h light/8h dark photoperiod. This treatment extended the subculture interval to 30 months in four potato genotypes, selected from the groups *tuberosum* and *andigena* (Sarkar and Naik 1998a). Protocol refinement included applying alginate-silverthiosulfate to reduce the deleterious effects of ethylene as this stress hormone caused morphological abnormalities in microplants maintained in medium supplemented with sucrose and mannitol (Sarkar et al.

1999, 2002). Sarkar et al. (2005) cautioned that nutritional deficiency was a side effect of growth retarding treatments in osmotically stressed *S. tuberosum* microplants, advising that prolonged MTS produced poor quality microplants due to calcium depletion; this was corrected by supplementing with 5-7 mM calcium salts. This treatment enhanced potato plant health by minimising morphological abnormalities, hyperhydricity and flaccidity. Sarkar et al. (2001) explored the use of ancymidol (α-cyclopropyl-α [4-methoxyphenyl]-5-pyrimidinemethanol) as an alternative growth retardant to mannitol for potato microplants maintained under cold storage at 6°C. Ancymidol has dual efficacy, it is a potent antioxidant and can potentially minimize stress and aberrant phenotype production. Sarkar et al. (2001) also noted ancymidol inhibited growth, probably by impairing gibberellic acid bioactivity; this effect persisted throughout a 16-month subculture interval. Optimizing growth limitation in potato was achieved by combining treatments of 10 μM ancymidol, 60 g/l sucrose at 6°C; hyperhydricity and flaccidity were not observed in ancymidol-treated cultures.

The Instituto Nacional de Investigaciones Forestales y Agropecuarias (INIFAP), Mexico has tested alternative MTS regimes in which low temperature (8°C) storage in the presence of 100 μM acetylsalicylic acid was substituted for mannitol treatments (Lopez-Delgado et al. 1998). Subculturing was prolonged to 6 months, using either mannitol or acetylsalicylic acid; microplants of *S. tuberosum* cultivars cultured in acetylsalicylic acid had a reduced number of phenotypic abnormalities compared to those maintained on mannitol.

Since the early 1950's the clonal potato collection of Groß-Lüsewitz, now the Institute of Plant Genetics and Crop Plant Research (IPK), has held the largest and oldest potato collection of Germany (Keller et al. 1999). The programmes at Braunschweig (West Germany) and Gatersleben (East Germany) were initially independent (Mix-Wagner 1999), now potato is the largest clonal crop collection held at IPK, comprising >2800 accessions of *S. tuberosum* and related wild species. The IPK uses *in vitro* plantlets and microtubers as source material for MTS; plants are grown in the field and passed through a phytosanitary phase to eradicate viruses after which they are initiated *in vitro* (Thieme 1992; reviewed by Keller et al. 2006). Once confirmed virus-free, material enters a slow growth maintenance phase comprising: (1) a warm phase with long days at 20°C for 2-3 months; (2) short day micro-tuber induction at 9°C for 2-4 months and (3) cold storage of microtubers at 4°C for 16-18 months.

Various studies have researched the effects of MTS on the genetic stability of recovered potato plants. Using a slow growth regime of 6% (w/v) mannitol for 6 months, Harding (1991) found two out of the sixteen *S. tuberosum* plants recovered had RFLP changes as revealed by a hybridization probe for ribosomal genes. Harding (1994) observed epigenetic changes in *in vitro* potato plants of *S. tuberosum* cultured under the same conditions. Methylated DNA was detected using the methylation sensitive restriction enzymes Hpa II/Msp I and Eco RII/Bst NI. This study showed methylation to be higher in slow-grown cultures compared to controls, suggesting that epigenetic changes might be induced by stress during MTS. This finding concurs with Joyce and Cassels (2002) who used methylation-sensitive restriction enzymes to assess quality in potato microplants. Thus, for cultures recovered from slow growth, it may be cautionary to confirm if DNA methylation changes are transitory and disappear on return to standard conditions or, if they persist (Harding 1994; Scowcroft 1984). Sarkar et al. (2001) comment that some growth-limiting treatments might have mutagenic effects and thus replaced mannitol with ancymidol; potato microplants conserved in medium containing this additive did not manifest any detectable genetic variability using RAPD analysis of genomic DNA. Sharma et al. (2007) undertook

genetic and phenotypic stability assessments of *S. tuberosum* plants regenerated via a number of routes (somatic embryos, shoots proliferated from axillary buds, microtubers and True Potato Seed [TPS]) and using flow cytometry to assess gross ploidy status they found the plants to be stable. However, a low level of AFLP marker variation was observed in plants generated from somatic embryos and microtubers and significantly only AFLP markers using methylation sensitive restriction enzymes revealed these polymorphisms.

2.1.2 Long-term storage of potato

Cryopreservation for the LTS of potato can be considered in terms of cryogenic and non-cryogenic factors, both are influential to survival. Cryogenic factors concern cryoprotection and low temperature treatments, non-cryogenic factors include genotype variability, physiology, all other associated treatments and technical and operator issues.

2.1.2.1 Non-cryogenic critical point factors: before cryopreservation

These factors are *in vitro* culture, pre- and post-treatments and physiological, genetically predetermined natural adaptations, they do not include cryoprotection and cryogenic treatments *per se*.

Source material

Potato meristems used for cryopreservation are derived from apical and axillary shoots from *in vitro* micropropagated plants and tuber and microtuber sprouts. Bajaj (1985) compared donor material from three *S. tuberosum* cultivars by using two different cryoprotectant regimes (10% DMSO or 5% each of DMSO, glycerol and sucrose) and ultra rapid freezing. The order of survival was apical = axillary shoots >tuber sprouts. Schäfer-Menuhr (1993) advised that apical meristems from 25 different potato cultivars, generally gave higher levels of survival after droplet freezing, as compared to nodal meristems. Manzhulin (1983) and Manzhulin et al. (1983) used heterogeneous tuber sprouts of *S. tuberosum* as source material and discovered that the morphogenetic state of apices influenced survival after controlled rate cooling. This factor was more important than size and shoot regrowth after cryostorage was improved by selecting axillary shoots which had new leaf primordia. For the encapsulation-vitrification of *S. tuberosum* meristems, Hirai and Sakai (1999) used apical buds of *in vitro* plantlets that comprised 3-4 nodes. In this protocol, nodal segments were transferred to basal medium and cultured to induce axillary buds from which meristems were excised for cryopreservation. The number of days (3-7) of nodal preculture had no or little effect on the cryopreservation responses of nodal segments sampled from the 1st to 3rd node from the apical bud.

Halmagyi et al. (2005) used *in vitro* cultures from tuber sprouts for cryopreserving *S. tuberosum* by PVS2-droplet vitrification. In contrast, Towill (1981b) sourced shoot meristems of *S. tuberosum* from glasshouse-grown seedlings derived from TPS. In order to assess their viability, terminal sections of axillary shoots were surface sterilized and incubated for a two-day period on medium containing benzylamino purine (BAP) and indoleacetic acid (IAA), thereafter followed the cryogenic pretreatment and cryoprotection with DMSO. Towill (1981b) adopted a different procedure for cryopreserving cultivars of *S. tuberosum*, by using non-*in vitro* material, thus axillary shoot tips (0.5 to 1.0 mm) containing 2-5 leaf primordia were excised from surface-sterilized glasshouse-grown plants and immediately processed for cryopreservation. Due to contamination problems with glasshouse-sourced explants, Towill (1984) subsequently used micropropagated potato

plants. Villafranca et al. (1998) observed that the physiological age of donor tubers influenced the performance of *in vitro* cultures that were initiated from them.

Bouafia et al. (1996) improved recovery in potato shoot tips cryopreserved by encapsulation-dehydration by using meristems excised from two-week old, *in vitro* nodal cuttings taken from micropropagated plants. Schäfer-Menuhr et al. (1996) obtained donor germplasm from an *in vitro* collection of old potato varieties that had been previously maintained under slow growth conditions and propagated via nodal segment cuttings. For droplet freezing experiments, plants were grown in 12 cm jars with good aeration to ensure the quality of the starting material; only plantlets 10 cm in height were used as shoot tip donors. Using the same cryopreservation protocol, Keller and Dreiling (2003) compared source material used by Schäfer-Menuhr et al. (1996) with apical shoot meristems from 5 cm microtuber plantlets propagated *in vitro* in the absence of hormones. Micro-tuber derived plantlets gave better results than did those grown as shoot cultures for several years; Keller and Dreiling (2003) also found survival and regeneration after cryopreservation was affected by culture vessel size, although the results were genotype dependent.

Bajaj (1987) commented that the ability to withstand freezing is influenced by genotype and that different species, cultivars, and plants grown under various conditions or, in winter and summer can react differently to freezing. This might suggest that potato plants grown in different seasons could yield germplasm that reacts differentially to cryopreservation. Henshaw et al. (1985) suggested a seasonal component may affect variable responses to a basic ultra rapid freezing protocol and surmised that environmental conditions could affect shoot size and water content. Mix-Wagner et al. (2003) found no apparent seasonal affect on the recovery of potato shoot meristems from droplet freezing, albeit, they suggested this factor should be checked more thoroughly. Henshaw et al. (1985) achieved survival rates of ca. 50% for several genotypes, but concluded variability between experiments was unacceptable for a routine procedure, they found the physiological state of the donor to be a critical factor.

Harding et al. (1991) evaluated the effect of culture age on the capacity of *S. tuberosum* shoot meristems to survive and regrow shoots after ultra rapid and controlled rate cooling. Meristems taken from long-term (in culture for 3 years) and short-term (in culture for 6-8 weeks) cultures of the cultivars 'Desiree' and 'Golden Wonder' responded differentially to cryopreservation. Younger cultures performed better with respect to survival and shoot production; in contrast long-term maintenance in culture reduced the capacity to recover after cryogenic storage. Halmagyi et al. (2005) sampled meristems from 1-2 month old *in vitro* potato cultures for the cryopreservation of *S. tuberosum* cultivars using PVS2-droplet vitrification. In contrast, Sarkar and Naik (1998b) applied PVS2 to apical shoot tips from 30-day-old plantlets of *S. tuberosum* that had been maintained by *in vitro* nodal cutting propagation for several years, this study achieved 50% post-cryopreservation shoot regeneration in five cultivars.

Towill (1981a, b, 1983, 1984) used glasshouse-grown potato plants maintained under relatively high light conditions (ca. 2000 μE $m^{-2}s^{-1}$) as sources of meristems for cryopreservation. Consequently, Benson et al. (1989) studied light as a factor in *S. tuberosum* cultivars 'Golden Wonder' and 'Desiree' which responded differently to cryopreservation. 'Desiree' was less tolerant to both controlled rate and ultra rapid cooling and recovery was significantly influenced by pre-light regime, growing plantlets of this cultivar under high light before freezing produced almost three times the level of recovery observed in low pre-

light treated plants. The converse was the case for 'Golden Wonder', thus indicating these genotypes have different pretreatment light requirements for sustaining post-cryopreservation survival (Benson et al. 1989). Keller et al. (2006) comment that the culture facility may be a determinant for successful potato meristem cryopreservation using droplet freezing as variation in performance after cryopreservation was observed between donor plants sourced from different types of culture rooms. These varied with respect to light, aeration, and temperature regime, this finding endorses the need to standardize growth conditions in genebanks. Keller et al. (2006) also found different culture vessels produced different growth habits and that shoot meristems sourced from these might have a negative impact on post-cryopreservation survival, they postulated that changes in vessel aeration may cause ethylene accumulation and change the microclimate of the vessel.

Yoon et al. (2006) investigated the effect of the subculture of mother plants on the recovery of *S. tuberosum* and *S. stenotomum* genotypes following PVS2 droplet vitrification. To ascertain optimum duration, donor mother-plants were subcultured for 3-9 weeks before shoot tip excision. Subculture duration significantly influenced survival in both species, for example, in *S. stenotomum* STN13 survival increased from 15% after a 3-week subculture interval to 71% after a 5-week interval. Yoon et al. (2006) concluded that the subculture of mother plants and the preculture of shoot tips are important determinants in the recovery of potato genotypes after droplet-vitrification. However, Yoon et al. (2006) evaluated short-term recovery at 14 days, recording the number of shoot tips that were green and swollen (≥3mm). Longer-term assessments and confirmation of new shoot regrowth, rather than viability are advised to support definitive assessments of their protocol's promising efficacy.

2.1.2.2 Acclimation and pregrowth treatments

In the context of this review, acclimation and pregrowth treatments are considered as procedures that enhance the overall ability of germplasm to survive after cryopreservation, but do not impart total cryoprotection when used alone. These treatments have been used most effectively to assist the cryopreservation of woody perennial species (Johnston et al. 2009; Reed 1988, 2008a). Acclimation and pregrowth can also involve cold treatment cycles or cold-simulated acclimation (i.e. pregrowth) in the presence of osmotica (sorbitol, mannitol, sucrose). Some treatments combine the pregrowth of excised, cold-acclimated meristems with exposure to lower concentrations of colligative cryoprotectants such as DMSO. For example, Reed (1988) grew *Rubus* meristems excised from cold-acclimated shoot cultures on 5% DMSO, and then returned the cultures to cold acclimation conditions for 2 days before cryopreservation.

Steponkus et al. (1992) define the operational process of 'loading' in vitrification procedures, this is necessary to increase the solute concentration of the cell and it involves the application of permeating cryoprotectants (DMSO, ethylene glycol and glycerol) although permeability may vary between species and cell types. Steponkus et al. (1992) comment that for some plants, shoot meristems are precultured on medium containing low concentrations of penetrating cryoprotectants for several days before vitrification; in this case the process may be considered a pregrowth treatment. This is in contrast to other crops, for which loading does not involve preculture, but rather, a short-term exposure to loading additives over hours, as is the case for *Musa* (Panis 2008, 2009; Panis and Thinh 2001). After preloading germplasm, vitrification is usually achieved after exposure to dehydrating, osmotically active cryoprotectants (Steponkus et al. 1992). Pretreatments and acclimation can also involve the addition of anti-stress agents and hormones (proline, abscisic acid, DMSO,

antioxidants). Some of these may simulate natural cold acclimation processes or, they may have a dual function such as DMSO, which can act as a colligative cryoprotectant as well as an antioxidant (Benson 2008a).

Clearly, the basis for acclimation and pregrowth is complicated by the use of the different terminologies that collectively describe acclimation, pregrowth and pretreatments. For practical purposes there now exists a wider range of options which provide considerable scope for using cold acclimation, simulated acclimation and pregrowth additives to enhance tolerance to further levels of cryoprotection and improve overall cryopreservation outcomes. Thus, Kaczmarczyk et al. (2008) enhanced post-cryopreservation recovery of *S. tuberosum* meristems by using an alternating lower temperature (22/8°C day/night) for a one-week preculture period.

Pretreatment of alginate encapsulated potato shoot meristems with 0.75 M sucrose before evaporative desiccation and cryopreservation by ultra rapid cooling was found to be a critical factor for survival (Benson et al. 1996; Bouafia et al. 1996; Fabre and Dereuddre 1990). Grospietsch et al. (1999) noted high survival (ca. 79%) of cryopreserved, encapsulated 'Desiree' shoot tips required pretreatment of the donor plants with 2 M sucrose for 5 days, followed by a 0.7 M sucrose preculture of the excised shoot tips. They concluded that this procedure simulated drought hardening and that it could replace cold acclimation regimes for plants sensitive to low temperatures. Before encapsulation-vitrification, Hirai and Sakai (1999) cold hardened *S. tuberosum* 'Danshakuimo', for 3 weeks using a 12 h light/8 h dark photoperiod at 20 μmol m^{-2} s^{-1} at 4°C. Halmagyi et al. (2005) increased the tolerance of *S. tuberosum* shoot tips to cryopreservation by using PVS2-droplet vitrification combined with sucrose pretreatment.

Removal of plant growth regulators from source material one week before cryopreservation improved shoot production and reduced callusing in meristems of five potato cultivars following their cryopreservation by encapsulation-dehydration (Bouafia et al. 1996). Conversely, Schäfer-Menuhr et al. (1996) incubated shoot tips before cryopreservation, in a medium containing zeatin riboside, GA_3 and IAA (based on Towill, 1983). Sarkar and Naik (1998b) similarly applied 8.7 μM of GA_3 to excised shoot tips of five cultivars of *S. tuberosum* before they were cryopreserved using PVS2. This treatment was administered concomitantly with sucrose or mannitol, for 2 days using a 16 light/8 h dark photoperiod; combining pregrowth treatment with mannitol and sucrose enhanced survival and shoot regeneration.

Shoot tip meristem dissection

Schäfer-Menuhr et al. (1996) and Mix-Wagner et al. (2003) categorized large and small meristems, dependent upon genotype, across the size range of ca. 0.5 to 3 mm in length and ca. 0.1 to 0.5 mm diameter. Hirai and Sakai (1999) selected axillary meristems of *S. tuberosum* nodal segments for dissection and excised 1 mm apices comprising five leaf primordia. Manipulations immediately before and following potato shoot tip dissection have been found to improve meristem survival after cryopreservation (Bajaj 1985; Benson et al. 2007; Henshaw et al. 1985; Towill 1981a, b). Treatments include: (1) excision of nodal segments from *in vitro* shoots; (2) maintenance in culture medium for 5-7 days [to allow meristem development following their release from apical dominance]; (3) shoot meristem excision and capture on filter papers soaked with liquid culture medium to avoid desiccation and (4) pregrowth in 2-5% (v/v) DMSO for 1-2 days before cryopreservation.

Halmagyi et al. (2005) excised shoot tips with 2-4 leaf primordia from *in vitro S. tuberosum* cultivars and tested the effects of shoot length (1-2 mm, 3-4 mm, 5-6 mm) and position of apices (numbered from the apical to basal meristem) on recovery after PVS2-droplet vitrification. A linear progression in meristem survival demonstrated that apical shoots were better able to survive, compared to those from lower ranking apices, thus, position of meristems on donor stems affected survival after dehydration and cryopreservation. Highest survival occurred in apical meristems from 3-4 mm long apices and significantly lower survival was observed for shoot tips of 5-6 mm.

Towill (1981b) found a post-dissection recovery treatment with DMSO yielded higher survival in *S. tuberosum*, compared to cryopreserving freshly excised shoot meristems. This procedure was also adopted by Grout and Henshaw (1978) and Benson et al. (1989) who used meristems comprising the apical dome with 2-4 leaf primordial. Henshaw et al. (1985) enhanced survival in *S. goniocalyx* shoot meristems from zero to 50%, and in *S. tuberosum* ssp. *andigena* from 0 to ca. 30%, by using respectively, a 24-48 h and 72 h post-dissection treatment with DMSO before ultra rapid freezing. Henshaw et al. (1985) concluded that allowing excised shoot meristems 1-3 days to recover from dissection trauma in a DMSO solution was a definitive requirement. This is corroborated by the testing of variable cryogenic parameters (cooling, and terminal temperature to LN transfers) across different potato genotypes (Benson et al. 1989; Henshaw et al. 1985; Manzhulin 1983; Towill 1981a, b). Manzhulin et al. (1983) studied the effects of source material on the survival of potato meristems after controlled rate cooling, and found necrosis and wounding after dissection to be important factors in recovery. As an enhancer of membrane permeability (Williams and Barry 2004) DMSO pretreatment might be expected to improve colligative protection at a later stage of the protocol, this may be significant for shoot tips comprising different cell types, with variable water contents and vacuole sizes. As DMSO is a potent antioxidant, it will also help to alleviate dissection stress caused by physical injury before cryopreservation (Johnston et al. 2007). Similarly, DMSO is also highly bioactive (Hahne and Hoffman 1984; Nilsson 1980) and when used as a pretreatment for meristems it may confer developmental and metabolic advantages during post-storage shoot regrowth. However, in contrast, Fabre and Dereuddre (1990) found DMSO pretreatments to cause abnormalities and tissue necrosis in *S. phureja* shoot tips. Hirai and Sakai (1999) pretreated excised meristems from *S. tuberosum* for 16 h on medium containing: 0.3 M sucrose, 1 mg/L GA_3, 0.01 mg/L 6-BAP and 0.001 mg/L NAA at 23°C before cryopreservation using encapsulation-vitrification. Similarly, Halmagyi et al. (2005) applied a 24 h hormone pretreatment of 0.4 mg/L GA_3, 0.5 mg/L zeatin and 0.2 mg/L IAA at 23°C to excised shoot tips before cryopreserving *S. tuberosum* cultivars using PVS2-droplet vitrification.

2.1.2.3 Cryogenic critical point factors and performance indicators

Two performance indicators are used to assess recovery after cryostorage, meristem survival (viability) and shoot regrowth which is the preferred indicator of a successful outcome. Loss of totipotency, failure to develop shoots and delayed death of survivors can occur at later stages of recovery (2-8 weeks). These responses are particularly evident in potato and seemingly they are protocol independent (Benson et al. 1989; Harding et al. 2008, 2009; Keller and Dreiling, 2003; Schäfer-Menuhr et al. 1996, 1997, 1998).

Ultra rapid freezing

Bajaj (1987, 1985) collated literature on the early development of potato cryopreservation using ultra rapid freezing, a process that involves the direct immersion of cryoprotected shoot meristems in LN. Various containment and delivery procedures have been devised to introduce samples into liquid phase LN, ranging from enclosure in cryovials to direct exposure of non-contained, cryoprotected shoot meristems (Henshaw et al. 1985). Bajaj (1977) reported the first survival (ca. 26%) of potato meristems following ultra rapid freezing, this was achieved by using 5% each of DMSO, glycerol and sucrose. Grout and Henshaw (1978) obtained ca. 20% survival in *S. goniocalyx* shoot meristems, by cryoprotecting in 10% DMSO followed by direct immersion on hypodermic needles into LN. As reviewed by Henshaw et al. (1985), ultra rapid freezing of potato caused irreparable damage to the original meristem as revealed by transmission and scanning electron microscopy. Maintenance of structural integrity was a critical factor for ensuring normal shoot regeneration as regrowth via a dedifferentiated callus state was undesirable for reasons of instability. Benson et al. (1989) and Harding et al. (1991) compared ultra rapid freezing with controlled rate cooling in *S. tuberosum* cultivars, applying protocols respectively developed by Grout and Henshaw (1978) and Towill (1981a, b, 1983) and using DMSO as the cryoprotectant. Ultra rapid freezing consistently supported higher shoot regeneration at ca. 20% for the more tolerant 'Golden Wonder', compared to freeze-sensitive, 'Desiree'. In contrast, the same ultra rapid freezing method (Benson et al. 1989) applied to four *S. tuberosum* dihaploids, *S. microdontum* and *S. pinnatisectum* supported survival and shoot regeneration to a maximum of 48% in *S. microdontum* (Ward et al. 1993).

An ultra rapid freezing method for potato shoot meristems was developed by Schäfer-Menuhr et al. (1996, 1997, 1998) and Mix-Wagner et al. (2003) based on the protocol that Kartha et al. (1982) first developed for cassava. The procedure, now termed droplet freezing (Schäfer-Menuhr et al. 1996) involves 2 h cryoprotection in 10% DMSO, followed by dispensing 2.5 µl droplets of DMSO onto aluminium foils to which the shoot tips are transferred. Subsequently, the foils are placed in cryovials filled with LN, the lid is loosely closed and the cryovial is plunged into a LN storage container. Two hundred and nineteen varieties of potato were originally cryopreserved by this method and the latest reported status is 1,017 cryobanked varieties at IPK (Joachim Keller, personal communication; Keller et al. 2008a). Most potato genotypes produced high levels of survival, averaging 80% and plant regeneration ca. 40% (Schäfer-Menuhr et al. 1996). Barandalla et al. (2003) applied the ultra rapid freezing method to ten potato cultivars, survival was 50% and in one case 100%; shoot regeneration was 2.5 to 22%, dependent upon cultivar and the growth regulator composition of the recovery medium.

Colligative cryoprotection and controlled rate cooling

Towill (1981a) applied a two-step controlled rate cooling method for shoot meristems derived from TPS-seedlings of *S. etuberosum*. The protocol used 10% DMSO as the colligative (penetrating) cryoprotectant and a cooling rate of 0.3°C/min to -40°C, (with a manual seeding step at -5°C) followed by immersion in LN. Control shoots regenerated with a consistent pattern of recovery, leaf expansion occurred at 2-4 days and shoot regrowth to 1-2 cm following further culture. Control recovery was consistently high at ca. 96% over an 8-month test period and shoot meristems exposed to LN gave good survival rates (40 to 75%) under optimized cooling conditions; although recovery via multiple shoot masses risked SCV

generated via adventitious development (Scowcroft 1984). Henshaw et al. (1985) endorsed the two-step controlled rate cooling method for potato as developed by Towill (1981a) in preference to the ultra rapid freezing protocol, on the basis that it combined the advantages of slow cooling with protective freeze-dehydration and colligative cryoprotection. Towill (1981b) suggested that the intermediate transfer temperature during controlled cooling was a determinant of both survival and morphogenetic response in *S. tuberosum* shoot meristems. Higher levels of shoot regeneration occurred to -20°C, but at lower transfer temperatures both survival and recovery were compromised; callus proliferation implied structural integrity was damaged in *S. tuberosum* meristems exposed to controlled rate cooling. This finding contrasts with the successful application of the same method to *S. etuberosum* (Towill 1981a) however, improved survival (29-75%) in *S. tuberosum* cultivars was achieved using controlled rate cooling and DMSO cryoprotection optimized for terminal transfer temperature (Towill 1983) although only a few survivors formed shoots. Towill (1984) applied the two-step cooling protocol using DMSO as the cryoprotectant to shoot meristems of *in vitro* plants from representatives of *S. andigena, S. phureja, S. stenotomum* and *S. tuberosum*. High levels of survival (up to 100%) were achieved for many genotypes but shoot regeneration was highly variable and associated with callus formation. Benson et al. (1989) and Harding et al. (1991) compared ultra rapid freezing with controlled rate cooling using the methods of Grout and Henshaw (1978) and Towill (1981b, 1983, 1984) as applied to *S. tuberosum* cultivars 'Desiree' and 'Golden Wonder', consistently, finding that controlled cooling compromised survival and shoot regeneration. Although some minor improvements could be made to the protocol the problems of delayed development and lack of shoot regeneration persisted.

Encapsulation-dehydration

Gonzalez-Arnao et al. (2008) have reviewed the status of encapsulation-dehydration as applied to crop plant germplasm. Fabre and Dereuddre (1990) first developed the alginate encapsulation-dehydration method for shoot meristems derived from *in vitro* plants of *S. phureja* for which controlled rate cooling and ultra rapid freezing were considered as approaches to cryopreserve the encapsulated potato apices. Following preculture in 0.75 M sucrose for one day, about 20% survival was achieved after ultra rapid cooling, although the shoots did not regenerate. Controlled rate, two-step cooling supported improved survival (ca. 41%) and some direct (<10%) shoot regeneration. By optimizing treatments, Fabre and Dereuddre (1990) achieved 40% direct shoot regrowth in *S. phureja* using ultra rapid cooling and critical factors were identified as preculture in sucrose and evaporative bead desiccation. Applying the same method to shoot meristems of *S. phureja*, *S. tuberosum*, *S. brachycarpum*, *S. acaule, S. guerreroense* and *S. iopetalum*, Benson et al. (1996) demonstrated that all genotypes were capable of surviving within the range of 9-73%; recovery progressed by direct shoot regeneration (4-73%) without callus or adventitious development. Variable responses between individual experiments were observed, ranging from 0-100% survival and these were attributed to the physiological status of donor material. Harding and Benson (2000, 2001) applied encapsulation-dehydration to *S. tuberosum* cultivars 'Brodick' and 'Golden Wonder' and were able to produce plantlets from cryopreserved meristems within 1-2 subculture cycles with a maximum shoot regrowth of 40-60%.

Bouafia et al. (1996) desiccated encapsulated shoot meristems of potato over silica gel before plunging into LN and identified several critical factors: using shoot tips excised from precultured nodal segments, duration of preculture, sucrose concentration and bead water content. Following optimization, shoot regrowth was ca. 60% and in some cases, higher

shoot recoveries of 70-90% were achieved across diverse genotypes comprising three hybrid clones of *S. phureja* and two cultivars of *S. tuberosum*. Grospietsch et al. (1999) focused on reducing osmotic stress in the encapsulation-dehydration protocol and achieved highest survival (ca. 79%) of cryopreserved, encapsulated 'Desiree' shoot tips using a 5-day, pretreatment with 2 M sucrose applied to donor plants. Regeneration was ca. 59%, indicating that this refinement improved the conversion of surviving meristems to shoots.

Vitrification

Sarkar and Naik (1998b) applied the PVS2 protocol to excised shoot tips of five cultivars of *S. tuberosum* and tested various sequences of cryoprotectant loading by either direct exposure or gradual addition; incorporating mannitol in the preculture medium aided survival. The optimal cryoprotection strategy involved: (1) preculture on medium containing 0.2 M and 0.3 M sucrose; (2) loading with 20% PVS2 for 30 min; (3) loading with 60% PVS2 for 15 min and (4) loading with 100% PVS2; cryoprotection was undertaken in an ice bath at 0°C, shoot tips were placed in 1 ml cryotubes and plunged directly into LN. Cryotubes were rewarmed in a water bath at 35°C for 1 min, PVS2 was then removed and the shoot tips dispensed into 1.2 M sucrose unloading solution followed by transfer to hormone-supplemented recovery medium. Survival at 4 weeks amounted to 54% and an almost total conversion (50%) of the survivors to regenerating shoots. Sarkar and Naik (1998b) cautioned against retaining recovering shoots for extended periods on medium containing osmotica as abnormal shoot development and callusing was induced. Zhao et al. (2005) developed a modified PVS2 protocol for *S. tuberosum*, using the ice-blocking agent, Supercool X1000 which is a partially hydrolyzed polymer of polyvinyl alcohol; it is considered to act like an antifreeze protein. Two cultivars ('Superior' and 'Atlantic') were cold acclimated, and their axillary buds precultured and cryoprotected with PVS2 to which Supercool X1000 was added. Antifreeze treatments improved survival to 55-70%, after cryopreservation and vitrified shoots resumed growth in a week.

Hirai and Sakai (1999) applied encapsulation-vitrification to *in vitro*-grown meristems of *S. tuberosum;* their protocol combined various stages of pretreatment in sucrose-supplemented medium to enhance dehydration tolerance. This step was followed by treatment of alginate-encapsulated shoot tips with a mixture of 2 M glycerol and 0.6 M sucrose for 90 min. Meristems were exposed to PVS2 solution for 3 h at 0°C, after which they were transferred to 1.8 ml cryotubes containing 1 ml of chilled, fresh PVS2 solution and plunged directly into LN. Hirai and Sakai (1999) found vitrified meristems recovered without callus formation within 3 weeks and produced about 70% shoot regrowth. The combination of vitrification and encapsulation produced higher levels of shoot development than did encapsulation-dehydration alone. Hirai and Sakai (1999) thus recommended encapsulation-vitrification as a useful method for cryopreserving potato germplasm, on the basis that it is easy to handle, allows large numbers of meristems to be processed and recovery is rapid. Furthermore, conversion of surviving apices to shoots is higher, although this is dependent upon optimizing the preculture and acclimation stages of the protocol. Hirai and Sakai (2000) reiterated the efficacy of their potato encapsulation-vitrification protocol, cautioning critical factors as osmotic pretreatment to ensure apices withstand PVS2 and gradual cryoprotectant loading to avoid osmotic stress.

Halmagyi et al. (2005) applied droplet-vitrification (see Panis et al. 2005) to three cultivars of *S. tuberosum* using PVS2 and direct exposure to LN after which regrowth of apices ranged from 46-55%. Following preculture for 24 h in sucrose, shoot tips were cryoprotected in 4 μl droplets of PVS2 placed on aluminium foil strips (0.6 cm x 1.5 cm) for 10-30 min at

ambient temperatures, after which the foils were transferred to precooled cryovials and directly immersed in LN. In this system, higher recovery and regeneration were obtained using sucrose as the pregrowth additive, which was considered a critical factor for survival. Kim et al. (2006) identified other critical factors in the recovery of wild and cultivated potato genotypes following PVS2-droplet vitrification as, duration of exposure to PVS2 and unloading in sucrose solution on rewarming; their optimized protocol supported short-term survival (64-94%) in 12 accessions. As surviving meristems often manifest delayed recovery, a lack of conversion to shoots and delayed-onset death it is cautionary to monitor recovery over an extended time course of about 6-8 weeks (Harding et al. 2008, 2009).

Thawing and rewarming

Thawing after cryopreservation using ultra rapid freezing and controlled rate cooling is usually performed at 35°C to 40°C in a water bath (Bajaj 1985). Encapsulated-dehydrated shoot meristems can be rewarmed at ambient temperatures (Benson et al. 2007). After encapsulation-vitrification, Hirai and Sakai (1999) rewarmed germplasm in cryovials in a water bath at 38°C. Recovery of potato shoot meristems cryopreserved using droplet-vitrification and droplet freezing entails their direct immersion in liquid recovery medium or unloading solution (Panis et al. 2005; Benson et al. 2007).

2.1.2.4 Non-cryogenic critical point factors: after cryopreservation

This section concerns the effects of non-cryogenic factors that are applied after cryopreservation (e.g. during recovery). After their retrieval from cryogenic storage, cryopreserved potato shoot meristems often enter a lag phase before visible signs of viability and regrowth are manifest (Harding et al. 2009). Initial survival is usually observed as greening, leaf expansion and a swelling of the apical dome, but some survivors may not produce shoots and their development is limited to leaf expansion. Although green and viable, these surviving meristems can be incapable of producing plants, in other cases survivors perish after an initial recovery phase which can extend to several weeks of advancing necrosis before death (Bajaj 1985; Benson et al. 1989; Harding et al. 2008, 2009). Recovery assessments should thus be undertaken over 4-6 weeks, or to the point at which definitive, sustained shoot regrowth is observed, this is imperative before any conclusions are made as to the success of a potato cryopreservation protocol.

Recovery and recovery media

Bajaj (1977) observed survival differences in potato shoot meristems that had been cryopreserved using various combinations of glycerol, DMSO and sucrose and freezing by ultra rapid and slow immersion in LN. Meristems recovered on filter paper wicks soaked in liquid medium responded more vigorously than those on standard semi-solid agar medium. Towill (1981a) reported recovery in *S. etuberosum* shoot tips on medium containing BAP and IAA after they had been cryopreserved using DMSO and controlled rate cooling. In contrast, recovery of *S. tuberosum* shoot meristems on medium containing 0.5 mg/L IAA, 0.2 mg/L GA_3 and 0.4 mg/L kinetin produced variable recovery and callus rather than shoots. Substituting, zeatin for kinetin improved shoot regeneration efficiency in *S. tuberosum* cryopreserved by controlled rate cooling and colligative cryoprotection using DMSO (Towill, 1983). This infers that zeatin is critical for initiating morphogenesis in potato shoot meristems cryopreserved using this controlled rate cooling protocol, for which up to 100% recovery was achieved. However, Towill (1983) also cautioned that a single recovery medium may not be suitable across all genotypes because of variable shoot regeneration. Manzhulin et al. (1983) observed

regeneration of shoots from *S. tuberosum* cryopreserved using an adapted controlled cooling method and medium formulation similar to that used by Towill (1983), although only limited numbers of normal regenerants (14% total shoot regrowth) were recovered via non-adventitious routes.

Henshaw et al. (1985) screened various recovery media comprising different combinations of auxins (naphthalene acetic acid, [NAA] cytokinins (N^6 benzyl adenine [BA], N^6 (2 isopentyl) adenine, [2iP], zeatin and gibberellic acid [GA_3 isomer]). Some combinations doubled survival, but they predisposed the recovering meristems to callusing; survival was observed on hormone free medium but this supported very limited recovery after ultra rapid freezing. Bouafia et al. (1996) used a phased recovery strategy for encapsulated-dehydrated shoot meristems of five potato cultivars. This involved initial recovery on medium containing BA and NAA and after one week, transfer to medium containing only GA_3. Sarkar and Naik (1998b) similarly applied a phased approach for recovering cryopreserved shoot tips of five cultivars of *S. tuberosum* cryoprotected with PVS2. This involved initial recovery for one week on medium containing 5.8 μM GA_3 and 1.0 μM BA, after which the shoot tips were transferred to medium containing 2.9 μM GA_3. The procedure also involved the gradual reduction in sucrose from 0.2 M to 0.09 M, resulting in 54% survival and a 50% conversion of survivors to shoots. Hirai and Sakai (1999) similarly used biphasic recovery on different levels of hormones for *S. tuberosum* shoot tips recovered after encapsulation-vitrification. This involved recovery for one day on medium containing 1 mg/L GA_3, 0.01 mg/L 6-BAP and 0.001 mg/L NAA, followed by transfer to medium containing 0.0005 mg/L GA_3.

Composition of recovery medium used for cryopreserved potato shoot meristems can have long-term effects on both the recovery and development of plants regenerated from cryobanks. Harding (1996, 1997), Harding and Benson (1994) and Harding and Staines (2001) demonstrated significant variability in plant height, time to maturation, mode of recovery and timelines of development in plants regenerated from shoot meristems of *S. tuberosum* 'Golden Wonder' and 'Desiree'. Their shoot meristems were recovered on different media following cryopreservation using ultra rapid freezing and 10% DMSO as the cryoprotectant. Plant growth regulator composition of the initial recovery medium can thus affect the long-term development of plants retrieved from cryobanks (Harding and Benson 1994; Harding et al. 2008, 2009).

The light regime applied during the initial phase of recovery may reduce photooxidation in germplasm exposed to cryogenic temperatures. Grout and Henshaw (1978) recovered *S. goniocalyx* shoot meristems (cryopreserved by ultra rapid freezing) in low light (500 lux) for 5 days before transferring to standard illumination at 4000 lux. Noting that without light quality specifications it is not possible to convert this retrospective measurement to a contemporary unit of photon flux density. Sarkar and Naik (1998b) similarly applied a phased, one-week recovery in low light (6 μmol m^{-2} s^{-1}) for *S. tuberosum* shoot tips cryo-preserved using PVS2. After which they were transferred to standard light (40 μmol m^{-2} s^{-1}) which supported ca. 50% survival and shoot regeneration. Benson et al. (1989) found that post-recovery light regimes differentially influenced recovery in two genotypes of *S. tuberosum* that had been cryopreserved using ultra rapid freezing and controlled rate cooling. In 'Golden Wonder', highest recovery was achieved when ultra rapid freezing was accompanied by a relatively increased level of post-freeze light (45 μmol m^{-2} s^{-1}) as compared to low levels of survival when controlled rate freezing was combined with recovery under

minimal light conditions (15 μmol m^{-2} s^{-1}). In 'Desiree' there was no interaction between light and mode of freezing with respect to recovery after cryopreservation.

Schäfer-Menuhr et al. (1996, 1997) recovered potato shoot meristems after droplet freezing in agarose droplets to which 1 to 1.5 ml of liquid culture medium was added, survival and recovery was assessed 4 weeks after rewarming. Recovery of potato shoot meristems treated with vitrification solutions requires their gradual removal or dilution to avoid osmotic damage. Following encapsulation-vitrification, Hirai and Sakai (1999) drained PVS2 from the cryovials and replaced the cryoprotectant with a 1.2 M sucrose unloading solution using 2 washes with an incubation of 10 min.

2.1.2.5 Genotype factors

Bajaj (1985) reviewed the effect of genotype on recovery after cryopreservation, noting across various studies that different species of *S. tuberosum* (Bajaj 1977; Towill 1983), *S. goniocalyx* (Grout and Henshaw 1978), *S. etuberosum* (Towill 1981a) and *S. phureja* (Fabre and Dereuddre 1990) all responded differently. Within *S. tuberosum*, cultivar differences can be very significant as shown for 'Golden Wonder' and 'Desiree' (Benson et al. 1989), interestingly, for some protocols 'Desiree' has proven to be a cryopreservation sensitive cultivar. Grospietsch et al. (1999) overcame this propensity by inducing/simulating drought-hardening tolerance in 'Desiree' by pretreating donor plants with 2 M sucrose for 5 days, this resulted in higher levels of survival of ca. 79% and shoot regeneration of ca. 59%. The ultra rapid freezing method first developed by Grout and Henshaw (1978) was applied by Ward et al. (1993) to four *S. tuberosum* dihaploids and the wild species *S. microdontum* and *S. pinnatisectum.* Shoot survival and regeneration occurred in all genotypes, however, maximum and minimum levels of viability varied from 16% to 76% and shoot regeneration from ca. 5 to 48%. Benson et al. (1996) observed variable levels of survival and regeneration in shoot meristems from *in vitro* plantlets of potato species with different ploidy levels, including *S. phureja*, *S. tuberosum*, *S. brachycarpum, S. acaule, S. guerreroense* and *S. iopetalum;* all survived and produced shoots and plants after cryogenic treatment using encapsulation-dehydration. Bouafia et al. (1996) found resistance of encapsulated shoot tips to osmotic dehydration was significantly different across genotypes of three dihaploids of *S. phureja* and two tetraploid clones of *S. tuberosum*. Dihaploid clones were more tolerant of LN than tetraploids although survival differences between clones could be moderated by optimizing the dehydration step of the protocol. Schäfer-Menuhr et al. (1996, 1997) reported the first large-scale cryopreservation of potato by using droplet freezing to cryoconserve 219 genotypes. After long-term storage, average survival was 80%, average shoot regeneration was 40% and overall genotype dependency ranged from 5% to 100%. Hirai and Sakai (1999) applied encapsulation-vitrification to 14 cultivars of *S. tuberosum*, consistently finding >50% shoot regeneration.

2.1.2.6 Operator and technical expertise

The long-term study of Schäfer-Menuhr et al. (1996, 1997) concerning potato cryopreservation by droplet freezing offers a unique perspective for exploring the influence of technical expertise on routine cryobanking performance. This was revealed by Mix-Wagner et al. (2003) in which a random sample of 51 potato varieties were assessed for survival and regeneration after short-term storage and then following storage after several years. Their assessment was possible because data were derived from a cumulative study performed from 1992 to 1999 and during which time technical skills improved through experience. An apparent detrimental effect was observed for short-term storage tests of small

apices, but this was not the case for shoot meristems held in cryostorage for longer periods. Mix-Wagner et al. (2003) concluded that the effect was unlikely to be caused by time in storage, but rather it was due to an enhanced skill in handling smaller sized apices, which would be less competent in the earlier stages of the project. This study highlights the importance of ensuring operator competency, especially aptitude in shoot meristem excision.

2.1.2.7 Large-scale cryopreservation of potato

The first example of the systematic, large-scale cryopreservation of potato germplasm was achieved using the droplet freezing method adapted by Schäfer-Menuhr et al. (1996, 1997). The DSMZ and the Institute of Crop and Grassland Science of the Federal Agricultural Research Centre designed the protocol for routine use in the Braunschweig genebanks (Mix-Wagner et al. 2003). The work was first undertaken within the framework of an IPGRI project entitled 'Refinement of Cryopreservation Techniques for Potato'. To facilitate cryobanking, a user-friendly storage and documentation system was later developed at IPK in order to permit easy retrieval of germplasm from cryobanks after prolonged storage and staff changes. In total, 219 potato varieties and genotypes were cryopreserved (Schäfer-Menuhr et al. 1996, 1997; Mix-Wagner et al. 2003). Each was allocated to ca. 30 cryovials (equivalent to 300-400 shoot tips) using three independent freezing experiments, for every batch frozen, 12 shoot tips were withdrawn to check viability which was assessed in groups for 200 varieties constructed at 10% increments (minimum of 0-10%, maximum 90-100%). The majority that survived was in the 90-100% range and plant regeneration was similarly assessed, although conversion of survivors to plants was lower. Most genotype recovery was in the 20-30% range for plant regeneration with the mean conversion of cryopreserved shoot tips to plants being around 40%.

Keller and Dreiling (2003) report the creation of a large collection of cryopreserved potato germplasm at IPK and their study provides useful experience of technology transfer for routine, scaled-up cryobanking procedures using droplet freezing. These methods were first developed at DSMZ by Schäfer-Menuhr et al. (1996, 1997, 1998) and transferred thereafter from institutes in Braunschweig, to IPK in Gatersleben. Progress has continued as reviewed by Keller et al. (2005, 2006) and within the EU Cost Action project 'CRYOPLANET', coordinated by KULeuven (at: http://www.agr.kuleuven.ac.be/dtp/tro/cost871/Home.htm).

Keller (2007) summarises the status of large-scale genebanking of potato germplasm held by IPK and report a mean regeneration of 45% for 1004 accessions stored by droplet freezing. Keller et al. (2006, 2008a) updated on potato cryobanking status at IPK, and reported that 33.2% of the potato collection is now held in cryostorage using droplet freezing. Keller et al. (2008a) also note that 550 potato accessions from Braunschweig were merged with 391 from IPK in 2002. This provided a good opportunity to compare the convergence of cryopreserved collections by undertaking a re-testing of plant regeneration for all accessions. This unique study identified that various critical factors affected cryogenic storage, including, technical experience, accession duplication, and primary source material (tubers in Braunschweig; microtubers from slow growth cycles in IPK). Keller et al. (2008a) found that the mean survival between accessions was similar between initial and second tests performed up to 10 years later and where differences were observed, they were attributed to non-cryogenic causes. Barandalla et al. (2003) report on the cryobanking of potato genetic resources in Spain and Zámečník et al (2007) have cryopreserved 35 potato accessions in the Czech Republic using the vitrification protocol developed by Steponkus et al. (1990). This was applied with

ultra rapid freezing facilitated by direct exposure of cryoprotected shoot tips on aluminium foils to LN; mean survival of 23% was achieved.

2.1.2.8 Logistics, costs and efficiency of large-scale potato cryobanks

Schäfer-Menuhr et al. (1998) methodically formulated the logistics of cryobanking large accessions within a working week; the time required to conduct one freezing experiment, of 100-150 shoot tips by one person was calculated as between 6 h 10 min to 8 h 50 min, equivalent to one working day. As protocol steps cannot be undertaken in one day (due to overnight incubations) this logistically reduced freezing activities to a four-day week and reduced to two days per week for statutory holidays. Schäfer-Menuhr et al. (1998) also calculated one person could cryobank 150 batches per year, based on using a single cryopreservation method across all genotypes. This was rationalized on the basis that: (1) optimizing regeneration media for improved recovery would slow down cryobank processing and (2) when speeding up cryostorage processing time, compromises have to be made. Also, stock material is very homogeneous and can be derived from a few selected plants or *in vitro* tubers cloned many times. Thus, Schäfer-Menuhr et al. (1998) concluded that low survival is acceptable for this type of material, so long as some plants can be regenerated, however, they advised that the easiest means of obtaining survivors was to thaw the contents of several vials.

Keller et al. (2005) recommended potato accessions with more frequent requests should not be removed from *in vitro* slow growth storage. In examining maintenance in the field, slow growth and cryopreservation, Keller et al. (2005) envisaged a step-wise shift to using less expensive cryopreservation for long-term safe deposition. Keller et al. (2008a) performed an economic analysis on the cryobanking of crop germplasm at IPK and although this is based on European (€) costs it could be used as a comparative guideline for efficiencies and budgets by taking into account differentials in labour and consumables, equipment costs and overheads. Indeed, these can affect local changes as reported by IPK, when a change in LN application technique almost doubled storage costs. Keller et al. (2008a) summarise annual costs per accession as: (1) field maintenance, €50-60; (2) cryopreservation €6.5-12 and (3) an additional €8 for *in vitro* culture and soil transfer for requested material. IPK's genebank calculated it was more cost effective to store un-requested material in cryobanks than to maintain germplasm *in vitro* and/or in the field. Benson (2008b) also provides a comparative critique of cryobank costs and efficiencies indicating that once the start-up and accession deposition costs are accounted for, the maintenance of cryopreserved collections are comparatively cost effective (e.g. compared with field genebanks). However, this is on the basis that the germplasm is amenable to cryopreservation and that laborious procedures are not required to optimize protocols on a case-by-case basis.

Staff time is a major issue in the initial establishment of a cryobank, albeit after accessions are first placed in storage costs become less and are mainly allocated to running, maintenance and safety budgets. To enhance cost efficiency, Keller et al. (2008a) reviewed the number of accessions that were required to be cryopreserved, based on the probability tool of Dussert et al. (2003) and the safe regeneration of samples from cryopreservation. This was applied to IPK's in house procedures, for which original accessions were stored in three repetitions, each consisting of 120 explants, plus 12 explants as a regeneration control as according to Schäfer-Menuhr et al. (1998). A review of the process by IPK considered size of control samples too small to enable a sufficiently reliable estimate of regeneration capacity in cryopreserved accessions, whereas, the actual sample size of the cryopreserved accession

was deemed higher than necessary. Thus, IPK now operates on the basis that new accessions are cryobanked using two, and not three repeated cryopreservation runs. Each comprises 100 explants, plus 50 additional explants as regeneration controls. This decreased the workload by 25% and increased the regeneration control from 9.1 % of the collection to 33% of the total sample size (Keller et al. 2008a).

2.1.2.9 One cryostorage protocol fits all or different methods?

Schäfer-Menuhr et al. (1998) proposed that the optimal strategy for large-scale cryobanking efficiency is to apply one protocol across all accessions and genotypes on the basis that accommodating low survivors by further optimization of the protocol can be less efficient and reduces overall cost effectiveness. Schäfer-Menuhr et al. (1997) recommended, that in the case of homogeneous potato cultures derived from a few selected tubers, a lower rate of regeneration can be accepted as long as: (1) some of the shoots from every vial stored are regenerated and (2) these plants are genetically identical. However, they also comment that for cases of very low regeneration, recovery medium optimization may still be required.

Within the IPK potato cryobank, Kryszczuk et al. (2006) also considered the alternative strategy of testing different protocols and optimizing steps within protocols. Experiments using *S. tuberosum* were designed to compare: (1) droplet freezing (Schäfer-Menuhr et al. 1996) with PVS2 vitrification (Sakai et al. 1990); (2) cold acclimation of *in vitro* plants; (3) recovery and regeneration in liquid and on solid medium and (4) mode of DMSO sterilization (autoclave or filtered). Differences between regeneration of shoot tips after droplet freezing and standard PVS2 vitrification were statistically significant, with the PVS2 method producing enhanced recovery. After PVS2 vitrification, mean survival in the four genotypes tested was ca. 80%, with an average shoot regeneration of ca. 58%. In comparison, survival after the standard droplet freezing method for the same genotypes was ca. 37% and average regeneration ca. 14%. Statistically significant genotype differences in survival were also observed, after PVS2 vitrification for which variation in regeneration ranged from 18% to about 83%, by comparison, after droplet freezing recovery was from 0 to 25% (Kryszczuk et al. 2006). Cold preculture affected shoot meristem recovery and shoot regeneration differently across the two protocols. Cold preculture decreased average survival (from ca. 80% to 60%) and shoot regeneration (from ca. 58% to 46%) in potato genotypes cryopreserved using PVS2. In contrast, for droplet freezing, cold preculture improved survival (from ca. 37 to 54%) and shoot regeneration (from ca. 14% to 30%), this modification supported the survival of all genotypes. Kryszczuk et al. (2006) noted that liquid regeneration medium was supportive of regrowth in shoots cryopreserved using PVS2; in contrast, recovery on solid medium was largely via callus, whereas, the type of regeneration medium used did not have a significant effect on shoot tip development after droplet freezing. Method of DMSO sterilization had variable effects, for PVS2 treated shoot tips, filter sterilization of DMSO decreased survival from ca 80% to ca. 67%, as compared to autoclaved DMSO which was used in the original protocol. However, more survivors regenerated shoots and reached the same level of regeneration as those in the original PVS2 protocol. When DMSO used in the droplet method was autoclaved, instead of being filter sterilized (as in the standard protocol) no survival/regrowth was observed.

Kryszczuk et al. (2006) concluded that improved survival and regeneration rates can be achieved for: (1) different protocols and (2) modifications to existing protocols, for example, PVS2 resulted in a higher efficiency of response for the four genotypes tested, although the droplet freezing method was considered simpler to apply and more time efficient. These findings also need to be balanced with the potential for optimizing improved regeneration,

whilst retaining a simple technical procedure. This rationale concurs with Kaczmarczyk et al. (2008) in which case regeneration of *S. tuberosum* 'cv' 'Desiree' improved from 20% to ca 46% after droplet freezing, this was achieved by applying an alternating preculture temperature to plants before shoot tip isolation. Proteomics studies investigating stress physiology in potato cryopreservation may offer future insights into the basis of differential genotype responses and provide new approaches to protocol development (Criel et al. 2005).

2.1.2.10 Risks and safety

The main issues of risk and safety concern contamination, stability and ensuring sufficient survival and plant regeneration after cryostorage. This is important for potato which has highly variable recovery responses, both within and between experiments and across different genotypes (Golmirzaie et al. 1999, 2000a; Harding et al. 2008, 2009). Inconsistent responses can also persist during the long-term recovery of potato plants regenerated from cryopreserved meristems (Harding and Benson 1994; Harding and Staines 2001).

Contamination

Contamination is a critical factor in root and tuber crops and was identified by Towill (1983) as a serious limitation to cryopreserving shoot material sourced from glasshouse-grown plants. Some of the variation in survival between potato cryopreservation experiments was considered to be due to high levels of bacterial contamination and extreme variations in re-growth after cryogenic treatments were attributed to covert, endophytic contaminants being revealed at later stages of assessment. Towill (1983) resolved this problem by using *in vitro*-propagated plants in preference to surface-sterilized explants sourced directly from the glasshouse. In a long-term cryostorage study undertaken by Keller et al. (2008a), some accessions regenerated after several years of cryobanking were weak and in some cases regeneration was never achieved. It was assumed endogenous bacteria was the most likely reason for cryostorage failure and this highlights the importance of ensuring germplasm is free of covert and systemic organisms before it is cryopreserved.

Safety measures for ensuring survival and regeneration

This section considers the logistical, safety measures needed to ensure that cryopreserved collections produce an acceptable level of regenerants on the retrieval of samples from cryobanks. Schäfer-Menuhr et al. (1997, 1998) investigated the higher security of viable returns from the DSMZ-FAL Braunschweig potato genebanks by undertaking three separate droplet freezing experiments. They found regeneration was: (1) relatively independent of freezing experiment; (2) mainly dependent on genotype and (3) to be unknown and unpredictable. Furthermore, by using the same medium for all genotypes it will most likely be suboptimal for several genotypes and based on these assumptions, reducing the number of experiments for less responsive genotypes requires careful decisions. Schäfer-Menuhr et al. (1998) also considered the logistics and risks of long-term storage, with respect to loss of expertise as to how to recover materials from cryobanks once they are established.

Keller et al. (2005) report safety considerations for the unified Germany cryopreserved potato collections based on the original measure of cryopreserving 120 shoot meristems in separate triplicate experiments as undertaken by Schäfer-Menuhr et al. (1998). In practice this resulted in placing two aluminium foil strips each holding 6 explants in one cryovial and using 10 cryovials per series, with one control sample of 12 explants taken per storage series. Keller et al. (2005) verified recovery and regeneration (during 2002 and 2003) of cryopreserved potato meristems at IPK after their initial introduction into the cryobank in 1992. They found 6.3% of

the total accessions to have a regeneration of <10%. Applying the probability equations of Dussert et al. (2003) they calculated the lowest level of regeneration, allowing a target number of regenerants. Based on the assumption that only five plantlets would need to be recovered for an accession, Keller et al. (2005) ascertained all accessions with >10% regeneration are sufficiently safe in their cryobank, at a probability level of 95%. On this basis, they predicted 97% of the collection was safe and only 3% was recalcitrant, which for a large collection of ca. 1000 accessions was deemed acceptable. Keller et al. (2005) found the correlation between assessments, on introduction into the cryobank compared with the second verification after storage although the mean regeneration was similar at ca. 47%. They surmised this was due to a small sample size of taking out only 12 verification test shoot tips from the cryobank from 120 frozen samples. The study of Keller et al. (2005) was a transient evaluation and currently, the IPK uses logistics concerning regeneration safety as described by Keller et al. (2008a). Recent studies at IPK are investigating the basis of cryoinjury in potato with a view to improving recovery and regeneration (Kaczmarczyk et al. 2008).

2.1.2.11 Stability

Mode of regrowth and regeneration is an important consideration in potato cryopreservation due to the crop's propensity for SCV (Scowcroft 1984). It is cautionary to note that Towill (1981b, 1983) observed high levels of callus formation and multiple shooting which is suggestive of adventitious development in *S. tuberosum* genotypes recovered from controlled rate cooling. This is probably due to shoots regenerating from a few cells surviving in the apical meristem, which eventually initiate new meristematic regions via an intervening callus phase. Towill (1984) applied the same two-step cooling protocol using DMSO to shoot meristems of *in vitro* plants from *S. tuberosum* and representatives of *S. andigena, S. phureja, S. stenotomum* and *S. tuberosum*. High levels of survival (up to 100%) were achieved for most genotypes as regeneration and/or callus, however again the critical factor was shoot regeneration associated with callus. Microscopic examination confirmed that shoots were of adventitious origin and studies indicated that growth regulator composition may be a factor in predisposing plants regenerating after cryopreservation to instability.

Bajaj (1985) did not observe any changes in ploidy or tuberisation in plants of *S. tuberosum* 'Alankar' regenerated from shoot meristems held in cryostorage for 4 years. Ward et al. (1993) assessed post-cryopreservation ploidy stability in *S. tuberosum* plants regenerated from four different dihaploid cultivars, cryopreserved by ultra rapid freezing using DMSO as the pretreatment and cryoprotective additive. The objective of this study was to compare the relative stabilities of dihaploids regenerated via organogenesis, from protoplasts and after cryopreservation. Dihaploids are very unstable and prone to doubling to the tetraploid state (2n=4x=48) and other ploidy changes and as such they provide an interesting measure of assessing genetic stability in potato following cryopreservation. Comparisons of ploidy stability were also made with the diploid wild species *S. microdontum* and *S. pinnatisectum*. Flow cytometry was used to confirm ploidy status in both primary and secondary (possible adventitious or axillary) shoots regenerated from cryopreserved apices. The frequency of polyploidization was minimal in plants regenerated from cryopreservation compared to those from leaf explants and protoplasts and cryogenic treatments did not induce ploidy changes in sensitive dihaploids of *S. tuberosum* (Ward et al. 1993). Benson et al. (1996) evaluated the effects of alginate encapsulation-dehydration on stability by applying the method of Dereuddre and Fabre (1990) to genotypically diverse diploid, tetraploid and hexaploid genotypes of wild and cultivated *Solanum* spp. Using cytological approaches to

assess instability, ploidy status was found to be maintained in all plants regenerated from cryopreserved shoot meristems and importantly, there was no evidence of any chromosomal abnormalities. Barandalla et al. (2003) applied the ultra rapid freezing method to ten potato cultivars and used cytogenetic analysis and flow cytometry to confirm the absence of polyploidy in plants regenerated from cryopreserved shoot meristems.

Molecular stability assessments of potato plants regenerated from cryopreserved shoot meristems of *S. tuberosum* 'Golden Wonder' were first reported by Harding (1991) using the ultra rapid freezing method and DMSO as the cryoprotective additive. Ribosomal gene (rDNA) probes combined with RFLP analyses revealed that plants were unchanged after cryogenic treatments with respect to their ribosomal gene RFLP profiles. Harding (1997) assessed recovery times, plant heights and mode of regeneration in *S. tuberosum* 'Golden Wonder' and 'Desiree' recovered from the same cryogenic treatments on various media. No reduction in rDNA repeat unit or organizational changes in inter-genic spacer (IGS) length were linked to plants recovered from cryopreservation, although individuals did exhibit different plant heights. Signal intensities of the main hybridization fragments of both cultivars were stable, although a 2.55kb fragment varied between individual plants. Harding and Benson (2000) found identical DNA fragment profiles in *S. tuberosum* 'Brodick' plants regenerated from shoot tips cryopreserved using encapsulation-dehydration, this assessment comprised nuclear and chloroplast BamHI DNA-DNA hybridization analysis. Harding and Benson (2001) compared microsatellite (888, BDB-$[CA]^7$) profiles using PCR in DNA extracted from plants regenerated from cryopreserved *S. tuberosum* 'Brodick' and 'Golden Wonder' and found no differences in plants recovered from cryopreserved meristems, as compared to controls, plants regenerated from tubers and field-grown plants.

Benson et al. (1996) conducted long-term developmental studies of plants regenerated from cryopreserved encapsulated-dehydrated meristems of *S. phureja*, *S. tuberosum*, *S. brachycarpum, S. acaule, S. guerreroense* and *S. iopetalum*. All exhibited normal patterns of flowering, berry set and tuber formation, as appropriate to species. Biometric analyses of trueness-to-type using principle component analysis was performed by Harding and Staines (2001) on the phenotypic characters of plants regenerated from shoot meristems of *S. tuberosum* of 'Golden Wonder' cryopreserved using ultra rapid freezing. This study assessed plants from tissue culture, DMSO treatments and cryopreservation; all experimental groups were found different with respect to tuber weight, height and length of petiole as compared to field-grown control plants.

Schäfer-Menuhr et al. (1996, 1997) performed molecular stability assessments using RFLP-DNA fingerprinting and flow cytometry on 161 plants regrown from shoot meristems of an *in vitro* collection of old potato varieties. These had been cryopreserved using droplet freezing and no unusual ploidy changes or banding patterns were found. After 3-8 years in LN, potato shoot tips from this cryopreserved collection were removed and a random sample of 51 varieties thawed. A mean regeneration of 27% was achieved which was comparable to tests undertaken at the time of cryobanking; Mix-Wagner et al. (2003) concluded that time in LN did not produce any major changes in recovery response. The data for this study were analysed during 1991 to 2000 and was presented as strict paired comparisons with apices from the same cryopreservation batch. However, the authors caution there was a strong correlation of $r = 0.99$ between storage and the date of the short-term storage test and they note that survival or plant recovery due to length in storage time was confounded by changes in the experimental protocol as the project progressed. Therefore, they advise this data should not be used to

predict trends in long-term viability of potato germplasm under cryopreservation, particularly as the effects of storage time on recovery presented a number of anomalous results. For example, among individual experiments over varying storage periods, a few varieties exhibited no plant regeneration, despite good results in the short-term storage test, whilst others exhibited either a considerable drop in regeneration or an improvement. One variety showed higher regeneration from a long-term storage experiment compared to two short-term storage experiments.

In the same study, Mix-Wagner et al. (2003) examined the effects of apical size on storage period, sizes were categorized as small (0.5 to 1 mm length) and large (2 to 3 mm length) and both groups showed comparable survival rates over time. However, plant regeneration after short-term storage was considerably lower (10%) in varieties with small apices compared to those with larger apices (40%). Variations in recovery were also attributed to a bias in the improvement of technical expertise as no significant linear time trend was detected for time in storage. Although, overall there was a significant reduction in shoot tip survival (mean 19 ± 3.5%) and a slight increase in plant regeneration (mean 9 ± 3.6%) when short-term storage data was compared with long-term storage data and out of the 51 varieties tested, only one failed to survive storage. Mix-Wagner et al. (2003) consider it is unlikely that frozen apices will continue to deteriorate in LN storage over time, despite the confounding effects of operator technical skills improvement influencing data interpretation. Keller et al. (2005) report subsequent stability checks of the unified, cryopreserved potato collections in the IPK genebank. This is based on data re-checked at Gatersleben, for storage longevity during 2002 and 2003 and it provides for the first time, direct comparisons of potato regeneration over storage periods of 7-10 years. The survey demonstrated no obvious decline in cryopreserved sample viability.

2.1.3 Lessons learnt from potato

In the case of MTS, collective observations suggest one of the key research priorities is to optimize slow growth strategies that avoid harmful stressors. For example, by applying anti-stress treatments (e.g. ethylene inhibitors), substituting different types of growth retardants (e.g. ancymidol), optimizing nutrient status and incorporating timely regeneration/ rejuvenation cycles. It may also be prudent to study epigenetic changes in cultures maintained in slow growth (Lopez-Delgado et al. 1998; Sarkar et al. 2002, 2005). Taking practical measures to prevent this problem is justified on the basis that it is a quality control measure and it is particularly advisable for developing treatments that minimize stress during MTS. The profiling of DNA methylation in plants of genotypes susceptible to genetic instability or off-type production following their recovery from slow growth may also be prudent. For example this may be used as a quality control indicator to confirm if epigenetic changes are a persistent, or transitory response to stress; a number of methods are available for detecting DNA methylation (Harding 1994, 1996; Joyce and Cassells 2002; Johnston et al. 2005). Ensuring stability in plants regenerated from cryopreserved germplasm is similarly significant for potato as the crop is susceptible to SCV, epigenetic change and field off-types (Joyce and Cassells 2002).

Although developing cryobanking strategies for potato has not been easy, an important, positive outcome has been the significant knowledge base created for this crop (Benson 2004). In this case study, research outcomes for cryogenic and non-cryogenic factors have been assessed sequentially throughout different storage protocols. Cryogenic factors concern tolerance to cryopreservation and include cryoprotection and exposure to, and recovery from

ultra low temperatures. Non-cryogenic factors include plant physiology, culture and preculture conditions, recovery hormones, stress responses and contamination. One of the most important lessons learnt from this case study is the highly influential role that donor plant and explant physiology has on survival after cryogenic storage. These factors affect short-term meristem survival and shoot growth, as well as the long-term developmental competency of regenerated plants (Harding and Benson 1994, Harding and Staines 2001, Harding et al. 2008, 2009). Whilst considerable emphasis has been placed on the optimization and refinement of cryoprotection and cryogenic treatments, closer scrutiny suggests that choice of meristem (e.g. apical or axillary), size of meristem and time in culture are equally as influential. A greater focus should therefore be placed on selecting the appropriate source and type of meristem, rather than on the continued, laborious refinement of the cryogenic components of already existing protocols. This approach may offer scope for improving the conservation of genotypes that have highly variable cryogenic stress responses. However, the wide genetic diversity of potato remains a major challenge to developing cryostorage protocols for the many different genotypes and accessions held in large clonal crop genebanks. Therefore, it is particularly important to develop several robust, routine storage methods that offer different options for cryopreserving genotypes that may respond badly to one protocol but are tolerant of another. Taking this approach may offset the need to optimize steps within a protocol on a case-by case basis. The lessons learnt from existing large-scale potato genebanks (Keller et al. 2008a) provide valuable insights into the logistics of running and maintaining an operational cryobank on this basis. To conclude this section, progress in potato cryopreservation has been substantial, resulting in the large-scale cryobanking of germplasm in some genebanks (Gonzalez-Arnao et al. 2008; Keller et al. 2008a, b). Keller et al. (2008b) describe in full the cryopreservation protocols developed for potato and other herbaceous dicot crops, these include technical details and guidelines for vitrification, encapsulation-dehydration, DMSO droplet freezing and droplet-vitrification. Analysis of the detailed critical point factors presented in this case study will help to highlight the priority research needs for LTS development in other crops (see Section 5).

2.2 *In vitro* conservation of cassava

The *in vitro* conservation of cassava is well researched, with the most substantial studies being undertaken at CIAT (Gonzalez-Arnao et al. 2008; IPGRI/CIAT 1994) and IITA (Ng and Ng 1997, 2000; Ng et al. 1999). Ng and Ng (2002) compiled lists of global and national genebanks conserving the crop in MTS and LTS. This section appraises the progress of cassava storage in the wider conservation community as documented by CIAT (2007a, b, c), Charoensub et al. (2007), Sarakarn et al. (2007), Pillai et al. (2007) and Unnikrishnan et al. (2007). Bajaj (1977) established *in vitro* cassava cultures from sprouted dormant lateral buds and applied 10% glycerol and 5% sucrose to excised buds before direct plunging into LN. The buds were thawed in water at 35-37°C and the meristems recovered in culture medium containing IAA and kinetin; dependent upon the hormone regime regrowth proceeded via callusing and/or shoot proliferation. Although a high level of survival was achieved (ca. 85%) only 13% of the cryopreserved meristems regenerated cassava plants. Bajaj (1983a, b 1985) recovered plants and callus from cassava meristems cryopreserved using the cryoprotection method of Bajaj (1977). Meristems maintained in LN for 3 and 4 years had a maximum survival of 34% after 4 years of storage.

Kartha (1985) reviews the early application of cryopreservation to cassava based on the method of Kartha et al. (1982) who developed droplet freezing for meristems using the surface sterilized sprouted buds of dormant cuttings as source material. Meristems of 0.4-0.5 mm in length, comprising a pair of leaf primordia and subjacent tissue were treated with a final cryoprotectant loading of 15% DMSO applied in culture medium containing 0.3 M sucrose which was delivered gradually to avoid osmotic injury. After 30 min equilibration, the meristems were distributed on aluminium foil in 2-3 µl droplets placed in a Petri dish, this apparatus was transferred to a programmable freezer and cooled to various terminal transfer temperatures before plunging into LN. Thawing involved immersion of the foils in liquid medium and recovery on cassava regeneration medium supplemented with BA, NAA and GA_3. Kartha et al. (1982) tested this method on four different genotypes and all were found to behave similarly. Terminal transfer temperature was the critical factor, which was optimal at -20°C, when preceded by a cooling rate of 0.5°C/min. Up to 100% plantlet regeneration was obtained at the intermediate transfer temperature, however, transfer of meristems from -20°C to LN resulted in low recovery and callusing. Variation in survival ranged from 16 to 80% and only a few survivors formed plants, this lead Kartha et al. (1982) to conclude that whilst meristems remained viable, only partial meristem survival occurred resulting in callus production without shoot recovery. Several different cryoprotectants were tested as alternatives, including glycerol, ethylene glycol, polyethylene glycol (mol wt 6000), however, none supported acceptable recovery in the absence of callus (Kartha 1985). A pilot study was undertaken to compare droplet freezing (Kartha et al. 1982) and encapsulation-dehydration (Fabre and Dereuddre 1992) protocols for the cryopreservation of cassava meristems from *in vitro* cultures (Benson et al. 1992; Engelmann et al. 1994). This investigation indicated encapsulation-dehydration was the preferred method, for which 60% survival was obtained. Transferring recovering meristems to hormone-free medium 3 weeks after rewarming reduced callus formation and increased shoot production.

The Kasetsart University Research and Development Institute, Thailand investigated the application of vitrification-based protocols to cassava shoot meristems. Charoensub et al. (1999) used *in vitro* grown plantlets as the source material for shoot tips precultured in 2 M glycerol and 0.4 M sucrose and cryoprotected with PVS2. After transfer to cryovials containing 0.5 ml PVS2, the shoots were plunged in LN; rewarming at 45°C was followed by unloading in 1.2 M sucrose solution and transfer of meristems to sterile filter paper discs overlaid on culture medium. After one day the shoot tips were transferred to fresh medium and shoot regrowth recorded after 5 weeks, vitrified meristems resumed growth within one week and developed shoots without callus formation. Mean shoot production was 75% for the genotype tested and the PVS2 vitrification method was subsequently developed for routine use by Charoensub et al. (2003, 2007), testing satisfactorily (average recovery 70%, shoot regrowth 32-90%) for 10 cultivars of cassava. Charoensub et al. (2004) proceeded to apply the encapsulation-vitrification protocol to four cultivars of cassava using *in vitro* plants as source material for meristems. The procedure involved culturing 5 mm nodal cuttings on medium for 28 days, after which, excised axillary shoot tips were precultured on 0.3 M sucrose-enriched medium for 16 h. Thereafter, shoots were encapsulated in alginate and osmoprotected in a mixture of 2 M glycerol and 0.6 M sucrose for 90 min at 25°C, followed by cryoprotection in PVS2 at 0°C for 4 h; meristems were transferred to cryovials containing PVS2 and plunged directly into LN. Shoot tips sampled from 21-day old plantlets produced the highest level of survival at 80%, although this was dependent upon day of excision, with

differential responses ranging from 38 to 89%. RAPD analysis (using 200 sets of 10 primers) was performed on recovered, cryopreserved plants, confirming that there were no major differences in stability profiles. This protocol was successfully applied to four cultivars with a mean survival of 80% assessed as normal shoot development. Charoensub et al. (2004) recommended encapsulation-vitrification as a promising approach for the large-scale cryopreservation of cassava, particularly as it gives high levels of normal shoot regrowth within 3 weeks of recovery. However, physiological condition, age of donor nodal cuttings and optimization of osmotic treatments were identified as critical factors to success.

Alternative strategies for both the *in vitro* conservation and cryopreservation of cassava have also been considered. Aladele and Kuta (2008) investigated the use of screen houses to maintain *in vitro* cultures at a reduced cost. The effects of environmental and genotypic factors on *in vitro* growth rate of ten varieties of cassava were evaluated, as compared to culture room maintenance using five different culture media. The project concluded that cassava tissue cultures could be cost effectively propagated under screen house conditions, so long as the plant materials were preconditioned in the culture room first. Stewart et al. (2001) used primary somatic embryos as an alternative germplasm source for cassava cryopreservation and tested desiccation and chemical-based cryoprotectant treatments by using microscopy to assess dehydration and cryoinjury. Subsequently, Danso and Ford-Lloyd (2002) suggested the high-frequency production of cassava somatic embryos as an alternative source material for cryopreservation.

2.3 *In vitro* conservation of sweetpotato

The Crop Diversity Trust strategy for sweetpotato (Roca 2007) identified 36 collections, holding 29,016 accessions of sweetpotato genetic resources, with 70% of the total held in 7 collections of which one is CIP. This section reviews the *in vitro* conservation of sweetpotato in the wider community which mostly hold collections in the field, rather than *in vitro* (Roca 2007). The first practical manual for handling sweetpotato germplasm maintained *in vitro* was compiled by S.V. Love, B.B. Rhode and J.W. Moyer for IBPGR (IBPGR 1987). This includes all aspects of conservation, tissue culture, phytosanitary management and disease indexing. Slow growth methods for sweetpotato were initiated at IITA during the 1970s (IITA 1980; Ng and Hahne 1985), minimal growth was achieved using low temperature and mannitol treatments which extended subculture intervals for up to 2 years. Cultures required routine checks for necrosis and contamination and a brief period of acclimatization in standard culture to establish growth after transfer from slow growth to *ex vitro* conditions. Jarret and Florowski (1990) considered sweetpotato *in vitro* conservation compared to field maintenance, reporting that cultures could be maintained across a wide genotype range for up to 2 years in MTS (Frison 1981; Jarret and Gawel 1991). Mandal (1999) described sweetpotato MTS in India's National Bureau for Plant Genetic Resources (NBPGR) which uses mannitol and sucrose to extend storage for 12-14 months.

Cryopreservation of embryogenic cultures is an alternative approach to sweetpotato conservation and it has been assessed using two-step freezing (Blakesley et al. 1995), non-encapsulated desiccation (Blakesley et al. 1996); and encapsulation (Blakesley 1997; Bhatti et al. 1997). The United States Department of Agriculture (USDA) National Seed Storage Laboratory, Fort Collins, pioneered cryopreservation for sweetpotato shoot tips and Towill and Jarett (1992) were the first to describe survival of meristems after cryopreservation, but they found controlled rate cooling to be ineffective. Testing PVS2-based vitrification as an alternative, they

observed surviving meristems developed callus and that there was a high level of variation across experimental replicates. Pennycooke and Towill (2000) concentrated on optimizing the condition of donor plants and cryogenic factors; this improved recovery for the PVS2 protocol which was adapted as a droplet-vitrification method. Thus, 4-8 week old *in vitro* sweetpotato plants were used as donors and 0.5 to 1.0 mm shoot tips were excised, each comprising 2-3 leaf primordia and an apical dome. The shoot tips were precultured in liquid medium containing 2% sucrose for 24 h, before they were transferred for a further 24 h to solid Murashige and Skoog medium containing 0.3 M sucrose. The meristems were then placed in a loading solution comprising 2 M glycerol and 0.4 M sucrose for 60 min and replaced with PVS2 for 10-26 min. For some treatments an intermediate concentration of PVS2 was applied; as sweetpotato is chill sensitive cryoprotective incubations were performed at 22°C (Pennycooke and Towill 2000).

For ultra rapid cooling (Pennycooke and Towill 2000) sweetpotato shoot tips were transferred to 10 µl droplets of PVS2 dispensed onto thin strips (40 x 2mm) of sterile aluminium foil, folded to enclose the shoots. The strips were immersed in partially solidified nitrogen for 10-30 min and then transferred to LN; rewarming involved direct immersion of the foils in 1.2 M sucrose and cultured on recovery medium containing NAA, BA and kinetin. Meristems were initially recovered in darkness for 2 days, under dim light (40 µmol m^{-2} s^{-1}) for 3 days before transfer to standard light (60 µmol m^{-2} s^{-1}). Optimization of the method involved an initial pretreatment in 0.3 M sucrose for 24 h at 22°C and survival was enhanced by excising the shoot tips meristems immediately after an 8 h dark period. Pennycooke and Towill (2000) reasoned this treatment converted starch to sugar which exerted a cryoprotective effect. Precultured shoot tips exposed to 2 M glycerol and 0.4 M sucrose loading solution for 1 h at 22°C, followed by cryoprotection with PVS2 for 16 min, (22°C) gave the best survival, with 66% normal shoot development after 8 weeks. Position of shoot on the *in vitro*-donor plant had a very significant effect on recovery, following PVS2 and LN treatments. Apical shoots from the main axis had the highest survival, recovery progressively diminished to zero tolerance in meristems taken from nearer the base.

Pennycooke and Towill (2001) made further improvements to their sweetpotato PVS2 (droplet) vitrification protocol by changing the nitrogen composition of the recovery medium. This increased viability three-fold when samples were initially recovered on ammonium-free medium for 5 days. The improved protocol was tested on four genotypes, demonstrating shoot regrowth of 62-83% to a maximum of 93%. The encapsulation-vitrification protocol was also tested in the same study, although it was not as successful, and achieved 67% regrowth of sweetpotato shoots following cryopreservation.

Hirai and Sakai (2003) optimized the encapsulation-vitrification method for sweetpotato by preculturing alginate-encapsulated shoot tips in 30g/L sucrose for 16 h, followed by a 3 h loading treatment with 2 M glycerol and 1.6 M sucrose and cryoprotection in PVS2 solution for 1 h at 25°C. The encapsulated shoot tips were transferred to cryovials containing 0.5 ml PVS2 and plunged directly into LN, following rapid warming in a water bath at 38°C for 2 min the PVS2 solution was drained and the shoot tips rinsed twice at 10 min intervals with 1 ml of 1.2 M unloading solution. Recovery was performed in two phases, with the first 7 days on medium containing 0.5 mg/l BA and 1 mg/L GA_3 after which, the recovering shoots were transferred to medium containing 0.5 mg/L GA_3; shoot meristems regenerated normal shoots within 3 weeks. The method was tested on three other cultivars for which recovery as normal shoot regrowth was ca. 80%. Hirai and Sakai (2003) recommend their optimized

encapsulation-vitrification methods for sweetpotato by testing the protocol for a wider genotype range, their modifications overcame the previous constraints of using PVS2 and the lack of cold adaptation in this crop (Takagi et al. 1998). Keller et al. (2008a, b) describe an encapsulation-vitrification protocol for sweetpotato based on the original method of Matsumoto et al. (1995) as optimized by Hirai and Sakai (2003).

2.4 *In vitro* conservation of yam

This section summarizes studies on yam undertaken by the wider conservation community. Vegetative propagation is possible using vine cuttings or tuber sets (Alizadeh et al. 1998) and somatic embryogenesis (Viana and Mantell 1989). Temporary immersion systems for the *in vitro* production and the MTS of yam tubers provide alternatives in commercial sectors (Jova et al. 2005). Malaurie et al. (1998a) developed successful MTS protocols for 20 yam species, using low mineral and sucrose medium, this method permitted maintenance for up to 2 years with subculture intervals of 6-8 months. Borges et al. (2004) used mannitol-based treatments to constrain growth in *Dioscorea alata* shoot cultures for up to 9 months, maintaining successful regeneration following transfer to standard medium. Keller et al. (2006) report the *in vitro* conservation of 41 yam accessions at IPK, Germany, using a culture rotation of 2 months on medium containing 3% sucrose. Malaurie et al. (1998a, b) investigated a range of medium and long-term protocols for yam genetic resources.

Popov's group at the K.A. Timiryaezey Institute of Plant Physiology, Moscow, pioneered cryopreservation of *in vitro* medicinal yam germplasm using colligative cryoprotection and two-step cooling cryopreservation. This approach was first applied to callus and cell suspension cultures (Chulafich et al. 1994; Federovskii and Popov 1992; Popov et al. 1995). Malaurie et al. (1998a, b) optimized the methodology for cryopreserving yam shoot tips using encapsulation-dehydration and applied the protocol to excised apices of *D. bulbifera* and *D. alata.* Shoot meristems were encapsulated in calcium alginate, pretreated with sucrose, desiccated over silica gel and cryopreserved by direct plunging in cryovials into LN. Osmotic pretreatment was a critical factor, in *D. alata*, which had the highest survival (67%) and plant regeneration (19%) using a 3-10 day culture in 0.9 M to 1 M sucrose. Osmotic treatments were followed by evaporative desiccation over silica gel for 11-16 h. In the case of *D. bulbifera*, highest survival (65%) and plant regeneration (60%) required pretreatment with concentrations of sucrose >0.75 M, combined with desiccation over silica gel for 14-16 h Malaurie et al. (1998a, b). Recovery of plants from cryopreserved apices occurred within three months of post-cryopreservation culture on hormone-free medium. Three critical factors: (1) pretreatment in sucrose liquid medium, (2) sucrose concentration and (3) duration of desiccation with silica gel were identified in the application of the encapsulation-dehydration protocol to yam (Malaurie et al. 1998 a, b, 2000). Survival was increased when dehydration was extended to a threshold of ca. 0.13 to 0.15 g H_2O/g dry wt, which was obtained after desiccation periods of 10-18 h. Cryopreservation of *D. rotundata* and other yam species was reported by Kyesmu and Takagi (2000) who used a vitrification protocol optimized for preculture duration and PVS2 exposure time, achieving 10% to 75% recovery.

Some of the most extensive studies performed on yam cryopreservation have been undertaken by Mandal and colleagues at NBPGR, India using *in vitro*-grown plantlets as source material (Mandal 1999; Mandal et al., 1996a, b). Encapsulation-dehydration was first tested on *D. alata*, *D. wallchii*, *D. bulbifera* and *D. floribunda*, by applying a 3-day pretreatment with 0.75 M sucrose to encapsulated apices, followed by 4 h desiccation in a sterile airflow

(Mandal et al. 1996b). *D. alata* and *D. wallchii* regenerated whole plants at respective levels of 21 and 37%, although other species only formed callus. Microscopic studies indicated problems with shoot regeneration and recovery which was due to shoot damage, if this was too extensive callusing occurred (Mandal et al. 1996a, b). A comparative study of three cryopreservation protocols, encapsulation-dehydration, vitrification (PVS2, PVS3) and encapsulation-vitrification was subsequently undertaken (Mandal 2000). Media with modifications made to plant growth regulator composition supported enhanced regeneration after encapsulation-dehydration, although the majority of plants produced shoots associated with callus proliferation. Vitrification was tested at NBPGR as an alternative to encapsulation-dehydration with the best results being obtained for *D. floribunda* shoot tips which were cryopreserved using PVS2 (87% survival, 30% shoot regeneration); recovery progressed in the absence of callus. This treatment also supported survival in *D. alata* and *D. wallchii* and all three yam species survived cryopreservation using encapsulation-vitrification for which recovery ranged from 20-50%, although shoot regeneration was lower (0-16%). Following comparative assessment of protocols, the PVS2-based method was selected to determine molecular, phenotypic and biosynthetic stability in *D. floribunda* plants regenerated from cryopreserved shoot tips (Ahuja et al. 2002). The optimized PVS2-based protocol supported 87% viable recovery in shoots, of which 30% were capable of producing sufficient plants for stability assessments. These involved RAPD analysis using 10 primers, which produced 64 reproducible bands, an assessment of 5120 bands revealed no significant difference between 60 plants recovered from cryopreserved shoot tips and 20 *in vitro* controls. Morphological assessments were undertaken on glasshouse-grown plants using 18 descriptors with no differences being observed. Diosgenin production of *in vitro* plants from cryopreserved germplasm was confirmed comparable to that of controls using HPLC analysis. Metabolite production was found to be stable in plants recovered from shoot tips of *D. deltoidea* that had been cryopreserved using vitrification and encapsulation-dehydration (Dixit-Sharma et al. 2003, 2005). Longer-term studies were performed on this species, they involved a comparison of shoot tip survival and regeneration after short term <24 h and one year of storage in LN (Mandal and Dixit-Sharma 2007). Survival and shoot regeneration of *D. deltoidea* shoots was maintained, producing a regeneration frequency of 76% using encapsulation-dehydration. In comparison, regeneration was 83% for shoot meristems cryopreserved using PVS2 and all plants regenerated without an intervening callus stage. However, Mandal and Dixit-Sharma (2007) found considerable genotypic variation in the response of yams to cryopreservation, particularly across distinct taxonomic groups. Mandal et al. (2008) reported an updated account of *D. rotundata* cryopreservation by comparing vitrification and encapsulation, both methods produced high levels of plant regeneration from cryopreserved shoot tips. For the PVS2-based protocol, 71% regeneration was achieved as compared to 67% for encapsulation-dehydration, although differences between these treatments were not statistically significant. This study included a stability analysis using RAPD markers; 5390 bands were obtained and no changes in RAPD banding patterns were observed. Mandal et al. (2008) concluded that the *in vitro* plants recovered from cryopreserved meristems were genetically stable at the molecular level they tested.

The IPK, Germany has undertaken detailed studies of yam cryopreservation which are mainly based on the doctoral studies of Leunufna (2004). Vitrification, droplet, and modified droplet protocols were tested for *D. bulbifera*, *D. oppositifolia*, *D. alata* and *D. cayenensis*

(Leunufna and Keller 2003, 2005). These were based on the methods of Sakai (2000) and a modified droplet protocol was tested for different vitrification solutions using larger droplets (7.5 µl) and various sucrose-unloading solutions (3-15%) on rewarming. Most protocols did not support acceptable recovery or shoot regrowth, although high survival (100%) and regrowth (52%) was achieved for *D. oppositifolia* using the modified droplet method. Overall, a higher average survival was observed for treatments using the droplet, as compared to the original vitrification method, the efficacy of these protocols was, however genotype-specific. Latterly, IPK has tested cold acclimation and sucrose pretreatment in *D. alata*, *D. bulbifera*, *D. polystachya* and *D. cayenensis*, cryopreserved using a modified PVS2-droplet method. Acclimation (alternating temperatures of 5°C at night and 28°C during the day) for 3 weeks provided the best treatment for all four genotypes. The highest level (47%) of plantlet development was found in *D. bulbifera* using a 10%-sucrose pretreatment. Efficacy of sucrose preculture was genotype dependent, ranging from high survival (67-70%) and shoot regrowth (30-50%) in *D. bulbifera, D. polystachya* and *D. cayenensis*, compared to 20% survival in *D. alata*. Keller et al. (2006) concluded yam cryopreservation is heavily genotype dependent and regardless of protocol modifications, the capacity of surviving shoots to convert to plantlets is largely genetically predetermined. Currently, IPK holds 52 vegetatively propagated accessions of yam, of which only one is cryopreserved (Keller et al. 2008a). Cryopreservation of *D. rotundata* and other yam species has also been undertaken by Kyesmu and Takagi (2000) using PVS2 cryoprotection. Gallet et al. (2007) are currently developing cryopreservation protocols for yam germplasm held in Guadeloupe at the Institut National de la Recherche Agronomique (INRA) in the French West Indies. They are investigating the various protocols and techniques for yam cryopreservation and preliminary findings confirm IPK's studies that responses to different cryogenic methods are genotype dependent.

2.5 *In vitro* conservation of *Musa*

The CGIAR centres, in particular the Bioversity International Transit Center have mainly developed methods for the *in vitro* conservation of *Musa*. Therefore, *Musa* conservation in the wider community will be considered collectively in later sections. A consultative document, supported in partial production by the Global Crop Diversity Trust has been prepared by INIBAP (INIBAP 2006) in collaboration with the *Musa* research and development community, this addresses the global conservation of banana and plantain. The importance of using *ex situ* approaches for the long-term conservation of *Musa* crops is reiterated in the document on the basis that as banana cultivars are usually seedless, there is a requirement for conserving their vegetative germplasm in both field and *in vitro* genebanks. Of the institutes surveyed (INIBAP 2006) 15 have *in vitro Musa* collections comprising 2000 accessions and Biodiversity ITC holds an additional 1176 accessions.

3. Status of mandate clonal crop *in vitro* conservation in CGIAR's genebanks

This section reviews the development of CGIAR's clonal crop *in vitro* genebanks and the status of their infrastructures, facilities and activities based on feedback in survey returns from Bioversity ITC, CIAT, CIP and IITA completed during 2007-2008 of the GPG2 programme. The milestone, cassava Pilot *in Vitro* Genebank Project (IPGRI-CIAT, 1994) undertaken by CIAT will be considered in more detail on the basis that it provides a significant building block in developing contemporary guidelines and best practices for the GPG2 programme. *In vitro* storage protocols for *Musa* have been mainly advanced through Bioversity ITC, formerly INIBAP (INIBAP 2006; Panis 2009), the activities of the ITC *Musa* genebank are thus considered together with the contributions of its associates. A Clonal Crop Task Force (CCTF) survey summarized in Tables 1-11 of this report highlights progress across the clonal crops held by CGIAR's *in vitro* genebanks. These include yam, sweetpotato potato and Andean Root and Tuber Crops (ARTCs) for which the routine implementation of cryopreservation is still under development. Pioneering research at CIP involves the *in vitro* storage of potato, sweetpotato and underutilized and neglected ARTCs (Herman and Heller 1997). The IITA holds responsibility for conserving three (cassava, *Musa* and yam) of the five main mandated crops.

3.1 Infrastructure status for conserving CGIAR'S mandate clonal crops

Of the three different CGIAR centres mandated to conserve and undertake root and tuber crop research, CIAT focuses on cassava for Latin America and Asia and CIP has a global mandate for potato, sweetpotato and the ARTCs. The IITA works mainly in sub-Saharan Africa on yam, cassava and *Musa*. The *Musa* International Transit Centre (ITC) is a component of Bioversity and INIBAP and is hosted by the Katholieke Universiteit Leuven (KULeuven); Bioversity ITC holds the largest proportion of the global *Musa* genepool, about 80% of the collection is held in trust under the auspices of FAO. The IITA maintains a collection of *Musa* germplasm, currently in MTS and is prospecting the development of a cryogenic capability.

3.1.1 Physical infrastructures

The physical infrastructures (Table 1) supporting CGIAR's storage repositories and associated black boxes comprise integrated operations connected by a process chain that includes phytosanitary testing and plant health facilities. The IVGBs are equipped with: (1) basic tissue culture equipment, growth rooms and support facilities (IPGRI-CIAT 1994; Withers 1985); (2) specialist storage equipment comprising: incubators, acclimatizing chambers, cold chambers, programmable freezers, LN storage Dewars and their physical inventories, supply Dewars [a LN-generator at CIP], (3) microscopes, analytical and molecular equipment for germplasm authentication, performance and stability testing and (4) safety equipment, storage alarms, LN level alarms, personnel O_2 safety alarms, intrusion alarms and smoke detectors; these are variously incorporated at the different institutions.

3.1.2 Virtual infrastructures

Different levels of interaction and operability support the virtual infrastructures of CGIAR's clonal genebanks. Each IVGB is equipped with, or is in the process of being supplied with

electronic database inventories and barcode systems (Table 1). These connect to wider genebanking operations and provide traceability for phytosanitary processing and germplasm exchanges. An evolving 'Genebank Knowledge Base' (KB) constructed during the GPG2 programme (see http://cropgenebank.sgrp.cgiar.org) services the CGIAR clonal crop genebanks, the role of the KB is to facilitate easy access to the knowledge and best practices underpinning genebanks. This service will aid more effective and efficient conservation as the KB provides information on best practices for genebank management, including registration, sample processing, quality testing, conservation, characterization, regeneration, safety duplication, dissemination, documentation, and equipment and supplies. Crop-specific information is also available on the KB, together with publications, training materials and a Knowledge Base Wiki. The GPG2 Knowledge Base is a one-stop, port of entry for information on policies, risk management, safety and back up, decision support tools, crop registry models and performance indicators. The main objectives of the KB are: (1) to provide a user-friendly one-stop shop for online access and procedures, standards and practices for both clonally propagated and seed crops held by the CGIAR and their selected partner genebanks; (2) compile and adapt best practices in a learning platform; (3) develop a query service for frequently asked questions on genebank management; (4) provide links to other related information and training sources; (5) develop mechanisms to update and develop new best practices for the management of other crops in genebanks and (6) build capacity of genebank curators and technical staff. The target audiences of the KB are genebanks, their curators, staff responsible for managing collections and beneficiaries, breeders, and academics involved in genetic resources conservation and training. For Activity 1.2 of the GPG2 Project, collections can be serviced via SGRP links to the four clonal crop centres' websites:

- Bioversity - ITC
- CIAT
- CIP
- IITA

which can be searched from http://singer.cgiar.org/index.jsp?page=collections or http://www.sgrp.cgiar.org/?q=node/164.

3.2 CGIAR's clonal crop genebanks *in vitro* conservation survey

The major objectives of the CCTF survey were to collate and make accessible information on the status of the CGIAR's *in vitro* conservation community and to facilitate cooperative action across the genebanks. The CCTF undertook their genebank evaluation in stages, commencing with a survey; this comprised four sections: (1) institutional information; (2) institutional facilities inventory; (3) generic methodologies and (4) specific conservation methodologies for MTS and LTS. The first clonal crop *in vitro* conservation survey was submitted to the CCTF, 31st August 2007, for completion by October 8th 2007. This allowed time to interrogate returns before a GPG2 workshop hosted by CIP, Lima, Peru during 12-16th November 2007. The event provided opportunities for the CCTF to arrive at consensus regarding clarity of the survey format and parity of reporting, omissions and superfluous entries. A revised clonal crop *in vitro* conservation survey (Phase 2) was re-submitted to the CCTF 20th December 2007 for completion. The CGIAR clonal crop centres completed the second survey by March 2008. These returns are collated in Tables 1-11 and they form the basis of this status report. A GPG2

workshop held by Bioversity, Rome in November 2008, identified the survey as a significant source of information for planning future validation exercises.

3.3 Developing *in vitro* genebanks for CGIAR'S clonal crops

This section provides wider context regarding the development of *in vitro* conservation best practices in CGIAR's clonal crop network, it reports on the background, status of facilities and conservation activities in each IVGB.

3.3.1 Bioversity International: *Musa* International Transit Centre (ITC)

The *in vitro* genebank of the International Transit Centre based at the KULeuven holds the International *Musa* Germplasm Collection. *In vitro* conservation of banana and plantain is a pivotal component of CGIAR's established *Musa* research and development programme (Sharrock and Engels 1996). Bioversity ITC currently holds the world's largest collection of *in vitro*-maintained *Musa* germplasm. Protocols for MTS and LTS were developed (INIBAP 2006) to complement traditional conservation strategies which use tissue culture to assist phytosanitary processing, disease indexing and the distribution of disease-free *Musa* germplasm (Sharrock and Engels 1996). Because domesticated bananas and plantains are seedless they must be vegetatively propagated, therefore *in vitro* conservation is crucial (Van den houwe et al. 2000). Methods and their rationales as applied at Bioversity ITC include:

1. Maintaining the *Musa* germplasm collection in MTS.
2. Rejuvenation of the *in vitro* collection:
    a. *Musa* accessions held in tissue culture are at risk from SCV.
    b. Rejuvenation of *in vitro* collections is thus a good management practice, the process involves growing plants *ex vitro* to assess trueness-to-type;
        i. Once this is confirmed new cultures are re-initiated;
        ii. After confirmation of trueness-to-type cultures are cryopreserved.
3. Screening and elimination of bacterial endophytes:
    a. A bacteriological test developed by the ITC is used to detect endophytes in banana shoot tips, it has been applied to the whole collection.
        i. As a quality assurance practice, screening for bacterial endophytes and their elimination is incorporated as a routine genebank activity.
        ii. This is undertaken at strategic points: (i) for newly acquired accessions; (ii) for any existing accessions subcultured for five storage cycles and (iii) for rejuvenated accessions before their reinitiation *in vitro*.
4. Cryopreserving the entire collection using the droplet-vitrification protocol (Panis et al. 2005) which is applicable to all *Musa*.
5. Germplasm distribution forms one of the most important functions of the ITC as it assures safe transfer of *Musa* germplasm.

Details of ITC methods are provided in Tables 2, 4 and 6 and their application is summarized in Table 9. In contrast to other crops, which have an extensive diversity, banana has a limited genetic base, with an estimated one thousand varieties. This makes it feasible to conserve the entire genepool (INIBAP 2006).

3.3.1.1 Medium-term storage

The use of tissue culture for the rapid clonal propagation and MTS of *Musa* was developed by Banerjee and de Langhe (1985) using shoot cultures established from excised shoot apices and applying reduced temperature (15°C) and low light intensity (1000 lux). Genotypes tested varied in their ability to withstand minimal growth temperatures, with most remaining healthy for up to 17 months. The AAB plantains ('Asamiensa', 'Agbagba' and 'Ntangu') and 'Bluggoe' (ABB genome) were relatively more tolerant to reduced temperature than Dwarf 'Cavendish' and 'Pisang nangka' (AAA genome). Vuylsteke (1989) produced the first practical manual pertaining to the *in vitro* culture, conservation and exchange of *Musa* germplasm. Currently, Bioversity ITC holds 1,182 accessions in MTS, no growth retardants are applied and growth limitation is achieved using low temperature (16°C) and a light regime of 25 μmol m^{-2} s^{-1} (24 h/24 h). Storage duration under MTS as previously informed by Banerjee and de Langhe (1985) and developed by Van den houwe et al. (1995, 2000) is dependent upon genomic group, ranging from 275 days for BBw to 390 days for AAA 'Lujugira-Mutika' subgroup; with a mean of 334 days across all genotypes. Surveillance of cultures held under MTS is undertaken on a monthly basis and cultures are assessed for viability, vigour, necrosis, chlorosis, blackening, hyperhydricity and contamination.

Screening for endophytic bacteria is a critical component of *Musa* MTS, particularly as growth limiting conditions can affect the detection of covert contaminants (Hamill et al. 2005, Thomas et al. 2008). At Bioversity ITC this involves the non-destructive testing of tissue on a broad-spectrum bacteriological medium with yeast extract and glucose (Van den houwe and Swennen 2000; Van den houwe et al. 1998). An incubation of 1-8 weeks at 28°C is implemented and testing is repeated at strategic points of the culture cycle. Decontamination by means of meristem culture is size-dependent for both *in vitro*-grown plants (1 mm shoots) and greenhouse plants (1-3 mm shoots). Monitoring genetic integrity involves visual observations performed on *in vitro* cultures and during the greenhouse regeneration of *in vitro* plants at 9 months. Regeneration is undertaken for *Musa* germplasm maintained in culture for >10 years and includes verification of identity and checking for SCV using field (type) comparisons with the original plant (if available) over two growth cycles. The process uses morphological descriptors, cytological assessment of ploidy status and molecular analyses. These assessments are precautionary measures, based on the knowledge that different types of SCV have been observed in *Musa* (Sahijram et al. 2003). Trueness-to-type testing at Bioversity ITC is combined with the rejuvenation of tissue cultures held in MTS. Critical factors requiring further consideration are: pre-storage conditions and cold acclimatization, optimization of the culture medium and temperature for certain genotypes. Tables 1, 2, 3, 4 and 6 summarize the current *in vitro* conservation methods used by Bioversity ITC.

3.3.1.2 Long-term storage

Cryopreservation of *Musa* germplasm has progressed using a range of explants (Panis 2009; Panis and Thinh 2001) and it supports both biotechnological and genetic resources aspects of *Musa* management and improvement (Abdelnour-Esquivel et al. 1992; Côte et al. 2000; Panis et al. 1990, 1996; Villalobos and Abdelnour 1992). Whilst LTS methodologies have been developed for cell suspensions (Panis 2008, 2009) the emphasis of the GPG2 Project is on cryopreserving *Musa* meristems. This is achieved via two morphogenetic routes: (1) proliferating, cauliflower-like meristem clumps, also termed 'scalps' and (2) apical

meristems excised from rooted *in vitro* plants (Panis 2008, 2009; Panis and Thinh 2001). Preliminary studies performed at KULeuven and summarized by Panis et al. (2000) reported slow cooling using DMSO as the cryoprotectant to be ineffective when applied to proliferating banana meristems, this was thought to be due to lethal extracellular ice crystallization. Similarly, encapsulation-dehydration was not a suitable method due to limited maximum recovery of only ca. 8%. It was thus concluded that shoots of *Musa* are sensitive to dehydration and controlled rate cooling and thus other cryopreservation strategies were considered.

Panis et al. (1996) developed a rapid freezing approach using meristematic clumps of proliferating *Musa* meristems cultured on medium supplemented with BAP and IAA. Three to six meristems were excised from 'cauliflower-like' clumps (scalps) and transferred to proliferation medium containing 0.1-0.75 M sucrose and/or placed on sterile, dry filter paper for evaporative dehydration for 2-4 h (to optimize pretreatment). Following osmotic conditioning the clumps were transferred to cryotubes and plunged into LN and thereafter rewarmed in a water bath at 40°C. Following protocol optimization, Panis et al. (1996) obtained post-thaw viabilities of 12-72% in the seven cultures tested. Genotype variation was attributed to morphological and physiological differences; ability to withstand freezing was linked to differential sensitivity to high sucrose correlated to drought tolerance in field conditions.

A PVS2-based protocol was applied to *Musa* shoot tips by Thinh et al. (1999) using shoot tips isolated from four-week old shoot cultures, for which apical morphology and stage of development was found to be a critical factor. Those more tolerant to PVS2 and LN had apical domes partially covered by 1-2 leaf primordia and these were preferentially selected thereafter. Dissected shoot tips were wrapped in tissue paper soaked in 5 ml of loading solution comprising 2 M glycerol and 0.4 M sucrose, after which they were transferred to PVS2 for cryoprotection and then plunged directly in LN. Rewarming and recovery entailed transfer to a 40°C water bath for 80 s, unloading in 1.2 M sucrose for 15 min and recovery on sterile filter papers (for 2 days) overlaid on basic medium with 0.3 M sucrose. Subsequently recovery was progressed on 3% sucrose in basic culture medium, for one month in reduced light, before transfer to standard conditions. Cryoprotective treatments were optimized for the loading solution (20 min at 25°C) and PVS2 treatments (20-30 min at 0°C), resulting in ca. 69% average survival, this was assessed as new shoot development. Microscopic examination revealed that the apical dome remained intact during the process and shoot regeneration occurred without callus. Ten genotypes were used to test the efficacy of the optimized protocol; all survived and demonstrated a shoot regeneration of 41 to 92%. As an alternative strategy, Thinh et al. (1999) recommended their optimized PVS2 protocol on the basis it is simple, and does not require a lengthy pretreatment phase on sucrose. Moreover, it supported high levels of shoot regeneration in surviving meristems, so long as explant preparation and cryoprotectant treatments are optimal.

Van den houwe et al. (2000) and Panis (2008, 2009) report the development of LTS protocols for *Musa* using three approaches. The first utilizes cauliflower-like, scalp meristems and the simple, rapid freezing protocol described by Panis et al. (1996). This produces a highly variable post-thaw recovery response which is genotype dependent ranging from 0% (for AAA highland bananas) to 75% (for ABB cooking bananas), the advantage of this method is its simplicity. The second protocol uses very small (1 mm in diameter) apical shoot meristems excised from rooted *in vitro* plants subjected to the PVS2 procedure developed by Thinh et al. (1999). The third protocol is a combination of the two and involves preculture of proliferating cauliflower-like meristem clumps on high sucrose medium followed by

treatment with PVS2 (Panis et al. 2000). Recovery ranged from 0-75% for method one, which is termed the simple freezing method (Panis et al. 1996) as applied to proliferating meristems. For method two, as applied to shoot meristems, recovery was 41-91% (Thinh et al. 1999) and for method three, as applied to proliferating meristems, 14-74% survival was achieved (Panis et al. 2000).

Subsequent studies by Helliot et al. (2002) demonstrated that PVS2 supported the survival of only a small number of cells in the meristematic dome and base of the primordia. Panis et al. (2000) highlighted the importance of limiting polyphenolic oxidation on proliferating meristems cryopreserved by the simple freezing method, for which recovery was improved by transfer to liquid medium which reduced the amount of non-regenerable callus. Panis et al. (2002) subsequently optimized the simple cryopreservation method for proliferating meristem cultures of banana by using sucrose preculture as the main cryoprotective strategy. This protocol was then applied to 26 banana accessions giving regeneration frequencies of 0-66%, although these outcomes were highly dependent upon the genomic constitution of the cultivar.

Ramon et al. (2002) correlated ratio of unstaturated:saturated fatty acid and putrescine content with enhanced survival of proliferating cultures of different banana cultivars associated with sucrose pretreatment. Proteomics is currently being applied to study responses of *Musa* germplasm to cryopreservation (Carpentier et al. 2006). In a study of banana meristems Carpentier et al. (2007) compared protein profiles of a dehydration-tolerant variety of *Musa* with that of a susceptible variety, finding a number of genotype-specific and differentially responsive proteins. This indicates that acclimation of the meristem proteome to osmotic stress involves an altered carbohydrate metabolism; the energy conserving glycolytic pathway possibly helps to maintain an osmoprotective level of intracellular sucrose. Carpentier et al. (2007) report sugar metabolism, cell wall integrity and ethylene signalling are involved in the osmotic protection of banana meristems. These factors might explain the genotype-specific differences regarding tolerance to dehydration that are incurred during cryogenic treatments and that may be associated with specific isoforms of enzymes involved in energy metabolism and proteins associated with stress adaptation.

The updating of technical guidelines for the cryopreservation of *Musa* developed by Panis and Thinh (2001) is completed (Panis 2009) and the recent version describes the droplet-vitrification method in detail. The PVS2-based protocol of Thinh et al. (1999) did not support sufficiently high levels of recovery and shoot regeneration for *Musa* germplasm held by Bioversity International ITC. The droplet-vitrification protocol involves pretreatment with sucrose and/or preloading with 2 M glycerol and 0.4 M sucrose, followed by cryoprotection in PVS2 micro-droplets. These are deposited on sterile aluminium foil surfaces which are directly exposed to liquid phase LN; the foils are subsequently transferred to cryovials pre-filled with LN. Rewarming involves placing the cryovials in a water bath at 40°C and applying a 1.2 M sucrose unloading solution, after which the shoot tips are recovered on filter papers. These are transferred to the surface of semisolid medium, on which they are maintained for 2 days before placing in standard culture medium; for the first week after retrieval from LN the shoot tips are maintained in the dark. The droplet-vitrification method was initially tested on sweetpotato shoot tips (Pennycooke and Towill 2001) before it was applied to *Musa* shoot tips. Agrawal et al. (2004) performed a comparative study of PVS2-based methods, the fast freeze/thaw method, now termed droplet-vitrification by Panis et al. (2005) was selected as the method of choice for *Musa* cryopreservation on the basis it is simple, user friendly and not labour intensive and, has comparable levels of survival to other

methods. Droplet-vitrification has been applied to all Musaceae (Panis et al. 2005) yielding on average, ca. 53% regeneration across 56 accessions, it is the method of choice at Bioversity ITC. Tables 1, 6 and 9 summarize the current LTS methods used by Bioversity ITC.

3.3.2 Centro Internacional de Agricultura Tropical (CIAT)

A milestone, four decades of agricultural research was celebrated by CIAT in 2007 (CIAT 2007a, b, c) concomitant with changes in the delivery of the Institute's activities as described in CIAT's Medium-Term Plan for 2008-2010. The status of CIAT's *in vitro* clonal crop conservation activities are collated in Tables 1-4, 5 and 10. The Pilot IVAG Project (IPGRI-CIAT 1994) remains a valuable source of information concerning the establishment and practical management of an *in vitro* conservation facility. It is an important precursor for GPG2's contemporary quality systems and best practices providing experience of "lessons learnt" to assist the *in vitro* conservation of other clonal crops. CIAT has also developed distance-learning packages for plant genetic resources conservation (Baena et al. 2007; CIAT 2007d). Mafla et al. (2007) have produced an on-line practical manual of general *in vitro* conservation procedures and it reports contemporary methods used by CIAT.

3.3.2.1 Medium-term storage: CIAT's pilot *in vitro* active genebank revisited

The collaborative project between CIAT and IPGRI (IPGRI-CIAT 1994) had the remit to report the activities of managing an *in vitro* genebank, providing a baseline of experience to assist other collections and crops. Importantly, the original logistics, planning and experimental design of CIAT's Pilot IVAG provides a tested template on which to develop contemporary best practices. It is thus justifiable to reiterate in this report, the original objectives of this landmark project, which were:

1. Selecting a condensed and representative sample of cassava genotypes from the 'World Cassava' collection held by CIAT and processing these samples *in vitro* under conditions of slow growth and to characterize the clones using morphological and biochemical traits.
2. Monitoring diseases, genetic stability and viability during slow growth in *in vitro* storage.
3. Determining the needs of laboratory facilities, equipment, consumable items and technical staffing involved throughout the operation of an *in vitro* genebank.
4. Providing guidelines and testing parameters for establishing and running an IVAG on the basis of experience gained from cassava.

The CIAT, Pilot-IVAG study (IPGRI-CIAT 1994) was undertaken on 100 cassava clones with various traits and representing different eco-geographical regions. They included all the morphological descriptors known for the crop to ensure the widest possible diversity for testing; on the recommendation of IPGRI, five replicates per accession were used to assess genetic stability. Disease indexing within the *in vitro* genebank involved predetermining the phytosanitary status of cassava mother plants using thermotherapy, meristem-tip culture and combinational (e.g. symptomology, ELISA, graft inoculation) virus testing. Morphological characterization was performed in the field genebank and in tissue culture and special attention was given to *in vitro* assessments by using descriptors for tissue cultures such as pigmentation, etiolation, different leaf shape, shooting and rooting. This was deemed important for monitoring health status of plants maintained under active and slow growth as it provides performance indicators for suboptimal storage. Isozyme electrophoresis was used to discriminate between the 100 clones and selected material from

in vitro conditions and intensive micropropagation was performed on disease-free material derived from meristem-tips that had undergone thermotherapy producing 50 replicates per clone for testing slow growth storage. Taking into account losses, 2,220 cassava cultures were introduced into *in vitro* storage of which 48 clones were represented in slow growth studies using low temperature and illumination as the limiting factors. Viability was evaluated systematically, every month, using performance indicators for contamination, browning (phenolic oxidation), defoliation, bleaching and death. Of the 48 cultures, 50% had to be subcultured after one year, 6 clones after 8-9 months and the remainder at 15 months. Genetic stability monitoring was a longer-term component of the project. General recommendations (IPGRI-CIAT 1994) were made as to the management of an IVAG using cassava as the model system, they give valuable insights into developing generic, technical multi-crop guidelines (Benson et al. 2011a, b) for the current GPG2 programme and include:

- Prospecting costs, efficacy and the value of using *in vitro* conservation before starting a programme and complementarity with other strategies.
- Thorough knowledge is required of *in vitro* behaviour and species-specific requirements are a prerequisite.
- Depending on the size, agronomic and economic importance of the collection, two levels of *in vitro* conservation are envisaged: (1) a fully implemented system and (2) a minimal system.
- The operational plan for the *in vitro* storage system should be designed to account for all steps procedures and data management required, including laboratory logistics and timing of technical help and requirements.
- Introduction of accessions into *in vitro* collections should be equated against risks of losing accessions and the possibility of introducing contaminated accessions into storage.
- If there is a phytopathological bottleneck due to the normally slow process of disease elimination, high multiplication rates need to secure pathogen-free collections.
- From the start, good quality phytopathological processes are advised.
- Genetic stability is an important condition of any *in vitro* conservation strategy.
- Should variants appear *in vitro* it should be determined if they are due to labelling and identity errors or genetic instability.
- Trueness-to-type should be corroborated by going back to type-field for which the Associated Field Genebank (AFG) provides the reference material.
- A field collection should exist for as long as the *in vitro* genebank has not been duplicated elsewhere for security reasons.
- Decisions regarding: number of replicates, size of vessels, risks of loss during *in vitro* multiplication, subculturing and storage will depend upon collection size.
- In the case of cassava, a minimum of one and a maximum of three culture replicates were lost, indicating a replication of 3-5 per accession is required.
- Depending upon collection size, a more sophisticated information system is required for labelling and tracking.

Whilst some of these recommendations are superseded by contemporary developments in technology and genebank management, they still offer a unique framework for

developing multi-crop guidelines for best practices (Benson et al. 2011 a, b). Furthermore, the Pilot-IVAG project and its continuing activities have generated a significant literature (Mafla et al. 1993; Roca et al. 1984, 1989, 1992). Including: (1) the assessment of factors important in minimal growth storage (Roca 1984; Roca et al. 1984) and the optimization of growth limitation to ameliorate stress in storage (Mafla et al. 2000, 2004; Roca et al. 1984); (2) a long-term stability study of cassava plants after retrieval from 10 years of *in vitro* MTS storage which confirmed the stability of plants held in the IVAG (Angel et al. 1996); (3) a cost analysis for maintaining cassava genetic resources in the field and *in vitro* (Epperson et al. 1997); (4) an assessment of the impacts of *in vitro* biology on small-scale cassava farmers in Latin America (Thro et al. 1999) and (5) a prototype for the larger-scale *in vitro* conservation of clonal crops (IPGRI-CIAT 1994; Roca et al. 1992, 2000). The current protocols used for MTS by CIAT are shown in Tables 1, 2, 4 and 10.

3.3.2.2 Long-term storage

The routine application of cryopreservation protocols for cassava shoot tip germplasm derived from *in vitro* cultures has been pioneered by CIAT (Escobar et al. 1997; Gonzalez-Arnao et al. 2008). Roca et al. (2000) outlined the progression in the technology at CIAT which was initiated in 1985 and resulted in a collaborative project with IBPGR for the cryopreservation of zygotic embryos and whole seeds of cassava. This was followed by the application of slow cooling for cassava shoot tips and a programme of activity during the period 1993 to 1998 in which both controlled rate and rapid cooling methods were tested on the cassava *in vitro* collection (Escobar and Roca 1997; Escobar et al. 1997); recovering plants were used for field and genotype stability testing. Thereafter, encapsulation-dehydration and vitrification-based methods were tested (Gonzalez-Arnao et al. 2008). This legacy provides a substantial knowledge base for developing cryopreservation methods for other clonal root and tuber crops for which the protocols tested by CIAT include the following outcomes:

- Colligative cryoprotection and controlled rate cooling
  - supporting plant recovery
- Colligative cryoprotection and rapid cooling
  - supporting plant recovery
- Droplet freezing
  - callus formation only
- Vitrification
  - phytotoxic/phototoxic
- Encapsulation-dehydration
  - supporting plant recovery
  - implemented for the core collection (619 clones)
- Encapsulation-vitrification
  - Under development for the lowest responding clones.

Different levels of genotype-dependent recovery were categorized by CIAT into cryopreservation response groups as follows:

1.	High Response Group	70% shooting, 26% of core collection
2.	Intermediate Response Group	30%-70% shooting, 30-70% of core collection
3.	Low Response Group	<30% shooting, 34% of core collection

The colligative cryoprotection, controlled rate cooling protocol was formulated by testing a range of different cryogenic and non-cryogenic parameters, optimized by Escobar and Roca (1997) and Escobar et al. (1997) as follows:

1. Explant: shoot tips 2 mm in height.
2. Preculture: in medium (C4) comprising 1 M sorbitol, 0.117 M (4%) sucrose, 0.1 M DMSO for 3 days in the dark at 26-28°C.
3. Cryoprotection with 1 M sorbitol, 0.117 M (4%) sucrose, 10% DMSO for 2 h on ice;
4. Tissue dehydration on filter paper for 1 h.
5. Controlled rate programmable freezing (CryoMed 1010) starting from a 5°C chamber temperature, a rate of 0.5°C/min to -15°C, and thereafter at a rate of 1°C/min to -40°C.
6. Immersion in LN.
7. Thawing at 37°C.
8. Sequential transfer recovery (2-days each) on medium containing (1) 0.75 M sucrose with 0.2% activated charcoal and (2) half-strength MS medium with 0.35 M sucrose and 5.56 x 10^{-3} M inositol in the dark; and standard culture medium under a light intensity of 15 μmol m^{-2} s^{-1}.
9. Evaluation of tissue viability and shoot growth after one month.

Growth conditions of the donor *in vitro* shoot cultures were modified using a lower temperature (21-23°C) and higher illumination (75 μmol m^{-2} s^{-1}) than for standard culture (Gonzalez-Arnao et al. 2008). Shoot tip size was a critical factor in recovery, which increased significantly when small (1-2 mm) shoot tips were used; dehydration at 26-28°C before freezing enhanced survival and shoot formation. Improved responses (18 to 20%) could be achieved by further manipulation of DMSO and sorbitol levels during preculture. Using this method, Escobar et al. (1997) repeatedly achieved 50-70% shoot regrowth and complete plant formation for several cassava cultivars. Genotype differences were attributed to preculture, cryoprotection and post-cryopreservation recovery rather than the freezing protocol. This methodology was reiterated by Escobar et al. (2000a) who comment that cryopreservation responses of cassava could be related to edaphoclimatic origin. Cultivars that survived cryopreservation were either more drought tolerant or, were adapted to subtropical conditions. Prolonged exposure to osmotic additives in the preculture medium reduced both shoot recovery and variation in different cultivars; these findings were linked to stress factors involved in the cryogenic treatments and different plant growth regulators, particularly the choice of cytokinin which affected plant recovery (Escobar et al. 2000a).

Escobar et al. (2000b) investigated the use of rapid freezing, vitrification and encapsulation-dehydration as alternative cryopreservation strategies, on the basis that rapid freezing is more cost and time effective as it involves direct plunging into LN as compared with controlled rate cooling. It is important that the cryoprotective strategy predisposes cassava shoot tip germplasm to vitrification otherwise survival after ultra rapid freezing may not be so effective. In the case of encapsulation-dehydration (Escobar et al. 2000b) cassava shoot tips were encapsulated in 3% calcium (Na) alginate beads, pretreated in sucrose medium for 3 days, desiccated over silica gel and plunged directly into LN. Subsequently the method was tested on five cassava cultivars, of which two genotypes recalcitrant to controlled rate cooling/colligative cryoprotection proved consistently amenable to

cryopreservation using encapsulation-dehydration. Less callusing occurred when this approach was used, implying that the route of recovery was less injurious, albeit sucrose preculture in liquid medium affected recovery and cassava shoot tips had a lower response when exposed to sucrose. To avoid this deleterious effect, sequential exposure to increasingly higher concentrations of sucrose was undertaken, resulting in enhanced shoot regeneration. Escobar et al. (2000c) recommend the encapsulation-dehydration protocol as an alternative to slow cooling using colligative and osmotic cryoprotectants because it is simpler, consistent and supported improved shoot growth from cryopreserved shoot tips. Ultra rapid freezing is considered economically effective, saving on personnel time and reducing costs of introducing the entire cassava collection into cryopreservation.

In addition to studies undertaken on vegetatively propagated cassava germplasm, cryostorage has also been applied to zygotic embryos and seeds of cassava (Marin et al. 1990). Both slow and rapid cooling gave high levels (ca. 97%) of survival for seeds and excised zygotic embryos although careful thawing was required to ensure seed shattering was minimized. Recovery of whole plants was achieved for the cryopreserved seeds; however excised zygotic embryos only produced 25-34% of plants compared to controls. The reason for this was attributed to dissection and manipulation stress rather than to cryogenic factors.

Roca et al. (2000) presented an integrated strategy for using cryopreservation as a complementary approach to managing cassava genetic resources at CIAT. It was envisaged that only core and sub-core collections are maintained in the field at any one time, while all the clonal collections are maintained all the time, in the IVAG under conditions of slow growth. In addition, cassava can also be conserved as base collections containing seed and pollen germplasm, thus providing another means of securing the genepool. In this design, cryopreservation is an integral component in maintaining the base collections as all types of genetic resources can be secured in cryobanks, including: shoot tips, pollen, seed and frozen leaf tissue and other samples that can provide sources of DNA for DNA libraries. The status of cryopreservation methodology at CIAT is shown in Tables 1, 2, 7 and 10.

3.3.3 Centro Internacional de la Papa (CIP)

In February 2008, CIP was awarded the Accreditation Certificate Testing Laboratory No. 4299 by the UK Accreditation Service (UKAS) in accordance with the International Standard ISO/IEC 17025:2005. This is a major milestone for developing Quality Assured (QA) practices in CGIAR's genebanks. Progress in establishing quality systems at CIP has been substantial and supported by the installation of high-performance computer facilities and information management systems using barcodes to track the movement and processing of germplasm (Table 1). Advances in the formulation and application of *in vitro* storage technologies at CIP span several decades (CIP, 2006, 2007; Espinoza et al. 1986, 1992; Estrada et al. 1986; Golmirzaie and Panta 1997a, b, 2000; Golmirzaie and Toledo 1998, 1999; Golmirzaie et al. 1999, 2000a, b; Gonzalez-Arnao et al. 2008; Lizarraga et al. 1989, 1992; Panta et al. 2006, 2007a, b). These activities are linked with phytosanitary control and disease indexing for the safe conservation and exchange of clonal genetic resources (Lizarraga et al. 1991) and successful protocols have been devised for the storage of *in vitro* tubers and the slow growth of shoot cultures. However, shoot meristem cryopreservation still requires more development, this is mainly due to the highly variable genotype responses to different protocols. The status of CIP's *in vitro* clonal crop conservation activities are presented in Tables 1-3, 5, 8 and 11.

3.3.3.1 Medium-term storage

Medium-term storage of potato germplasm at CIP is undertaken using sorbitol (2-4%) as the osmotic growth retardant and cultures are maintained at 18-22°C, either for 1 year or for 2 years at 6-8°C, in a 16 h light/8 h dark photoperiod (Golmirzaie et al. 1999). Medium-term storage for sweetpotato uses a medium supplemented with ascorbic acid and putrescine, in combination with a relative low temperature treatment of 18-22°C for 10-14 months. For oca (*Oxalis tuberosa*), ulluco (*Ullucus tuberosus*), and mashua (*Tropaeolum tuberosum*), MTS is undertaken using 3% sorbitol and culture at 18-22°C for about 1 year. Performance and post-storage assessments for MTS are undertaken for contamination, rooting and viability. These are assessed every 3-4 months using plant health descriptors and semi-quantitative observations; currently genetic stability assessments are not undertaken routinely for potato held in MTS. Genotype variability is a common limiting factor, affecting the capacity to conserve potato, sweetpotato, oca, ulluco, mashua, achira, yacon and arracacha in MTS. Polyphenolic oxidation is particularly problematic in oca, as is hyperhydricity in potato and sweetpotato; poor rooting occurs in sweetpotato and arracacha, and endophytic contamination prevails in sweetpotato, achira and yacon. Protocols are established for identity verification (authentication) in CIP's MTS genebank which uses SSR or AFLP-based DNA fingerprinting and comparisons of clones of virus tested, versus original material maintained *in vitro* and/or the field and greenhouse. DNA-authenticated clones are virus tested and confirmed by trueness-to-type inspection. Clones with different DNA fingerprints undergo a morphological comparison test using CIP descriptors, databases, bibliographic sources and donor information. Decisions as to retention and distribution in the genebank are based on true-to-type and virus elimination outcomes. Specifically, potato germplasm is assessed using 10 SSR primers using an LI-COR high-throughput genotyping system and 22 morphological descriptors; sweetpotato is monitored using 3 AFLP primer combinations with silver staining and 17 morphological descriptors; oca and ulluco are assessed using 14-15 and 28 morphological descriptors respectively. The status of MTS protocols at CIP is summarized in Tables 1-4, 8, 10 and 11.

3.3.3.2 Long-term storage

The development of LTS for potato germplasm held by CIP has progressed through several phases (Golmirzaie and Panta 2000; Gonzalez-Arnao et al. 2008) commencing in the period 1993-1995 during which time a collaboration with Cornell University applied a vitrification protocol developed by Steponkus et al. (1992). This method involved cryoprotection using a mixture of 50% ethylene glycol, 15% sorbitol and 6% Bovine Serum Albumin (BSA) and vitrification in 0.25 ml polypropylene straws containing 70 µl of cryoprotectant solution (Golmirzaie et al. 1999). The protocol was tested on a range of genotypes with various ploidy levels and by making modifications to the original protocol, 75% of the tested genotypes were successfully recovered with an average survival of 46% (Golmirzaie and Panta 1997a, b). Survival after 3 months of cryogenic storage was the same as for initial survival on first exposure to LN and storage stability was thus confirmed for 80 genotypes. A major limitation of this protocol was variability across diverse genotypes of which about 30% did not survive and 30% had levels of survival <15%. After cryopreserving 100 accessions, Golmirzaie et al. (1999) identified various limiting factors in the cryopreservation of potato at CIP, including plant vigour and type and condition of shoot tips (apical were more resilient than axillary) and all these factors were major bottlenecks to LN storage. Seven genotypes were used to test the effects of vigour, showing that survival could be increased from 31 to

67% by selecting apical shoots from vigorous plants. A delayed decline in post-recovery responses was observed in shoot meristems of some surviving genotypes and this proceeded for up to 45 days after rewarming. Golmirzaie et al. (1999) and Golmirzaie et al. (2000a, b) suggested death could still occur several weeks after an initial survival response; supplementing the recovery medium with vitamins and amino acids improved recovery by 10% in seven genotypes. Structural studies revealed abnormal cytoplasm and cellular structures in cryopreserved shoot tips (Golmirzaie et al. 2000a, b). In 1996, CIP undertook a collaborative project with DSMZ-FAL Germany, IPGRI and GTZ for the transfer of the droplet freezing method. During 1997-1999 CIP tested various cryopreservation protocols, creating a cryopreserved experimental collection of 385 accessions and including longer-term studies of storage stability after one year (Golmirzaie et al. 1999, 2000a, b).

During 2000 to 2002, genetic stability assessments of cryopreserved genotypes were initiated at CIP and in the period 2003-2005 a cryopreserved collection of native cultivars was established. Currently, (2003-to date) the droplet-vitrification method is being assessed (Table 5). The protocol involves selecting 3-week-old plantlets grown at 22°C and excising 1.8-2.5 mm shoot tips which are treated with a loading solution for 15-20 min (see Table 5). This is followed by 50 min exposure to PVS2 at 0°C to 4°C and ultra rapid cooling on aluminium foils as described by Panis et al. (2005). On rewarming, shoot tips are incubated in sucrose unloading solution followed by step-wise reduction in sucrose in culture medium over several days. Panta et al. (2006) undertook a comparison of the original vitrification of Steponkus et al. (1992) with the droplet-vitrification method developed by Panis et al. (2005). This included a comparison of delivery in straws and direct exposure to LN in aluminium foils; post-thaw recovery was genotype dependent, varying from 47% for *S. tuberosum* 'cv' 'Desiree', to 8% for Wila Yari, but independent of freezing method. This study also found cold acclimation enhanced recovery and that sugar treatments had no or a negative effect. Based on the outcomes of Panta et al. (2006), CIP is currently using PVS2-droplet vitrification (Panis et al. 2005) as a routine protocol (Panta et al., 2007a, b). This has been applied to four genotypes of oca and ulluco, achieving 8% recovery with a PVS2 treatment of 60 min and to *Ullucus tuberosus* 'olluco', securing 32% recovery after 60 min exposure to PVS2. It is projected at least 30% (equivalent to 1,300 accessions) of the potato collection maintained at CIP will be cryopreserved (Gonzalez-Arnao et al. 2008). A systematic study of sweetpotato cryopreservation was introduced by Golmirzaie et al. (1999, 2000a, b) based on methods developed by Towill and Jarret (1992), Blakesley et al. (1995, 1996), Steponkus et al. (1992) and Schnabel-Preikstas et al. (1992). Outcomes affirmed method modification and optimization based on Schnabel-Preikstas et al. (1992) was the most promising approach which involved preculture in sucrose and using the vitrification solution of Steponkus et al. (1992). Currently studies are testing droplet-vitrification for the cryopreservation of sweetpotato at CIP.

Clonal true-to-type verification of 22 potato accessions held by CIP has been undertaken using plants regenerated from *in vitro* and cryopreserved germplasm (Perazzo et al. 2000). Two accessions showed differences in multiple morphological characters, suggesting cases of misidentification, of the remaining accessions differences were observed for 10 descriptors associated with flowering and colour.

3.3.4 International Institute of Tropical Agriculture (IITA)

Advances in technology and capacity building for the conservation and use of *in vitro* propagated clonal crop germplasm at IITA were highlighted as major achievements in the Institute's review (IITA 2007a). IITA has a pivotal role in the GPG2's collective action across the CGIAR's clonal crop repositories as it shares in common, three out of the five major clonal crop groups. Safeguarding clonal crop germplasm is part of IITA's mission to enhance the food security and the income and well-being of people in sub-Saharan Africa (IITA 2001). Currently, IITA's genebank holds over 28,000 accessions (http://www.iita.org 20.6.2008) of which those attributed to the clonally propagated *in vitro* crops include: cassava (2,712 accessions); yam (3,200 accessions) and *Musa* (250 accessions). During the GPG2 project, IITA has transferred over 2,300 accessions of cassava, 500 accessions of yam and 250 accessions of *Musa* from the field to the *in vitro* genebank which currently maintains 4,186 accessions of these crops (IITA 2007a). Safe duplication of 2,350 accessions of the *in vitro* collection has also been established by IITA in the Bénin genebank based in Cotonou. Concomitantly, during 2007 the wild Manihot field genebank was field-rejuvenated and the Germplasm Health Unit of IITA has developed and applied phytosanitary procedures and molecular diagnostics for the storage, production and distribution of *in vitro* plants. A significant quality assurance outcome has been the formulation of best practice manuals for the *in vitro* processing and genebanking of cassava (IITA 2007b) and yam (IITA 2007c). The status of IITA's *in vitro* clonal crop conservation activities are presented in Tables 1-4, 8-10.

3.3.4.1 Medium-term storage

The IITA has engaged in *in vitro* conservation and distribution of root and tuber crop germplasm for several decades (Ng, 1991). Protocols for MTS are applied to yam and cassava and a cryopreservation capability is currently under development. Field genebanks are the traditional conservation approach for yam genetic resources, although they are constrained by space, maintenance time, disease, and pest problems. These can cause a significant loss of genetic resources and Ng and Ng (1996) were thus instrumental in developing *in vitro* approaches to circumvent these problems, including *in vitro* tuber production (Ng 1988) and reduced growth storage (Ng and Ng 1991; Ng and Ng 1997; Ng et al. 1999). There have been contemporary developments in *Musa* technology transfers to IITA, as related to the improvement of banana and plantain production in sub-Saharan Africa (IITA 2007a).

Medium-term storage for yam at IITA was largely undertaken by S.Y.C Ng and N.Q. Ng (Ng 1991, 1992; Ng and Ng 1997, Ng et al. 1999), they used osmotica, low temperature and nutrient limitation to extend subculture intervals to ca. 12-13 months. Summaries of the MTS protocols used to maintain root crop germplasm at IITA are provided by Ng (1991, 1992). Disinfected explants, meristems and node cuttings are first placed in culture medium (Ng and Hahne, 1985) and cultured at 25-30°C and 4000 lux in a 12 h light/2 h dark photoperiod. After 3-4 weeks nodes develop into plantlets which are transferred to reduced temperature culture rooms (18-22°C) at 3000 lux with the same 12 h photoperiod, noting that without details of light quality it is not possible to convert older units to contemporary photon flux measurements. The cultures are stored for 8-24 months and checked for deterioration which is managed by transfer to new medium. Ng (1991) used meristem culture techniques for the elimination of viruses and reported field accessions of sweetpotato and cassava maintained *in vitro* for 6-7 years had the same morphological characters as control materials. Cassava and yam regimes involve plantlet culture at reduced temperatures at 18-22°C (day-night); in contrast, to sweetpotato (Ng 1991) plantlets are maintained on 3% sucrose and 3% mannitol

and/or with a reduced temperature regime of 18-22°C (day-night). Sucrose applied in combination with low temperatures effectively reduced growth, although significant genotype differences were apparent for the various treatments. Contamination and blackening of yam explants are significant problems in establishing cultures and using excised meristems reduces contamination. On first transfer to the genebank, yam cultures are inspected every week for signs of necrosis and contamination; once stabilized, checks are normally undertaken for cassava and yam at 6-weekly intervals. Subculturing is required every 1-24 months or, when accessions show obvious signs of deterioration and/or when stock becomes low and there is a need for multiplication (IITA 2007c).

Slow growth methods for cassava implemented at IITA are similarly based on protocols developed by CIAT (CIAT 1980; Mafla et al. 1993; Ng and Ng 1997; Roca 1984) and validation exercises between the institutes are in progress. Monitoring yam cultures in MTS is undertaken by visual screening of contamination and necrosis every 6 weeks, the average time between subculture intervals is 1-2 years. Black box monitoring at Bénin occurs at 6-8 week intervals. Monitoring is undertaken using the same regime as for yam and the time between subcultures ranges from 6-18 months (CIAT 2007b). Accessions showing no obvious signs of deterioration and/or when MTS stock becomes low they are sent for multiplication. Further optimization of *Musa* MTS is currently in progress at IITA. Status of protocols and storage regimes at IITA as compared to other CGIAR clonal genebanks are collated in Tables 1-4, 8-10.

3.3.4.2 Long-term storage

Cryopreservation of yam was initiated in IITA during 1996 (Ng and Daniel 2000; Ng et al. 1999) and cryopreservation of cassava in 1998 (IITA 1998; Ng and Ng 2000), methods included pretreatment and cryoprotection with DMSO and direct immersion in LN. Preliminary results were presented by Ng and Ng (2000) using cassava genotypes: TME2, TME3, 160142, 170775, 163397 and M86/00106 as well as yam genotypes: TDr179 and TDr608 (*D. rotundata*), TDb3058 (*D. bulbifera*) and TDa1170 (*D. alata*). Shoot tips of cassava were excised from *in vitro* plantlets precultured for 3 days on medium containing 0.7 M sucrose (Ng and Hahn 1985) and similarly for yam (Ng 1992) after which they were transferred to PVS2 solution containing 0.7 M sucrose and cryoprotected for 20 min before being transferred to cryovials and direct immersion in LN. Samples were rewarmed at ambient temperatures of ca. 28°C or in a water bath at 40°C and rinsed with washing medium and cultured on 0.7 M sucrose shoot culture medium for 3 days under dark conditions before transfer to standard growth regimes. Recovery after cryopreservation was genotype dependent and for cassava ranged from 60-85% and for yam 25-75%. Critical factors in the recovery of cassava were the duration of exposure to PVS2 and rewarming regime, for which faster rates enhanced survival. Explant physiology influenced survival and in general for cassava, shoot tip meristems had higher levels of recovery compared to nodal cuttings. Survival of yam shoot tips was also genotype dependent and dependent upon duration of cryoprotectant treatment. In addition to shoot meristem cryopreservation, Ng and Daniel (2000) reported the successful preservation of yam pollen at -80°C for 2 years.

Limiting factors presently identified for the development of yam *in vitro* conservation include meristem culture, bacterial detection, offsetting losses, optimization of growth rates, stability assessment and cryopreservation. For cassava, optimization of growth rate in MTS, germplasm stability assessment and cryopreservation are key factors for future development. *Musa* MTS and LTS protocols require optimization for slow growth, germplasm stability assessment and cryopreservation. Protocols used by IITA are collated in Tables 1-4, 8-10.

4. Collective critical point analysis of *in vitro* genebanks

Critical point analyses aid cooperative action across dispersed communities of practice by highlighting decisive steps in conservation procedures (Day et al. 2007; Reed et al. 2001, 2004). This approach facilitates the effective implementation of protocols and methods (Reed 2008b) on a routine basis in large-scale genebank operations (Keller et al. 2008a). The aim of this section is to analyse GPG2 Clonal Task Force Survey returns. These are collated in Tables 1-11 and they can be used to help identify those components that are the most influential for achieving successful storage outcomes. Robust critical point assessment should encompass all aspects of genebanking practices and it is prudent to factor-in risk management (ISBER 2005, 2008; OECD 2007). Developing efficient and robust *in vitro* multi-crop storage methods (Benson et al. 2011a, b) requires an holistic strategy that evaluates the whole procedure, from germplasm selection to fit-for-purpose performance testing after recovery.

4.1 Facilities and instrumentation

All four clonal repositories meet basic growth room and MTS requirements (Table 1) although cryogenic facilities vary regarding type of equipment (e.g. cryotanks, LN supply Dewars, programmable freezers). Risk management is implemented with different stringency across the genebanks and several factors have been identified:

- Controlled environment surveillance, safety/hazard alarms.
- Contingencies in case of failure in growth, cold and growth rooms.
- Security and reliability of LN supply;
    - LN level monitors and surveillance routines for cryostores.
    - In house storage back up, in the event a main cryobank becomes compromised and requires its inventory to be transferred to another locally cited Dewar.
- O_2 atmosphere safety monitors for personnel.

Previous critical point assessments of storage validation exercises for other clonal crops pinpointed differences between growth room parameters can influence recovery after storage (Benson et al. 2011a). These factors may require careful consideration in developing multi-crop guidelines across different institutes (Benson et al., 1989, 2011a, b; Keller et al. 2006; Keller et al. 2008a; Harding et al. 2008, 2009). Differences between programmable freezer models and manufacturers affect cryopreservation protocol validation (Benson et al. 2005; Reed and Uchendu 2008; Reed et al. 2001) and this should be considered when controlled cooling methods are applied for some genotypes across different institutions (Escobar et al. 1997; Roca et al. 2000). Diagnostic, molecular and analytical amenities for phytosanitary treatment authentication and genetic stability testing are variable across the genebanks (Table 1). They are undertaken in house at CIAT and CIP; outsourced at Bioversity ITC and under are development at IITA. Differences between types of facilities, instruments and procedures can be accommodated by careful validation exercises which account for local variations (Benson et al. 2011a; Day et al. 2007; Reed et al. 2001, 2004). These variables should not be considered as limiting factors, rather, they can help to develop robust protocols and best practices that are capable of withstanding differences in local practices and facilities.

4.2 Data and process tracking

Table 1 indicates parity and/or moving towards equivalence (e.g. IITA) across genebanks with respect to operating systems, physical and virtual infrastructures, however, brands of database, inventories and barcode systems are not common across genebanks. Harmonization of different tracking and inventory systems may become a critical point in inter-genebank collective actions for commonly held crops. Implementation of electronic tracking systems in CGIAR's clonal crop genebanks is supportive of developing quality assured systems and directly links records keeping to traceability.

4.3 Generic methodologies and procedures

These methods (Table 2) comprise basic germplasm selection procedures, phytosanitary and tissue culture manipulations (inclusive of *in vitro* and *in vivo* monitoring) and they are applied to germplasm before and after MTS and LTS. Critical point factors include germplasm source, mother plants and type of explant and all can affect responses to different storage regimes, especially the conversion of surviving meristem to plants following shoot tip cryopreservation (Bajaj 1987; Harding et al. 1991, 1994, 2008, 2009; Henshaw et al. 1985; Keller et al. 2008a, b; Yoon et al. 2006). Assessment of phytosanitary status is crucial and generic across all activities in the IVGB and a potential problem is that covert, endophytic and systemic organisms can disrupt procedures at all levels. It is noted that the monitoring routines for tissue culture performance are undertaken with variable stringency across the genebanks, this is achieved using descriptors for physiological condition and health status (Table 2) for example, *in vitro* blackening and browning, due to polyphenolic oxidation (see Benson 2000a, b) occurs to some extent in germplasm held by all genebanks. Genetic stability assessments using a range of molecular methods and *ex vitro* regeneration cycles are performed by Bioversity ITC and CIAT. Glasshouse regeneration of *Musa* tissue culture variants is performed by Bioversity ITC as a check for instability (Table 2).

4.4 Medium-term storage

Summaries of MTS activities across the centres are categorized into individual crops (Table 3) and crops held in common (i.e. *Musa* and cassava) by more than one CGIAR genebank (Table 4). Also indicated, are duplicate and Black Box collections that are safeguarded on a reciprocated basis by other CGIAR clonal crop genebanks and their associated partners. All mandated crops and the majority of their genotypes are conserved in MTS, with the exception of a few wild species which require optimization of culture regime to extend their subculture cycles (e.g. the ARTCs, Table 3). The most common, critical MTS point factor across the CGIAR genebanks is the detection and elimination of covert contamination before and during slow growth. This is most prevalent in yams, sweetpotato and the ARTCs (Table 3) and in *Musa* held by IITA (Table 4) and this indicates the importance of bacterial indexing from the point of culture initiation, before cultures are placed in MTS and thereafter. There is a need for regular and vigilant surveillance throughout slow growth cycles, optimally this should include regular bacterial indexing after several subcultures (e.g. Bioversity ITC, *Musa*, Table 4). Lack of rooting and stress-induced symptoms of hyperhydricity, deleterious oxidation phenomena (variously described as blackening, browning, and polyphenol oxidation), etiolation, chlorosis and necrosis, together with loss of vigour and shoot/root proliferation are problematic (see Benson, 2000a, b). These physiological indicators are semi-qualitative; quantitative descriptors and

regular monitoring offsets germplasm loss through contamination and for sensitive accessions stress symptoms are performance indicators that may help to improve suboptimal protocols.

Variation in genotype response is limiting for some *Musa*, sweetpotato and yam, (Table 3) and several cassava accessions are recalcitrant to standard storage protocols (Table 4). Notwithstanding the wide genetic base of crops across the CGIAR's clonal crop genebanks, variable responses to MTS might be best considered an inherent issue. This should be addressed to ensure optimal protocols are developed for a comparatively small fraction of the more problematic germplasm as compared to the greater proportion of responsive MTS accessions. This would be expected for the conservation of diverse genotypes within the ARTC collection and other crop wild species and landraces. Genetic stability assessments, verification and authentication have various levels of stringency and they are variable in their implementation across the IVAGs (Tables 3 and 4). They range from long-term assessments, including descriptor, phenotypic, biochemical and molecular analyses undertaken by CIAT, to new monitoring strategies currently in progress at IITA.

4.5 Long-term storage

The routine implementation (Tables 5-7) of cryopreservation is undertaken for *Musa* at Bioversity ITC; cassava at CIAT and potato at CIP; a cryobanking capability is currently under development at IITA (Tables 5-7). Historical progress in the use of different LTS protocols across the CGIAR's IVBGs has paralleled advances in cryopreservation research, consequently different cryogenic methods have been variously applied at different times. The longest-established cryobank at CIAT (Table 7) has achieved greatest success using encapsulation-dehydration which is now a routine procedure for the cassava core collection (Roca et al. 2000). Some recovery has been realized for cassava using controlled rate cooling/colligative cryoprotection (Escobar et al. 1997); in contrast, vitrification and droplet freezing methods proved less amenable because they do not support acceptable levels of survival and regenerant quality is poor. CIAT is currently investigating encapsulation/vitrification as an alternative protocol for less responsive cassava genotypes. Bioversity ITC uses the droplet-vitrification protocol (Panis 2008, 2009; Panis et al. 2000, 2005) for routinely cryopreserving *Musa* shoot meristems derived from two different sources (Table 6). Due to the ease and efficiency of *Musa* meristem processing the preferred explant is proliferating meristem clumps (Panis and Thinh 2001). This choice contrasts with using meristems from original apical shoots, for which excision is laborious and requires good technical competency. A number of different cryopreservation protocols have been applied to potato, but none offer a desirable level of success or reproducibility. Droplet-vitrification is now used as the method of choice (Table 5) for the CIP's base collection although several genotypes remain recalcitrant. Research projects are currently examining cryopreservation for sweetpotato and ARTCs at CIP. Four genotypes of oca, ulluco and *Ullucus tuberosus* Loz. 'olluco' have been tested by using a 60 min exposure to PVS2; this treatment supported 8% recovery in oca and ulluco and 32% recovery in *U. tuberosus*.

4.6 Collective actions for multi-crop *in vitro* conservation strategies

A main objective of the GPG2 Project is consolidating collective actions for the validation and implementation of *in vitro* conservation protocols as best practices across the CGIAR's clonal crops IVGBs. This is integral to, and the charts progress towards developing multi-crop guidelines (Benson et al. 2011b). To facilitate the process, cooperation across the repositories is shown in Table 8; primarily they involve reciprocation of partial duplicated back up collections (Black Boxes), collaborative research, training and informal technology transfers.

4.6.1 Potato, *Musa* and cassava

Tables 8, 9 and 10 collate activities related to these crops across the CGIAR system. For cassava, MTS black box duplication is either in place or in progress between CIAT, CIP and IITA; duplication for *Musa* is in progress and/or established in Bioversity ITC and their associated partners. Inter-centre research collaborations are active across all the CGIAR centres mandated to conserve potato, banana and cassava. This provides opportunities to facilitate formal protocol validation and cross-cutting exercises for best practices and risk management. The status of the number of accessions stored *in vitro* in the CGIAR IVGBs is shown in Table 9 (for *Musa*), Table 10 (for cassava) and Table 11 (for potato).

4.6.2 ARTCs, yam and sweetpotato

The *in vitro* conservation status of ARTCs, yam and sweetpotato in the CGIAR's clonal genebanks is collated in Tables 8, 9 and 10. Black Box duplication for sweetpotato MTS is established between CIP and CIAT. A yam black box is established between IITA-Nigeria and IITA-Benin. Initiatives are currently ongoing for collective action for these crops across the CGIAR network (Table 8). Status of the number of ARTCs, sweetpotato and yams accessions stored *in vitro* in the CGIAR IVGBs is shown in Table 11.

4.7 Multi-crop research, training and technology transfer

Collaborative research across the IVGBs is underpinned by training and technology transfers (Table 8) and is optimally implemented using virtual and practical technology transfers via the GPG2 virtual Knowledge Base, reinforced by training materials and handbooks (Baena et al. 2007; CIAT 2007d). It is important for CGIAR's clonal crop community of practice to be updated with state-of-the-art methodologies and best practices from both within and outside the system.

4.8 Consensus for validation and best practices

The significance of validating best practices across biorepositories is highlighted by Smith and Ryan (2008) in the context of the Organization for Economic Co-operation and Development (OECD) guidelines (OECD, 2007). The International Society for Biological and Environmental Repositories (ISBER) has produced generic guidelines for various types of biorepository (ISBER 2005, 2008). Collectively, these bodies offer common guidance for biorepositories to ensure their biological materials are authenticated and of the highest quality. It is thus timely for the CGIAR genebanks to consider their basic standards for example:

1. Compliance with national and international rules, regulations and policies.
2. Good laboratory design and procedures.
3. Handling, authenticity, preservation and distribution procedures.

4. Data recording, validation, access and accuracy of labelling.
5. Auditing and accreditation procedures.

Tables 2-7 suggest various permutations of protocol validation are possible within the CGIAR's IVGBs. Exemplars based on the standard validation formats of Smith and Ryan (2008) may include:

1. Protocol validation between IVGBs sharing common crops ('internal' validation).
2. Protocol validation across IVGBs which do not share common crops ('external' validation).
3. Reciprocated validation of different protocols developed by different CGIAR IVGBs and applied to the same crops.
4. Reciprocated validation of different protocols developed by different CGIAR IVGBs and applied to different crops.
5. External third party validation of CGIAR IVGB protocols outside the CGIAR network.
6. Internal third party validation by the CGIAR IVGBs of protocols developed by non-CGIAR Centres.

Validation facilitates reaching consensus on the technical detail of crop-specific best practices and the formulation of multi-crop generic guidelines and critical point analyses can help to calibrate protocols to local conditions (Benson et al. 2011a). This concurs with the recommendation of the OECD (2007) that task forces involved in developing biorepository guidelines should first undertake pilot studies. These should be constructed by collective consultation across communities of practice in order to assist:

1. The validation of best practices and assessment of applicability in each biorepository.
2. The assessment of the impacts of best practices on existing operations.
3. The identification of the range of available options for each individual IVGB in order to adopt protocols as best practices, and for which local calibration may require some changes to ensure effective and efficient in house use.
4. Preliminary cost-benefit analyses for adopting best practices, particularly those imported by partner and associated institutions which may have different economic constraints (e.g. for labour, consumables, equipment and LN supplies).
5. Risk management of newly imported best practices.

4.8.1 Consensus for risk management: critical points for IVGBs

Achieving consensus in risk control is important across CGIAR's clonal crop repositories working towards creating robust multi-crop guidelines. Three main critical points can be identified: (1) special storage facilities; (2) containment and contamination and (3) *in vitro* stability/authentication.

The first relates to maintenance of stringent environment control (particularly temperature) in culture rooms, cold stores holding germplasm in MTS, and cryostore LN tank level and supply from top up Dewars (Table 1). Reaching consensus in the type and stringency of safety facilities (e.g. surveillance, alarms) required and adverse incident mitigation procedures is prudent. For example, an onsite generator assures CIP's LN supply,

so long as the facility is adequately maintained. At Bioversity ITC, the external supplier is reliable, however, CIAT cautions that their regular LN supply can sometimes be problematic and this may have significance for the dual-phase inventory in both liquid and vapour phases of the cryobank. Vapour phase storage is a critical point factor for stabilizing glasses, particularly for PVS2-treated germplasm (Volk and Walters 2006) and consequently the security of LN supply and delivery is crucial. Deleterious Tg changes during shipment should not be overlooked as dry shippers operate in the vapour phase (Benson 2008b, Volk and Walters 2006).

The second critical point is containment and the limitation of contamination risks throughout all IVGB procedures (Chart 1). Risks due to pathogenic organisms, viruses and viroids will be largely offset by the stringent practices connected to national and international phytosanitary regulations and quarantine. However, nuisance covert and adventitious microorganisms can be highly persistent and are a pernicious problem in CGIAR's *in vitro* genebanks. This is due to a resilience to standard sterilization and culture-detection treatments which makes covert contaminants a potential problem (Cassells 1991). Indexation, eradication and monitoring for covert micro-flora and security of containment at each stage of *in vitro* genebanking are essential. Charts 1-3 highlight potential contamination risks at critical points of basic procedures used in IVGBs; for continuity, the risks are identified in the charts using collated information from the survey returns (Tables 1-11).

***In Vitro* Facility/Generic Procedure**	**Potential Contamination Risk & Consequences**
(1) Institution (2) Infrastructures-Facilities	Failure of autoclave, laminar flow bench, clean room containment, inadequate skills training in aseptic techniques
(3) Generic Methodologies (3.1) General Pre-Storage Assessments	Risk variable dependent on source: soil-derived high risk, *in vitro* source from another *in vitro* genebank low risk
(3.1.1) Source of Material & Processing	Non-axenic material potentially infected by pathogen/pest external microbial flora, epiphyte, endophyte (systemic), asymptomatic-covert infection, spores, endospores
(3.1.2) Starting Material	Variable propensity for successful surface sterilization, risk germplasm loss with severe treatments (phenolic oxidation) dormant tissues harbour persistent nuisance microflora
(3.1.3) Physiological Status	Older explants and cultures have complex flora, at cumulative higher levels persistent nuisance microflora and spores increase risk of covert infection
(3.1.4) Phytosanitary Status	Failure in compliance with quarantine, containment and phytosanitary regulations-health inspection, stringent virus indexing disease-free certification critical
(3.1.5) Standard Tissue Culture Maintenance Regimes	Older explants/cultures have complex and higher levels of persistent nuisance microflora, increased risk of covert and latent infections emergent months after initiation, revealed if culture cycle extended due to process bottlenecks and transfers to regeneration medium, covert contaminants interfere with viability-vigour assessment
(3.2) Generic Germplasm Treatments Post-Storage Assessments	
(3.2.1) Viability & Regeneration Assessments	
(3.2.2) Genetic Stability Assessments	Covert contaminants interfere with molecular analysis (PCR at risk)
(3.2.3) Field Performance Testing	Germplasm loss due to contamination disables field trials-covert pathogen released to environment

Chart 1. Critical point evaluation of contamination risks associated with generic procedures used for the *in vitro* conservation of clonal crop germplasm.

Covert and nuisance systemic (endophytic) infections that are not removed during pathogen testing are more problematic for materials sourced directly from the field (Towill 1984). Differences occur in the prevalence of nuisance microflora in germplasm derived from glasshouse, field and *in vitro* conditions (Niedz and Bausher 2002), disparities in contamination can affect storage efficiency of samples from different donor types. The most effective control of bacterial contamination requires a concerted strategy that includes indexing (Van den houwe and Swennen 2000), good cultivation practices and storage hygiene (Janse and Wenneker 2002). Endophytes harboured in explants from perennial tissues (Ulrich et al. 2008) have variable, idiosyncratic culture responses which can interfere with standard microorganism detection procedures (Cassells 1992; Thomas et al. 2008). Inadequate clean up during culture initiation causes germplasm loss down-stream, during slow growth; some microbial flora in plant cultures have complex symbiotic or commensal associations that can become deleterious to the plant during long-term culture under suboptimal conditions (Bunn and Tan 2004; Cassels 1991; Hamill et al. 2005). Chart 2 highlights potential contamination risks in MTS of *in vitro* plant germplasm held in IVAGs.

***In Vitro* Active Genebank (IVAG)**	**Potential Contamination Risk & Consequences**
(1) Institution (2) Infrastructures-Facilities	Failure of autoclave, laminar flow bench, clean room containment, inadequate skills training in aseptic techniques
(4) Specific Conservation Methodologies *In Vitro* Active Genebank (IVAG)	Failure of pre-genebank testing phytosanitary processing to eradicate pathogens, nuisance flora and covert/latent endophytes
(4.1) Crop Collections Maintained Under Medium Term Storage (MTS) Slow Growth	Integrity of the collection is compromised if any one of the accessions is contaminated, risking cross-contamination highest risk from covert, latent and systemic microflora
(4.2) Specific Protocols for MTS	Low temperatures, growth inhibitors and osmotica change (asymptomatic symptoms) contaminant growth, covert organisms and spores remain quiescent, latent infections emerge with time stressed, necrotic, hyperhydric tissues alter microflora status, from benign endophytes to opportunistic pathogens and spoilage, organisms, sealants on culture vessels fail over long periods allowing entry of adventitious contaminants
(4.2.1) Storage Protocols & Procedures	
(4.2.2) Monitoring	Visual observations inadequate for detection of latent and covert organisms and spores, explant streak plates (swabs) may produce false negatives if contaminant growth is arrested by slow growth regime
(4.2.3) Biosecurity & Safety	
(4.2.4) Recycling after Storage	Risk of covert and latent cross-contamination spread in duplicate black box collections, failure to discard contaminated cultures
(4.2.5) Additional Information	Covert/latent contamination discovered on recycling in new medium, failure to remove-destroy contaminated cultures

Chart 2. Critical point evaluation of contamination risks associated with operations in the *In Vitro* Active Geneban

Cryopreservation protocols comprise multi-component manipulations, some of which are technically laborious such as meristem dissection; these procedures can potentially compromise containment, leading to adventitious contamination and the transmission of covert and endophytic infection between samples (Charts 1-3). As such LTS requires

stringent aseptic control, assurance of the physical integrity of the cryogenic supply chain is a critical point factor for IVBGs (Benson, 2008b). Recovery from cryopreservation is an inherently stressful process and it can confer an opportunistic advantage on covert, systemic and endophytic microorganisms (Chart 3) and this can lead to tissue necrosis and death (Liu et al. 2005). Long-term cryobanking risks the gradual accumulation of microbial flora in the cryotank (Benson 2008b) particularly in high humidity environments, as ice formed during LN dispensing and the removal and samples in and out of the cryotank can entrap microorganisms from the laboratory atmosphere. Morris (2005) recommends preferentially locating cryotanks in clean rooms, and advises the dispensing of top-up LN within the confines of clean facilities to reduce risks of adventitious contamination. In clonal crop cryobanking this may become significant for germplasm held in LTS and cryovial containment can be facilitated by sealants developed by manufacturers specializing in cryobank security (Chen et al. 2006). Dry shippers require careful operational procedures to ensure cold-chain integrity and disinfection between uses (Bielanski 2005).

***In Vitro* Base Genebank (IVBG)**	**Potential Contamination Risk & Consequences**
(1) Institution (2) Infrastructures-Facilities	Failure of autoclave, laminar flow bench, clean room containment, inadequate skills training in aseptic techniques, contaminated: cryogenic equipment, LN supply and dispensing area
(4.3) Crop Collections Maintained Under Long Term Storage (LTS) Cryopreservation	Failure of pre-genebank testing and phytosanitary processing to eradicate pathogens, nuisance flora and covert/latent endophytes
(4.4) Specific Protocols for LTS (4.4.1) Storage Protocols & Procedures explant type, protocol type, pre-growth, cryoprotection, cryogenic treatment, (various) storage phase vapour or liquid	Integrity of collection compromised if any one accessions is contaminated, risking cross-contamination highest risk from covert, latent and systemic microflora Pre-treatment- osmotica change alters growth of covert organisms lengthy, complex dissections, cryoprotective manipulations risks aseptic technique, increases cross-contamination risk, failure of cryoprotectant filter-sterilization sugars and DMSO promote growth of covert/latent microflora direct exposure to LN cryovial, cryotank rupture and (explosion) of cryovials risks adventitious and cross-contamination, accumulation of microflora in cryotanks on long-term storage LN liquid phase storage increases risks of adventitious and cross-contamination, increases with time in storage, inappropriate cryovial selection for LN-phase resistance risks loss/or explosion of contaminated cryovial in cryotank probabilistic tools do not account for loss from contamination
(4.4.2) Probabilistic Tools for Successful Storage	
(4.4.3) Biosecurity & Safety	
(4.4.4) Recovery from Storage	Pathogens, nuisance flora, covert/latent endophytes proliferate in stressed tissues which on recovery risks germplasm loss and cross-contamination
(4.4.5) Additional Information	Cryo-therapeutic treatments may give false negatives

Chart 3. Critical point evaluation of contamination risks associated with operations in the *In Vitro* Base Genebank (IVBG).

The third critical factor is genetic stability and authentication (Tables 2-7) for which three basic approaches are undertaken by CGIAR's clonal crop genebanks:

1. Pre-storage screening to confirm identity and assess the risk of SCV.

2. Responsive checks, when culture abnormalities or dubious growth habits suggest SCV or a mistaken identity;
   a. For clarification, a responsive check is action taken that is related to an incident; for example, the production of an abnormal culture without identifiable reason due to mislabelling, or a media problem.
   b. Responsive checks are in addition to standard, continuous surveillance procedures.
3. Routine and strategic checks to verify authentic status and stability after: (a) a set number of culture cycles; (b) on regeneration, and (c) before requested cultures are dispatched to a third party.

Authentication prioritization is undertaken at CIP for: (a) accessions most frequently requested for international distribution; (b) landraces requested for distribution and (c) breeding material requested for distribution. Routine monitoring of cassava by CIAT is after 1 hour and 1 month of cryostorage, followed by reconfirmation of authenticity/stability at 6 months. Sometimes, cassava longevity checks are more regular (3, 9, 12 months) and they are combined with genetic stability assessments using morphological descriptors, isozymes and AFLP analysis (Table 7). Genetic stability monitoring is not routine for cryopreserved potato or *Musa*.

4.8.1.1 Decision support tools for choice of storage protocol

Decisions as to which protocols to apply are important for both slow growth and cryopreservation; their cost effectiveness and safety incurs different advantages and disadvantages. Diverse genotypes will be adapted for intrinsic tolerances and sensitivities to storage treatments such as desiccation, dehydration and chilling. As no one method is applicable across all crops (Tables 6-7), an important critical point is making the appropriate choice of protocol(s) to ensure security and stability of the genetic diversity held within CGIAR's clonal genebanks. This is particularly important for cryopreservation for which variation in genotype responses has been accommodated by using three basic strategies:

1. Applying the same protocol to different types of germplasm, as is the case for *Musa* held by Bioversity ITC. Both direct and proliferating shoots are selected dependent upon genotype amenability to culture and cryopreservation (Table 6). For example, proliferating 'scalp' shoot meristem excision is less labour intensive and more cost effective. The standard droplet-vitrification protocol is considered effective for the majority of genotypes, but may be suboptimal for a few.
2. Using different cryopreservation protocols, on the basis that one or more will be applicable to the majority of the accessions; this is exemplified by the strategy used for cassava at CIAT (Table 7). Encapsulation-dehydration is used as routine for the core collection, but controlled rate cooling and encapsulation-vitrification are alternatives for accessions/genotypes that are not amenable to encapsulation-dehydration.
3. Optimizing protocols on a case-by-case basis for accessions/genotypes highly recalcitrant to cryopreservation (Tables 5 and 7). This approach takes into account physiological status, pretreatments and testing of different cryoprotection strategies. This approach is being applied to potato by CIP in the optimization of cold acclimation to enhance recovery; it is used in tandem with the first strategy described above for droplet-vitrification.

Justification of personnel time and resources also influences protocol choice and includes the overall cost-benefit analysis of using suboptimal procedures. But it is important to bear in mind that these may: (1) compromise genetic diversity through selection processes; (2) enhance an existing predisposition to epigenetic/genetic instability and (3) incur further losses of germplasm due to stress-intolerance. A useful probabilistic tool has been developed by Dussert et al. (2003) to facilitate decisions as to the minimal recovery for germplasm to be safely stored in cryobanks. This is routinely used by Bioversity ITC for *Musa* (Table 6) and for potato at CIP (Table 5) and is based on the probability of one or more plants (i.e. a target number) being regenerated as 0.95 per repetition. Viability is tested on the same day the germplasm is cryopreserved. In contrast, CIAT (Table 7) takes a different approach, in accepting 30% shoot regeneration for cassava as the minimal threshold for recovery from the cryobank. To facilitate decision-making for storage choice it may be helpful to examine the experiences of other bioresources sectors for example, Ryan et al. (2000) produced a decision-based key to determine the most appropriate protocol for preserving fungi.

The droplet freezing method has been applied to all potato genotypes held in a large-scale genebank (Keller et al. 2008a) on the basis that it is more efficient and cost effective to accommodate a cryostorage method, suboptimal for some genotypes but amenable for the majority, providing one or a few survive. However, it is prudent to manage very carefully the risks associated with germplasm conserved by suboptimal methods. For low recovery, it is important to offset further losses of a few survivors due to: (1) delayed onset culture/meristem death (Baust et al. 2007; Harding et al. 2008, 2009); (2) lack of shoot regeneration in survivors (Harding et al. 2009); (3) covert and non-culturable contamination (Benson, 2008b); (4) cryoselection and genetic instability (Scowcroft 1984) and (5) epigenetic changes whether persistent, deleterious or beneficial (Harding 2004; Harding et al. 2009; Johnston et al. 2009). Assessment of critical point factors may thus benefit from intercalating risk management with best practice development (Benson et al. 2011a, b).

5. Conclusions: Lessons learnt and priority research needs

One of the practical objectives of GPG2 Activity 1.2 is to '*Draw on the techniques and experience available for banana, potato and cassava, and analyse the lessons learnt and apply them to other crops. This involves the identification of priority research needs to further refine and standardize protocols, and apply them to overcome constraints in the storage of sweetpotato, yam and Andean roots and tubers (ART)*'. Tables 12 and 13 summarize the basic protocols currently used by CGIAR's *in vitro* genebanks and indicates the generic lessons learnt. All crops are routinely maintained in slow growth with the main variable in subculture extension being genotype. Strategies developed for cassava include the incorporation of silver nitrate in the MTS medium to improve culture performance. This approach is being tested for cassava, in a validation exercise that involves testing CIAT protocols in IITA. The Pilot Genebank Model developed for cassava at CIAT thus provides significant information as to the logistics of applying MTS.

The situation for LTS is more complex because many different protocols have been developed and tested on CGIAR's clonal crops but despite a substantial undertaking of cryopreservation research, its routine application in CGIAR genebanks still remains limited. However, contemporary research and protocol refinements offer potential to increase CGIAR's cryobank holdings. This is particularly evident using the lessons learnt regarding *Musa* droplet-vitrification (Table 13) although it is important to acknowledge the various levels of success achieved in non-CGIAR genebanks and research laboratories that have applied droplet-freezing, encapsulation-dehydration, vitrification and encapsulation-vitrification to potato, sweetpotato, yam and cassava. In the case of IPK, droplet freezing is used routinely for potato, but transfer of this and other protocols to CIP has been limited. This may be due to the wider genotype range and diversity of potato genetic resources held by CIP which severely limits the applicability of any one cryostorage protocol. Currently, the droplet-vitrification method developed for *Musa* by Bioversity ITC is being adapted for potato, sweetpotato and ARTCs at CIP and the protocol will be similarly tested for yam at IITA. The development of controlled rate cooling and encapsulation-dehydration protocols for cassava LTS at CIAT provides two complementary approaches for conserving germplasm from differentially responsive genotypes.

Priority research should also include fundamental studies into the reasons that underpin successful and unsuccessful responses to different storage protocols. Proteomics knowledge and techniques applied to *Musa* cryopreservation by Bioversity ITC is providing useful information on stress physiology and this may be applicable to other crops. Similarly, studies of non-cryogenic factors, particularly donor and explant physiology should be prioritized as these parameters can have a significant effect on survival and recovery.

An overarching priority is the construction and undertaking of validation exercises as these enable technology transfers of *in vitro* storage protocols and help to confirm that they are fit-for-purpose across all CGIAR genebanks. This process has been initiated within the remit of the GPG2 Project, although the possibility of one common protocol being applicable as a best practice across all crops is limited, this is mainly due to variable crop and genotype responses. It may therefore be more practical to develop a number of protocols as 'standard operating procedures' which can be validated for different crops across different genebanks;

on the basis that if one protocol is not effective, then alternatives are available. Finally, it will be important to prioritize activities that continue to support cooperative actions as this is crucial for robust risk management and the upholding of quality genebank standards, and best practices in the clonal crop community of practice.

Tables

Table 1. Summary of *in vitro* conservation facilities for MTS and LTS in CGIAR's clonal crop genebanks

Facility	Bioversity ITC	CIAT	CIP	IITA
General facility growth rooms	***Musa***: No.1 ground surface 9m², shelf surface 11.3m², 26-28°C; No.2 ground surface 16m², shelf surface 19.5m², 26-28°C	**Cassava**: No.1 17.2 m² (16 metallic shelves); No.2 6.3 m² (10 metallic shelves); No.3 8.37 m² (for cryopreservation)	**Potato**: 18-22°C, 41.62 m² **ARTC**: 18-22°C, 35.10 m² **Sweetpotato**: No.1, 23-25°C, 34.15 m²; No.2: 22-24°C 14 m² **Cassava**: **safety back-up** 22-24°C, 10.50 m²	**Cassava, *Musa* and Yam** 5 light-temperature controlled rooms, No.1: 12.6m², No.2: 17.55m², No.3: 17.25m², No.4: 14.88m², No.5: 15.62m² (aluminium-iron shelving)
Cold chambers	Ground surface 26m², shelf surface 40m², 15-17°C	No.1 47.92 m² (41 metallic shelves); 6.3 m² (10 metallic shelves); No.2 (safety back-up for sweetpotato)	No.1 **Potato and ARTC**: 6-8°C, 54.65 m² No.2 **Sweetpotato**: 19-21°C, 38.95 m²	2 cold rooms at 18°C ± 2 (35 m² total)
Acclimatizing chambers	None	No.1 Percival Model E-36L(MTS) No.2 Percival Model E-36L(LTS)	No.1 **Potato**: 32-34°C No.2 **Sweetpotato**: 34-37°C No.3 **ARTC**: 28°C No.4 *In vitro* quarantine	1 small pre-chamber 1 insect proof, air conditioned screen house (30 m²)
Alarm systems	Cold chamber alarmed	Smoke/temperature alarms	Yes	No, T°C data logger to check temperature stability
MTS storage capacity	No. accessions =1404 No. tubes/accession = 20	No. accessions = 9840 No. tubes/accession = 5	**Potato**: No. accessions = 11 231 No. tubes/accession = 3 **Sweetpotato**: No. accessions = 5541 No. tubes/accession = 6 **ARTCs**: No. accessions = 1137 No. tubes accession = 3 – 10	**Cassava, *Musa* and Yam** No. accessions = 7000 No. tubes/accession = 10 (seedlings each) for genebank area (cold rooms)

Facility	Bioversity ITC	CIAT	CIP	IITA
Table 1(cont'd)				
Inventory system (database) and tracking (bar-code) IT systems	MSAccess/SQL and VB for Pocket PC	ORACLE database bar-code system in place	MSSQL 2000 Database mobile computers PDAs - Pocket PCs Symbol Model: MC-50. System operative: Microsoft® Windows Mobile 2003 2nd Ed	Web-based Java application hosted on Ubuntu Linux server with replicated MySQL 5 for data storage, industry-strength Zebra label printers, Symbol **bar-code scanners and network**
Other facilities	Tropical greenhouse *In vitro* lab (culture media preparation room, transfer room, 1 laminar air flow)	None	Media preparation area 44.98 m^2; Subculture areas: No.1 **Potato**: 40.56 m^2; No.2 **Sweetpotato**: 43.37 m^2; No.3 **ARTC**: 39.09 m2 Washing area 30.18 m^2 thermotherapy area 17.82 m2	Media preparation laboratory: 40 m^2 Laminar flow room: 50 m^2 Storage room: 40 m^2
Cryogenic Programmable freezers	Cryocool CC-60, Exatrol and agitainer, Neslab, Portsmouth, New Hampshire, USA	Cryomed 1010	10K cryostorage system (Taylor Wharton)	None
LTS storage capacity (No. tubes)	2 x locator 6 plus Thermolyne (2 x 4860 2ml tubes) 1 x locator 4 plus Thermolyne (1 x 3240 2ml tubes)	1 x Tank Model CMS-450A	2 x Tanks 130 litres 1 x Tank 34 litres	**Cassava, *Musa* and Yam** Two LD50 50 litres each (6 canisters) One 750RS 750 litres (6 canisters)
Liquid nitrogen safety level alarms	LN level indicator + alarm on each tank	None		
Liquid nitrogen suppliers	Offsite commercial provider Air Liquide	Offsite commercial provider	Self-supply CRYOMECH LN Generator 40 litres/day	BOC Gases sourced from Ibadan or Lagos

Facility	Bioversity ITC	CIAT	CIP	IITA
Table 1(cont'd)				
Cryogenic (cont'd) Other facilities	*In vitro* lab (culture media prep room + transfer room with 2 laminar air flows)	None		
Analytical instruments	Fluorescence microscope FDA-viability tests, histological and microscopical facilities	None		
Molecular diagnostics genetic stability tests	Cytology/DArTs/SSR markers contracted to specialized laboratories	Genetic Quality Laboratory compares *in vitro* and field plants using morphological, biochemical (isoenzymes) molecular markers	2 high-throughput genotyping systems LI-COR, 1 molecular marker lab for DNA extraction and PCR, 2 labs for electrophoresis- silver staining	Molecular stability/identity diagnostics under development
Genotype verification authentication	Cytology/DArTs/SSR markers morphological characterization contracted to specialized laboratories	Genetic Quality Laboratory identifies genetic duplicates and redundant accessions using morphological, biochemical (isoenzymes) and molecular markers	Comparison of pathogen-tested clone with originals *in vitro*- and field-conserved clones using SSR and AFLP markers and morphological descriptors	As above
Phytosanitary control disease indexing	ELISA/PCR/ISEM/EM contracted to specialized laboratories	Germplasm Health Laboratory ELISA and grafting	For potato and ARTC: NASH, ELISA serological test, and indexation by indicators plants for detecting all known viruses. For sweetpotato: NCM-ELISA, symptomatology observation and grafting stem cuttings into the indicator plant *Ipomoea setosa*	Visual, biochemical and molecular tests
Virus therapy	Thermotherapy-chemotherapy contracted outside to specialized laboratories	Thermotherapy chamber	Thermotherapy and meristem culture	Meristem Thermotherapy in development (cryo and hot temperature)
Others	Bacterial indexing, diagnostics facilities	None	1 unit (Partec FloMax)	None

Table 2. Summary of generic *in vitro* conservation methodologies in CGIAR's clonal crop genebanks

Method/protocol	Bioversity ITC	CIAT	CIP	IITA
Pre-storage source	***Musa***: field genebanks, plants/seeds from collecting missions, as tissue cultures from in *vitro* genebanks	**Cassava**: apical buds of crop genotypes zygotic embryos wild species	**Potato**: apical or axillary buds **Sweetpotato**: apical or axillary buds **ARTC**: Achira, buds from rhizomes. Arracacha buds from corms Mashua, Oca, Ulluco, Yacon, apical or axillary buds	***Musa***: meristems taken from suckers directly from the field genebank **Cassava**: buds taken from either thermo-treated cuttings or from 4-6 week old screen house grown plants **Yam**: meristems, from screen house plants and *in vitro* seedlings; nodal cuttings from screen house plants and dry tuber sprouts
Germplasm type	Non-axenic and axenic (*in vitro*) meristems from original or proliferating shoots	Zygotic embryos, *in vitro* shoot meristems	Rhizome, corm, tuber, apical, and axillary buds	***Musa***: meristems from suckers **Cassava**: buds and meristems **Yam**: meristems and buds from *in vitro* and *in vivo* plants and tuber sprouts
Physiological status (time to produce *in vitro* cultures after initiation, germplasm storage selection criteria, monitoring performance indicators and observational descriptors for cultures)	4-6 months after initiation tested for microbial contamination negative result retained for culture selected for vigour, **descriptors: not selected if tissues have symptoms of: necrosis, are blackened due to polyphenolic oxidation, contaminated or chlorotic	3-4 months after initiation some wild species are tissue culture recalcitrant and have rooting problems bacterial indexation for microbial contamination and if a negative result retained for culture **descriptors: contamination, leaf senescence index (ratio green:dead leaves), No. green shoots suitable for further micropropagation, viable green nodes, relative to green stem elongation, rooting, callus, dead cultures	4-6 weeks after initiation tested for microbial contamination negative result retained for culture **Potato**: rooting problems in some cases **Sweetpotato**: bacterial contamination, retarded growth, poor rooting **ARTCs**: browning, poor rooting, retarded growth **descriptors: plantlets free of bacteria and fungi, good rooting and vigour	***Musa***: 3-4 months after initiation checked for contamination and necrosis **Cassava**: 3-6 months after initiation. Performance indicators/descriptors not applied, any accession or meristem failed to elongate within 3 months of introduction *in vitro* is discarded **Yam**: meristem cultures 6-18 months, nodal cuttings 3-6 months. Culture performance not tested, sub-optimal culture conditions for many accession and recalcitrance to existing media

Method/protocol	Bioversity ITC	CIAT	CIP	IITA
Table 2 (cont'd)				
Phytosanitary status	Sub-contracted outside *in vitro* genebank and in accordance with international plant protection guidelines	'In house' CIAT-Plant Health Laboratory, quarantine, in association with Colombian national authority and in accordance with international plant protection guidelines	'In house' CIP-Plant Health and Quarantine Unit, in association with Peruvian national authority and in accordance with international plant protection guidelines	'In house' by IITA's Pathology Unit and in accordance with Nigerian national authority and international plant protection guidelines
***Culture maintenance**	***Musa*** standard culture medium* photoperiod 24h light 63µmol $m^{-2}s^{-1}$ temperature 26-28ºC	**Cassava** standard culture medium* photoperiod 12h/12h light-dark, 18.5 µmol $m^{-2}s^{-1}$ temperature 26-28ºC	**Potato, Sweetpotato and ARTCs** standard culture media* photoperiod 16h/8h light-dark, 3000 lux **Potato-ARTCs** 18-22ºC **Sweetpotato** 22-23ºC	***Musa*, Cassava and Yam** standard culture media* photoperiod 12h/12h light-dark,, 30 µmol $m^{-2}s^{-2}$ temperature 24-28ºC
Culture assessments	Viability/regeneration **descriptor observations *n vitro***	Viability/regeneration **descriptor observations *in vitro***	Viability/regeneration **descriptor observations *in vitro***	Viability/regeneration **descriptor observations *in vitro*** Observation on full plant performed during indexing tests
Genetic stability	Cultures putative for somaclonal variation regenerated in glass house, observed for vegetative development over 9 months, cytological ploidy testing	Isozyme analysis, molecular markers, DNA fingerprinting of SSRs	None	Yam: Molecular tools for somaclonal variation detection under development
Field trials	None	Field regeneration assessed by standard descriptors	None	None

*Culture media are prepared in accordance with crop-specific technical guidelines published by the respective CGIAR IVGBs (Panis 2008; IITA 2007a,b; Mafla et al. 2007; Panta et al. 2007);

**Descriptor observations as described by *in vitro* physiological status assessments; SSR = Single Sequence Repeat markers.

Table 3. Summary of MTS methodologies for individual crops in CGIAR's clonal crop genebanks

Protocol step	CIP-Potato	CIP-ARTC	CIP-Sweetpotato	IITA-Yam
Germplasm type	*In vitro* cultures of wild species, landraces, improved varieties, breeding materials	*In vitro* shoot cultures of wild species and landraces derived from: **Oca**, **Ulluco**, **Achira**, **Yacon** and landraces of **Arracacha** and **Mashua**	*In vitro* shoot cultures of wild species, landraces, improved varieties and breeding materials	Seedlings
Temperature/light regime	6-8°C, 1000 lux	**Oca** 6-8°C, 1000 lux **Ulluco** 6-8°C, 1000 lux **Mashua** 6-8°C, 1000 lux **Achira** 18-22°C, 2000 lux **Yacon** 18-22°C, 2000 lux **Arracacha** 18-22°C, 2000 lux **All ARTCs** light/dark photoperiod 16h/8h	19-21°C, 2000 lux, photoperiod 16h/8h light/dark	18±2°C, light 30 µmol $m^{-2}s^{-2}$ photoperiod 12h/12h light/dark
Media (growth retardants)	20-30 g/L sorbitol	**Oca** 30 g/L sorbitol **Ulluco** 30 g/L sorbitol **Mashua** 30 g/L sorbitol **Achira** None **Yacon** None **Arracacha** None	None	None
Vessel	25 x 150 mm tubes	25 x 150 mm tubes	18 x 150 mm tubes	16 x 125 mm tubes 16 x 125 mm polyethylene bags
No. replicates/accession stored	3 (4 explants each)	**Oca** 3 (3 explants each) **Ulluco** 3 (3 explants each) **Mashua** 3 (4 explants each) **Achira** 10 (1 explant each) **Yacon** 7 (1 explant each) **Arracacha** 7 (1 explant each)	6 (2 explants each)	5 tubes and 5 polyethylene pocket per accession in genebank 5 tubes per accession Cotonou genebank (safe duplication)
Storage duration (time of subculturing extension) months	Average = 24 Min = 8 Max = 36	**Oca, Ulluco, and Mashua** Average = 18, Min = 12, Max = 20 **Achira, Yacon, and Arracacha** Average = 3, Min = 3, Max = 4	Average = 10 Min = 6 Max = 12	Minimum = 12 months Maximum = 24 months

Protocol step	CIP-Potato	CIP-ARTC	CIP-Sweetpotato	IITA-Yam
Table 3 (cont'd)				
Monitoring General performance	Every 2 months Stem necrosis: Good, 0-10%; Medium, 10-30%; Bad, 30-70%; Lost-dead, 100%	Monthly **Mashua**: Stem necrosis: Good, 0-10%; Medium, 10-30%; Bad, 30-70%; Lost-dead, 100% **Yacon, Achira and Arracacha:** Stem necrosis/defoliation: Good 0-10%/0-20%; Medium, 10-30%/20-50%; Bad, 30-70%/50- 70%; Lost-dead 100%/100% **Oca** and **Ulluco**: Oxidation-browning (in culture medium/foliage), stem necrosis: Good, none 0-5%; Medium-light, 5-30%; Bad, Medium–high 30-70%; Lost-dead High 100%	Monthly Shoot/stem necrosis/defoliation: Good, 0-10%/0-20%; Medium, 10-30%/20-50%; Bad, 30-70%/50-70%; Lost-dead, 100%	Every 6 weeks for necrotic and contaminated cultures
Contamination	Indexing for endophytic bacteria, evaluated every 3 months during multiplication and conservation. Positive visual result (cloudy, white or coloured colonies in medium) bacteria elimination process activated, accessions with doubtful contamination are submitted to bacterial detection: biopsy tissue from 1 tube/accession is tested, negative test outcome tube returned to storage, positive outcome test repeated with another clone of same accession			For severe contamination problems cultures dispatched to CABI if identification not resolved in house
Genetic stability Verification (authentication)	*High-throughput genotyping system 10 SSR primers using LI-COR and 22 morphological descriptors	***Oca:** 14-15 morphological descriptors **Ulluco:** 28 morphological descriptors	*3 AFLP primer combination using silver staining; 17 morphological descriptors	Under development
Regeneration frequency	Every 2 years	**Oca, Ulluco, Mashua,** every 18 months, **Achira, Yacon Arracacha,** every 3 months	Every year	1-2 years

Protocol step	CIP-Potato	CIP-ARTC	CIP-Sweetpotato	IITA-Yam
Table 3(cont'd)				
Biosecurity and safety	3365 accessions *in vitro* and duplicated at INTA-Castelar Argentina 465 accessions in cryobanks at CIP	CIP-Huancayo: field accessions: **Oca, Ulluco, Mashua** 80%, **Achira, Yacon**, 70% and 0% for **Arracacha;** *in vitro* accessions: **Oca** (521), **Ulluco** (500), **Mashua** (52), **Achira** (18), **Yacon** (36), **Arracacha** (10)	4493 *in vitro* accessions and duplicated at CIAT Colombia	5 tubes per accession backed up duplicated in Cotonou genebank and monitored every 6-8 weeks
Recycling after storage	I transfer (80% of collection) 2 transfers (20% of collection)	All ARTCs 1 transfer expected recovery time 1 month	I transfer (70% of collection) 2 transfers (30% of collection) expected recovery time 1 month	Seedlings roots in MTS culture, transferred to peat pellets in plastic bags, elongating within 3-4 weeks. Planted in plastic pot containing sterile soil, re-enclosed in bags, at 30-50 cm, opened, regeneration in screen house
Loss of accessions per year	4	**Oca** (1) **Ulluco** (1) **Mashua** (0) **Achira** (1) **Yacon** (1) **Arracacha** (0)	6	2-3 %
Limiting factors	Genotype response and hyperhydricity very important	**Oca**: genotype response very important, polyphenolic oxidation important **Ulluco, Mashua:** genotype response important **Achira:** genotype response important, endophytic bacterial contamination very important **Yacon:** endophytic bacterial contamination, and genotype response important **Arracacha:** poor rooting extremely important	Endophytic bacterial contamination, and hyper-hydricity very important genotype response and poor rooting important	Endophytic bacterial contamination

Protocol step	CIP-Potato	CIP-ARTC	CIP-Sweetpotato	IITA-Yam
Table 3 (cont'd)				
Sub-optimal regimes	Yes for potato hybrids	None	None	Yes for some genotypes
Strategies for MTS Improvement	Improve protocols for wild and hybrid accessions	**Oca, Ulluco**, **Mashua, Yacon, Achira**: optimization of low temperature and light intensity; **Achira, Yacon**: endophytic bacteria treatment; **Arracacha**: improve rooting	Cold acclimation, agar optimization	Meristem processing on a species-by-species basis

*At CIP, materials are subjected to identity verification according to priorities:
(1) Accessions most frequently requested for international distribution;
(2) Landraces requested for distribution;
(3) CIP's breeding material requested for distribution; using molecular and/or morphological methods the pathogen-tested clone is compared with originals *in vitro*- and field-conserved clones. When differences between clones are detected, mislabelled clones are identified by comparing *in vitro* and field clones with CIP germplasm descriptors database and donor information. Technical procedures are undertaken in accordance with crop-specific technical guidelines published by the respective CGIAR IVGBs (Panis 2008; IITA 2007a, b; Mafla et al. 2007; Panta et al. 2007).

**Descriptor observations as described by *in vitro* physiological status assessments; SSR = Single Sequence Repeat markers.

Table 4. Summary of MTS methodologies for crops held in common in CGIAR's clonal crop genebanks

Protocol step	Bioversity ITC-*Musa*	IITA-*Musa*	IITA-Cassava	CIAT-Cassava
Germplasm type	Multiple shoot cultures	Meristem derived seedlings	Meristem derived seedlings	Nodal cuttings, apical buds
Temperature/light regime	25 µmol $m^{-2}s^{-1}$ 24h illumination temperature 16ºC	30 µmol $m^{-2}s^{-1}$ 12h/12h light/dark temperature 18±2ºC	30 µmol $m^{-2}s^{-1}$ 12h/12h light/dark temperature 18±2ºC	18.5 µmol $m^{-2}s^{-1}$ 12h/12h light/dark temperature 23-24ºC
Media (growth retardants)	None	None	None	None ($AgNO_3$)
Vessel	25(diameter) x 150 mm (height) tubes	25 x 150 mm tubes or polyethylene bags	16 x 125 mm tubes or polyethylene bags	25 x 150 mm tubes
No. replicates/accession stored	12-20 tubes/accession	10 tubes/accession	10 tubes/accession	5 tubes/accession each with 2-3 explants/tube
Storage duration (subculture extension-months)	average = 12 min = 3 max = 22	3-4	6-12	average = 11 min = 3 max = 19
Monitoring General performance	Monthly: viability, vigour, necrosis, chlorosis, blackening, hyperhydricity, etiolation, contamination; visual observations	Every 6 weeks for necrotic and contaminated cultures	Every 6 weeks for necrotic and contaminated cultures	Weekly inventory using systematic descriptors: viability, contamination, leaf senescence (green:dead leaves), No. green shoots (for micropropagation), No. viable (green) nodes relative to stem (green) elongation ± roots, callus and dead cultures
Contamination	Bacterial indexing performed at tissue culture initiation and annual recycling (each 5 subculture cycles) using non-destructive testing explant streaking method for broad spectrum bacteriological agar	Visual check for bacterial contaminations	Visual check for bacterial contaminations	Elimination of bacterial contaminants by placing the explants in the 8S media (without agar) at low pH or antibiotics (Mafla et al. 2007)

Protocol step	Bioversity ITC-*Musa*	IITA-*Musa*	IITA-Cassava	CIAT-Cassava
Table 4 (cont'd)				
Monitoring (cont'd) Genetic stability Verification (authentication)	Observations, in case of abnormalities a sample is regenerated under greenhouse conditions, assessed for phenotype, ploidy, *ex vitro* sample regeneration under field conditions (observed during 2 cycles)	Not performed	Not performed	Phenotypic, biochemical (isozymes), molecular (DNA fingerprinting, RAPDs, SSR, RFLPs) applied to: (1) assess genetic stability in the cassava world-wide collection after 10-30 years of *in vitro* storage (2) verification of genetic integrity and management practices in *in vitro* and field accessions (3) Identification of genetic duplicates and redundant accessions
Regeneration frequency	After ten cycles = ±10 years continuously kept *in vitro* morphological description and molecular and/or cytological techniques applied to confirm morphological observations	Not started - Relatively young culture < 3 years	Not started - Relatively young culture < 3 years	Not performed
Biosecurity and safety	Accessions partially backed-up in cryobanks on-site and at one off-site location (IRD, Montpellier)	All accession maintained in the field	Duplication in a distinct unit in Ibadan Cotonou black box (same storage condition as the Ibadan bank) and monitored every 6- 8 weeks. In addition, all accessions are maintained in the field	74% of collection deposited as duplicates at CIP, 98.6% of core collection conserved in cryobanks at CIAT

Protocol step	Bioversity ITC-*Musa*	IITA-*Musa*	IITA-Cassava	CIAT-Cassava
Table 4 (cont'd)				
Recycling after storage	Subcultured on fresh multiplication medium for 3 weeks under normal growth conditions, performance assessed and if satisfactory transferred to cold room	Prior to acclimatization, seedlings subcultured on regeneration media to develop stem-root system, then directly transferred to pot containing sterile soil, enclosed in plastic bag and maintained in screen house until 30 to 50 cm in height	No need – maintenance medium is the standard multiplication medium	Subcultured on multiplication medium, expected recovery time 1 month
Losses of accessions per year	0.5%	10-20%	2-3%	In 27 years, 0.8% of accessions (acari) and 0.7% wild species
Limiting factors	Polyphenolic oxidation, genotype variability	Delayed (2-3 months) covert-latent bacteria	Undetected contamination	None
Sub-optimal regimes	*Ensete* spp. deteriorate easily, producing excessive polyphenols and occasionally etiolates wild relatives, generally cannot be stored as long as cultivated forms under the same conditions	No in terms of introduction to culture Yes in terms of subculture frequency	Yes on introduction to culture Some accessions recalcitrant to standard process	Some wild species are unresponsive with increase in subculture frequency
Strategies for MTS Improvement	Cold acclimation (pre-storage temperature regimes) lower storage temperature/light intensity	Early detection/diagnostics for covert bacteria Improved growth retardation	Avoidance of SCV Optimization of slow growth	Optimization of MTS for some wild species.

Technical procedures are undertaken in accordance with crop-specific technical guidelines published by the respective CGIAR IVGBs (Panis 2008; IITA 2007a b; Mafla et al. 2007; Panta et al. 2007);

SCV = somaclonal variation

Table 5. Summary of cryopreservation using droplet vitrification applied to potato at CIP

Protocol step	Crop specific protocol details
Germplasm type	Shoot meristems from 3-week old micropropagated *in vitro* shoot cultures
Cryopreservation protocol	Droplet-vitrification ([1]Panis et al. 2005)
Pre-treatments	*In vitro* plants micropropagate by single-node cuttings in Magenta jars containing MS propagation medium: MS salts plus 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.1 mg/l thiamine, 2.5% sucrose, 0.28% phytagel. Plantlets grown for 3 weeks (3000 lux/6 h light) incubated at both 22°C and cold acclimation regime at 6°C
Cryoprotection	Loading solution: MS salts containing 2 M glycerol and 0.4 M sucrose, pH 5.8. Replaced by 2 ml ice-cooled filter-sterilized PVS2 [30% (3.26 M) glycerol, 15% (2.42 M), ethylene glycol (EG) and 15% (1.9 M) DMSO, dissolved in MS salts containing 0.4 M sucrose, pH is adjusted to 5.8. Place the tubes at 0°C (on ice) for 50 min
Freezing/cooling regime	After PVS2 treatment, 10 shoot tips transferred to PVS2 droplet on aluminium foil (0.5 x 2 cm), strip quenched in LN. 2 foils containing/10 shoot tips each stored in cryovial filled with LN and transferred to cryobank
Thawing/rewarming regime	Strips removed from cryotube with a forceps, rinsed in 10 ml unloading solution in a small Petri dish at room temperature. Unloaded with 1.2 M sucrose dissolved in MS medium (pH 5.8), after a few seconds, released meristems are maintained for 15 min in unloading solution
Post-storage recovery	Plate shoot tips on filter paper on semisolid medium with 0.3 M sucrose, after 1-2 days, transfer to 0.1 M sucrose, 22°C in dark for 1 week. After 1 week, transfer shoot tips on standard medium without filter paper, at 22°C, 3000 lux and 16 h light
Probabilistic tools	[2]Dussert et al. (2003)
Minimal recovery (safely stored)	Accessions with ≥20% are included in the cryocollection based on the statistical tool of Dussert *et al.* (2003)
GMP	External Accreditation (Feb. 2008) Certificate Testing Laboratory No. 4299; UK Accreditation Service (UKAS) in accordance with ISO/IEC 17025:2005
Biosecurity/safety	Based on Dussert et al. (2003), it is estimated that at least one plantlet will be recovered from the cryobank
Cryobank black box duplication	None
Cryoshipment safety	No shipments have taken place
Monitoring	Viability: No. shoot tips remaining green after 4-6 weeks, recovery: No. shoots/plantlets growing from surviving shoot tips
Longevity checks	1 and 5 years
Genetic stability assessments	22 morphological descriptors, SSR using 18 primers no changes detected

Protocol step	Crop specific protocol details
Table 5 (cont'd)	
Accession processing metrics	50 accessions/year (preparation of starting material, media preparation, storage, post-storage screening)
Genotype variation	ca. 75% of genotypes survive, not all the accessions respond the same
Cryostorage recalcitrance	20% of genotypes do not respond to protocol, 5% do not survive no specific protocols for these accessions applied
Samples for stock renewal	Unknown
*Other cryopreservation uses	None
Special safety precautions	PPC and LN safety
Strategies for improvement	Improvement of recovery culture treatments; determination of biochemical components linked to cryoability, effect of dormancy controlling components on cryoresponse
Working status of cryobank (how is the cryobank utilized?)	In house storage and development only, not used to recovery lost accessions

GMP = Good Management Practice; PPC = Personal Protective Clothing

*other than for base genebanking (e.g. cryotherapy)

[1]Panis B, Piette B, Swennen R. 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Science 168:45-55.

[2]Dussert S, Engelmann F, Noirot M. 2003. Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. CryoLetters 24:149-160.

Technical procedures are undertaken in accordance with crop-specific technical guidelines (Panta et al. 2007).

Table 6. Summary of cryopreservation using droplet vitrification applied to *Musa* at Bioversity ITC

Protocol step	Crop specific protocol details
Germplasm type	Proliferating meristems or apical meristems excised from rooted *in vitro* plants. Excision of apical meristems is labour intensive and only applied to cultivars difficult to cryopreserve using proliferating meristems. For all other accessions, germplasm choice is based on: (i) quality of proliferating meristems obtained after 3 subculture cycles on a medium enriched with high cytokinin concentrations; (ii) survival of these meristems after a preliminary cryopreservation trial
Cryopreservation protocol	Droplet vitrification ([1]Panis et al. 2005)
Pre-treatments	Six weeks after previous subculture, proliferating meristematic clumps (4 mm diameter) containing at least four apical domes, are excised and precultured on multiplication medium + 0.4 M sucrose) for 2 weeks; at 25°C in the dark, after 2 weeks 1-2 mm tissues are excised for cryopreservation
Cryoprotection	Meristems immediately transferred to filter sterilized LS (2 M glycerol and 0.4 M sucrose dissolved in MS medium, pH 5.8) and incubated in the dark; LS replaced by ice-cooled filter-sterilised PVS2 (30% (3.26.M) glycerol, 15% (2.42 M) ethylene glycol (EG) and 15% (1.9.M) DMSO. Cryoprotected in PVS2 solution for 120 min at 0°C; [apical meristems subjected to the PVS2 solution for 30 min at 0°C]; 5 min before end of treatment, ca. 10 meristems (or clumps) transferred to 15 µl droplet of PVS2 solution (on a strip of aluminium foil (5 x 20 mm) maintained at 0°C by transfer to a plastic Petri dish over a cooling element
Freezing/cooling regime	After PVS2 treatment, aluminium strip plunged into LN aided by fine forceps, for permanent cryostorage, foil quickly transferred to a 2 ml Greiner cryotube filled with LN, closed and placed in cryotank
Thawing/rewarming regime	Strips removed from cryotube with a forceps, rinsed in 10 ml unloading solution in a small Petri dish at room temperature. Unloaded with 1.2 M sucrose dissolved in MS medium (pH 5.8), after a few seconds, released meristems are maintained for 15 min in unloading solution
Post-storage recovery	26-28°C phased light conditions; 1 week dark and then at 63 µmol $m^{-2}s^{-1}$, 24h illumination; rewarmed meristems are placed onto 2 sterile filter papers on top of a semi-solid hormone-free MS medium containing 0.3 M sucrose. After two days, meristems were transferred to regeneration medium without filter papers- both post-thaw survival and post-thaw regeneration were determined 4 to 6 weeks following cryopreservation
Probabilistic tools	[2]Dussert et al. (2003)
Minimal recovery (safely stored)	No independent experiments = 3
GMP	None
Biosecurity/safety	Based on Dussert et al. (2003) 1 plant can be regenerated with a probability of 0.95 (per repetition)
Cryobank duplication (Black Box)	2 x 96 duplicate accessions stored in IRD, Montpellier (one repetition), 400 to follow
Cryoshipment safety	Dry shipper; the first shipments by car, International Phytosanitary Certificate not needed within Europe

Protocol step	Crop specific protocol details
Table 6 (cont'd)	
Monitoring	Viability: No. shoot tips remaining green after 4-6 weeks, recovery: No. shoots/plantlets growing from surviving shoot tips
Longevity checks	Viability test executed once, same day cryopreservation takes place; only occasional longevity checks
Genetic stability assessments	Not tested
Accession processing metrics	50 accessions/year (preparation of starting material, media preparation, storage, post-storage screening)
Genotype variation	None
Cryostorage recalcitrance	If both proliferating and apical meristems are used there is no recalcitrant germplasm
Samples for stock renewal	Unknown
***Other cryopreservation uses**	Cell suspensions cryopreservation, trials with virus eradication, nematodes
Special safety precautions	PPC and LN safety, cryotube with internal thread
Strategies for improvement	Improve droplet vitrification of proliferating meristems to increase accessions cryopreserved using less labour intensive protocol
Working status of cryobank (how is the cryobank utilized?)	IVGB

GMP = Good Management Practice; PPC = Personal Protective Clothing

*other than for base genebanking (e.g. cryotherapy)

[1]Panis B, Piette B, Swennen R. 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Science 168:45-55.

[2]Dussert S, Engelmann F, Noirot M. 2003. Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. CryoLetters 24:149-160.

Technical procedures are undertaken in accordance with crop-specific technical guidelines (Panis 2008).

Table 7. Summary of cryopreservation protocols applied to cassava at CIAT

Protocol Step	Crop specific protocol details
Germplasm type	Shoot tips for genebank development, buds for lowest responding material to reduce propagation activities, embryogenic tissue to maintain new lines for genetic transformation, botanical seed for wild species. Donor materials undergo 3-4 propagation cycles to obtain at least 100 plants for shoot extraction based on 2 culture tubes with one plant in each. Young tissue is preferentially used for cryopreservation: 2-3 months for shoots, 2-3 weeks for buds, excised shoot tips (2 mm height)
Cryopreservation protocols	Several
Controlled rate cooling ([1]Escobar et al. 1997)	Shoot tips precultured: in medium (C4) comprising 1 M sorbitol, 0.117 M (4%) sucrose, 0.1 M DMSO for 3 days in the dark at 26 - 28ºC; cryoprotection with 1M sorbitol, 0.117 M (4%) sucrose, 10% DMSO for 2h on ice; dehydration on filter paper for 1h; controlled rate programmable freezing (CryoMed 1010) from 5ºC (chamber temperature); 0.5ºC min^{-1} to -15ºC, thereafter -1ºC min^{-1} to -40 ºC; immersion in LN; thawing at 37ºC; sequential transfer recovery (2 days each) on medium containing (1) 0.75 M sucrose + 0.2% activated charcoal and (2) half-strength MS medium + 0.35 M sucrose and 5.5.6 X 10^{-3} M inositol in the dark; (3) standard culture medium under a light intensity of 15 $\mu Em^{-2}.s^{-1}$. viability and shoot growth assessment after 1 month
Encapsulation-dehydration ([2]Escobar et al. 2000)	Shoot tips encapsulated in 3% calcium (Na) alginate beads, pretreated in sucrose medium for 3 days, desiccated over silica gel and plunged directly into LN. Recommend as an alternative to controlled slow cooling for genotypes unresponsive to this method
PVS2 vitrification	Phototoxic effects
Encapsulation-vitrification	Under development
Droplet freezing	Callus development only
LN storage and phase	Each vial contains 10 beads; 4 vials/clone for long-term storage 2 tubes/clone, for monitoring at 1 and 6 months after cryobanking LN liquid phase bottom inventory, vapour phase top inventory
Post-storage recovery	Viability = any growth (callus, roots, shoots); shoot formation = plants that could be grown transferred to field via direct recovery
Probabilistic tools	None
Minimal recovery (safely stored)	30% as shoot recovery after freezing phase
GMP	None
Biosecurity/safety	
Cryobank black box duplication	In process
Cryoshipment safety	None

Protocol Step	Crop specific protocol details
Table 7 (cont'd)	
Monitoring	Monitoring material cryopreserved during 1 h and 1, 3, 6, 9, 12 months 2 vials/clone assessed for survival after long-term LN treatment
Longevity checks	1 month cryobank storage and re-confirmation after 6 months
Genetic stability assessments	Morphological descriptors, yield comparisons, isozymes (8-systems), AFLP using 5 combinations and 372 bands
Accession processing metrics	Time of each activity within the encapsulation/dehydration (method of choice used for core collection cryobanking) protocol has been determined 150-250 clones/year can be processed for cryobanking
Genotype variation	One main protocol (encapsulation-dehydration) applied to the core collection for which two types: low, medium of cryogenic responses have been identified
Cryostorage recalcitrance	One group of genotypes is difficult to cryopreserve
Samples for stock renewal	Unknown
***Other cryopreservation uses**	Cryoconservation of embryogenic tissues for genetic transformation and botanic seed
Special safety precautions	PPC[3]; LN safety precautions; 1ml capacity NUNC cryovials preferred as other brand 'crash' during freezing and thawing
Constraints and strategies for improvement	Major constraint is processing manpower (2.5. people for implementation) and time for cryopreserving entire collection which is >6000 clones, lab equipment, infrastructure, continuous LN supply problematic
Working status of cryobank (how is the cryobank utilized?)	IVBG for core cassava collection

[1] Escobar RH, Mafla G, Roca WM. 1997. A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. Plant Cell Reports 16:474-478.

[2] Escobar RH, Mafla G, Roca WM. 2000b. Cassava cryopreservation - I. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, International Plant Genetic Resources Institute, Rome, IT. pp. 404-407.

[3] PPC = Personal Protective Clothing

Technical procedures are undertaken in accordance with crop-specific technical guidelines (Mafla et al. 2007).

Table 8. Status of CGIAR's clonal crop genebanks collective actions

Crop	Storage method	CGIAR inter-centre linkages	Type of activity	Potential for collective GPG2 actions		
				Validation	*Best practices (BP)*	*Risk management consensus*
Cassava	**MTS**	CIAT-CIP	Black Box	Inter-institute MTS procedures	Creation, tracking, monitoring, management and maintenance of MTS black box collections	Safe transfer, shipment and long-term storage risk assessment and mitigation
Musa	**MTS**	Bioversity ITC-IITA	Research collaboration	Testing of different improved protocols working towards formal validation exercises	Development for BP for extended culture cycles	Risk assessment and mitigation for phytosanitary, culture and genetic stability risks
Potato	**MTS**	None	None	Facilitation of new initiatives for inter-centre collective actions for MTS BP, validity and risk management for potato and yam		
Yam	**MTS**	None	None			
Sweetpotato	**MTS**	CIP-CIAT	Black box	Testing of different improved protocols working towards formal validation exercises	Creation, tracking, monitoring, management and maintenance of MTS Black Box collections	Safe transfer, shipment and long-term storage risk assessment and mitigation
ARTC	**MTS**	None	None	Facilitation of new initiatives for inter-centre collective actions for MTS BP, validity and risk management for ARTC		
Cassava	**LTS**	None	None	Facilitation of new initiatives for inter-centre collective actions for LTS BP, validity and risk management for cassava		
Musa	**LTS**	Bioversity ITC-IITA	Research collaboration	Testing of different improved protocols working towards formal validation exercises	Optimization of BP to local conditions	Risk assessment and mitigation for phytosanitary, culture and genetic stability risks
Potato	**LTS**	CIP- Bioversity ITC	Research collaboration	Testing of different improved protocols working towards formal validation exercises	Optimization of BP to local conditions	Risk assessment and mitigation for phytosanitary, culture and genetic stability risks
Yam	**LTS**	None	None	Facilitation of new initiatives for inter-centre collective actions for LTS BP, validity and risk management for yam		

Crop	Storage method	CGIAR inter-centre linkages	Type of activity	Potential for collective GPG2 actions		
				Validation	*Best practices (BP)*	*Risk management consensus*
Table 8 (cont'd)						
Sweetpotato	**LTS**	CIP-Bioversity ITC	Research collaboration	Testing of different improved protocols working towards formal validation exercises	Optimization of BP to local conditions	Risk assessment and mitigation for phytosanitary, culture and genetic stability risks
ARTC	**LTS**	CIP-Bioversity ITC	Research collaboration	Testing of different improved protocols working towards formal validation exercises	Optimization of BP to local conditions	Risk assessment and mitigation for phytosanitary, culture and genetic stability risks

Table 9. Collective CGIAR status of Musa in vitro conservation in Bioversity ITC and IITA

Genebank storage method	Crop	Landraces		Wild		Other		Total	
		Total	*Under IT*	*Total*	*Under IT*	*Total*	*Under IT*	*Total*	*Under IT*
Bioversity ITC-MTS	*Musa*	893	893	167	163	117	117	1177	1173
Bioversity ITC-MTS	Ensete	0	0	5	0	0	0	5	0
Bioversity ITC-LTS	*Musa*	383	383	71	70	51	51	505	504
Bioversity ITC-LTS	Ensete	0	0*	0	0	0	0	0	0
IITA - MTS	*Musa*	190	0	6	0	50	0	246	0

*Accessions placed in trust by Bioversity, IITA does not currently cryobank *Musa* germplasm.

Table 10. Collective CGIAR status of Cassava *in vitro* conservation in CIAT and IITA

Genebank storage method	Crop	Landraces		Wild		Other		Total	
		Total	*Under IT*	*Total*	*Under IT*	*Total*	*Under IT*	*Total*	*Under IT*
CIAT-MTS	Cassava	5212	5184	883	883	653	400	6748	6467
CIAT-LTS	Cassava	619	619	0	0	0	0	619	619
IITA-MTS	Cassava	1487	670	0	0	811	733	2298	1403

Table 11. Comparative status of CGIAR's potato, sweetpotato, ARTCs and yam *in vitro* conservation

Genebank storage method	Crop	Landraces		Wild		Other		Total	
		Total	*Under IT*	*Total*	*Under IT*	*Total*	*Under IT*	*Total*	*Under IT*
CIP MTS	Potato	4602	4442	23	23	6606	219	11231	4684
CIP LTS	Potato	465	465	0	0	0	0	465	465
*CIP MTS	Sweetpotato	3840	3717	6	6	1695	217	5541	3940
*CIP MTS	ARTC Achira	17	9	1	0	0	0	18	9
CIP MTS	ARTC Arracacha	10	2	0	0	0	0	10	2
CIP MTS	ARTC Mashua	52	28	0	0	0	0	52	28
CIP MTS	ARTC Oca	518	424	3	3	0	0	521	427
CIP MTS	ARTC Ulluco	498	386	2	2	0	0	500	388
CIP MTS	ARTC Yacon	35	29	1	0	0	0	36	29
**IITA-MTS	*D. abysinnica*				0	0	0	1	0
IITA-MTS	*D. alata*	463	463	0	0	0	0	463	463
IITA-MTS	*D. bulbifera*	40	40	0	0	0	0	40	40
IITA-MTS	*D. cayenensis*	13	13	0	0	0	0	13	13
IITA-MTS	*D. dumentorum*	10	10	0	0	0	0	10	10
IITA-MTS	*D. esculenta*	4	4	0	0	0	0	4	4
IITA-MTS	*D. manganotiana*	5	5	0	0	0	0	5	5
IITA-MTS	*D. preusii*			10	10	0	0	10	10
IITA-MTS	*D. rotundata*	1164	1164	0	0	0	0	1164	1164

*CIP does not currently cryobank ARTCs and sweetpotato germplasm; **IITA does not currently cryobank yam germplasm.

Table 12. CGIAR clonal crop genebank medium term storage: analysis of the lessons learnt from potato, cassava, *Musa* and their applicability to other crops (sweetpotato, yam, ARTCS)

Storage technique experience	Mandate crops							
	Exemplars					Other crops		
	Potato	Cassava		*Musa*		Sweetpotato	Yam	ARTCs
Temperature	At CIP 6-8ºC	At CIAT 23-24ºC	At IITA 18±2ºC	At Bioversity 16ºC	At IITA 18±2ºC	At CIP 19-21ºC	At IITA 18±2ºC	At CIP 6-8ºC or 18-22ºC
Light	1000lux 16h light/8h dark	18.5µmol m^{-2} s^{-1} 12h light/12h dark	30µmol m^{-2} s^{-1} 12h light/ 12h dark	25µmol m^{-2} s^{-1} 24h light	30µmol m^{-2} s^{-1} 12h light/ 12h dark	2000lux 16h light/ 8h dark	30µmol m^{-2} s^{-1}	1000-2000lux 16h light/ 8h dark
Growth retardants	None	None	None	None	None	None	None	None
Osmotica	Sorbitol	None	None	None	None	None	None	Sorbitol or none
Special additives	None	$AgNO_3$	None	None	None	None	None	None
Subculture extension (months)	Min=8/ Max=36	Min=3/ Max=19	Min=6/ Max=12	Min=3/ Max=22	Min=3/ Max=4	Min=6/ Max=12	Min=12/ Max=24	Min=3/ Max=20

For specific details of MTS protocols (used within CGIAR), see Tables 3 and 4.

Table 13. CGIAR clonal crop genebank long term storage: analysis of the lessons learnt from potato, cassava, *Musa* and their applicability to other crops (sweetpotato, yam, ARTCs)

Storage technique experience	Mandate crops					
	Exemplars			Other crops		
	Potato	Cassava	*Musa*	Sweetpotato	Yam	ARTCs
Controlled rate cooling	Several protocols developed by different research institutes and repositories outside CGIAR. Varied and limited success in technology transfer, genotype variability and lack of a robust, reproducible method has limited applicability in CIP.	A protocol developed at CIAT has been applied as standard to some accessions	Various protocols tested at Bioversity-ITC but found ineffective due to low survival and shoot recovery	Some protocol testing in research institutes outside CGIAR/CIP with only limited success due to poor survival.	Limited protocol testing on medicinal yam cell cultures outside CGIAR	Various limited studies. Current emphasis at CIP on droplet vitrification combined with fundamental research of ARTC responses to pre-growth and cryogenic treatments
Encapsulation dehydration		A protocol developed at CIAT is used as an alternative to cryopreserve genotypes unresponsive to controlled rate cooling	Protocol tested at Bioversity-ITC, found ineffective due to low survival and shoot recovery	Limited testing outside/inside CGIAR	Several protocols developed by different research institutes and repositories outside CGIAR. Some successful responses for certain genotypes.	
Droplet freezing	Protocol developed and routinely used in IPK had limited applicability when tested at CIP.	Protocol tested at CIAT was ineffective due to callus production	See droplet vitrification as preferred method		Protocol developed for potato tested for yam at IPK. High survival obtained but limited shoot regeneration.	

Vitrification (PVS2)	Applied with some success for certain genotypes by research institutes outside CGIAR. Variable genotype responses limited routine application in CIP	Applied with some success in various research institutes outside CGIAR. PVS2 toxicity has limited its application at CIAT for some genotypes	Successful protocols developed by different research institutes and repositories outside CGIAR had varied and limited success in their technology transfer to Bioversity-ITC.	Various PVS2-based protocols tested and optimized by repositories outside CGIAR with some successful responses for certain genotypes. One method became the prototype for *Musa* droplet vitrification protocol. Limited testing at CIP, droplet vitrification preferred method.	Several protocols tested, optimized and developed by different research institutes and repositories outside CGIAR. Some successful responses for certain genotype. Limited testing of protocols in CGIAR.
Encapsulation Vitrification (PVS2)		Under development			
Droplet vitrification	Protocol developed for *Musa* applied to potato at CIP. Currently being tested for routine use	Currently not tested/under development. Testing at IITA to be undertaken.	Successful protocol developed at Bioversity-ITC that is widely applicable to all *Musa*. Testing at IITA to be undertaken.	Testing of *Musa* droplet vitrification method in progress at CIP	IPK tested method in combination with refined pre-treatments with some limited success for certain genotype. Variability in responses limited routine a use. Testing at IITA to be undertaken.

For specific details of LTS protocols outside CGIAR see: Sections 2.1 (potato); 2.2. (cassava); 2.3 (sweetpotato); 2.4. (yam); 2.5 (*Musa*).
For specific details of protocols and activities within CGIAR see: Table 5 (potato-CIP); Table 6 (*Musa* – Bioversity-ITC); Table 7 (cassava-CIAT).

References

Abdelnour-Esquivel A, Mora A, Villalobos V. 1992. Cryopreservation of zygotic embryos of *Musa acuminata* (AA) and *Musa balbisiana* (BB). CryoLetters 13:259-164.

Agrawal A, Swennen R, Panis B. 2004. A comparison of four methods for cryopreservation of meristems in banana (*Musa* spp.). CryoLetters 25:101-110.

Ahuja S, Mandal BB, Dixit D, Srivastava PS. 2002. Molecular, phenotypic and biosynthetic stability in *Dioscorea floribunda* plants derived from cryopreserved shoot tips. Plant Science 163:971-977.

Aladele SE, Kuta DD. 2008. Environmental and genotypic effects on the growth rate of *in vitro* cassava plantlet (*Manihot esculenta*). African Journal of Biotechnology 7:381-385.

Alizadeh S, Mantell SH, Viana AM. 1998. *In vitro* shoot culture and microtuber induction in the steroid yam *Dioscorea composite* Hemsl. Plant Cell Tissue and Organ Culture 53:107-112.

Angel F, Barney VE, Tohme J, Roca WM. 1996. Stability of cassava plants at the DNA level after retrieval from 10 years of *in vitro* storage. Euphytica 90:307-313.

Ashmore SE. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. IPGRI, Rome, IT.

Baena M, Mejía M, Pineda B, Hidalgo R, Hesse E, Goldberg E et al. 2007. Delivering distance education on plant genetic resources. An evaluation of the collaborative association between CIAT, Bioversity International, UNC and REDCAPA on conducting a distance-education course on the *ex situ* conservation of plant genetic resources. Bioversity International, Rome, IT and CIAT, Cali, CO.

Bajaj YPS. 1977. Initiation of shoots and callus from potato-tuber sprouts and axillary buds frozen at -196°C. Crop Improvement 4:48-53.

Bajaj YPS. 1983a. Cassava plants from meristem cultures of freeze-preserved for three years. Field Crops Research 7:161-167.

Bajaj YPS. 1983b. Cryopreservation of potato (*Solanum tuberosum* L.) and cassava (*Manihot esculenta* Crantz): viability of excised meristems cryopreserved for up to 4 years. Indian Journal of Experimental Biology 23:285-287.

Bajaj YPS. 1985. Cryopreservation of germplasm of potato (*Solanum tuberosum* L.) and cassava (*Manihot esculenta* Crantz). In: Bajaj YPS, editor. Biotechnology in Agriculture and Forestry 32, Cryopreservation of Plant Germplasm I. Springer, New York, USA. pp. 398-416.

Bajaj YPS. 1987. Cryopreservation of potato germplasm. In: Bajaj YPS, editor. Biotechnology in Agriculture and Forestry 3, Potato. Springer, New York, USA. pp. 472-486.

Banerjee N, de Langhe E. 1985. A tissue culture technique for rapid clonal propagation and storage under minimal growth conditions of *Musa* (banana and plantain). Plant Cell Reports 4:351-354.

Barandalla L, Sanchez I, Ritter E, Ruiz-de-Galarreta JI. 2003. Conservation of potato (*Solanum tuberosum* L.) cultivars by cryopreservation. Spanish Journal of Agricultural Research 1:9-13.

Baust JM, Vogel MJ, Snyder KK, Van Buskirk RG, Baust JG. 2007. Activation of mitochondrial-associated apoptosis contributes to cryopreservation failure. Cell Preservation Technology 5:155-163.

Benson EE. 2000a. Special symposium: *In vitro* plant recalcitrance: an introduction. *In Vitro* Cellular and Development Biology Plant 36:141-148.

Benson EE. 2000b. Special symposium: *In vitro* plant recalcitrance. Do free radicals have a role in plant tissue culture recalcitrance? *In Vitro* Cellular and Development Biology Plant 36:163-170.

Benson EE. 2004. Cryoconserving algal and plant diversity: historical perspective and future challenges. In: Fuller B, Lane N, Benson EE, editors. Life in the Frozen State. CRC Press, London, UK. pp. 299-328.

Benson EE. 2008a. Cryopreservation theory. In: Reed BM, editor. Plant Cryopreservation: a Practical Guide. Springer, New York, USA. pp. 15-32.

Benson EE. 2008b. Cryopreservation of phytodiversity: a critical appraisal of theory and practice. Critical Reviews in Plant Sciences 27:141-219.

Benson EE, Harding K, Smith H. 1989. Variation in recovery of cryopreserved shoot tips of *Solanum tuberosum* exposed to different pre- and post-freeze light regimes. CryoLetters 10:323-344.

Benson EE, Chabrillange N, Engelmann F. 1992. Mise au point de méthodes de cryoconservation de méristèmes pour la conservation à long terme des resources génétiques du manioc (*Manihot* spp.). Rapport de fin d'étude, ORSTOM, Montpellier, FR.

Benson EE, Wilkinson M, Todd A, Ekuere U, Lyon J. 1996. Developmental competence and ploidy stability in plants regenerated from cryopreserved potato shoot tips. CryoLetters 17:119-128.

Benson EE, Johnston J, Muthusamy J, Harding K. 2005. Physical and engineering perspectives of *in vitro* plant cryopreservation. In: Dutta Gupta S, Ibaraki Y, editors. Plant Tissue Culture Engineering. Springer, NL. pp. 441-473.

Benson EE, Harding K, Johnston J. 2007. Cryopreservation of shoot tips and meristems. In: Day JG, Stacey G, editors. Cryopreservation and Freeze Drying Protocols. Methods in Molecular Biology, Vol. 368. Humana Press, (2nd ed.). Totowa, New Jersey, USA. pp. 163-183.

Benson EE, Harding K, Debouck D, Dumet D, Escobar R, Mafla G et al. 2011a. 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. Systemwide Genetic Resources Programme, Rome, IT.

Benson EE, Harding K, Debouck D, Dumet D, Escobar R, Mafla G et al. 2011b Refinement and standardization of storage procedures for clonal crops -Global public goods Phase 2: Part III: Multi-crop guidelines for developing *in vitro* conservation best practices for clonal crops. Systemwide Genetic Resources Programme, Rome, IT.

Bhatti MH, Percival T, Davey CDM, Henshaw GG. 1997. Cryopreservation of embryogenic tissue of a range of genotypes of sweet potato (*Ipomoea batatas* [L] Lam.) using an encapsulation protocol. Plant Cell Reports 16:802–806.

Bielanski A. 2005. Experimental microbial contamination and disinfection of dry (vapour) shipper Dewars designed for short-term storage and transportation of cryopreserved germplasm and other biological specimens. Theriogeneology 63:1946-1957.

Blakesley D. 1997. Cryopreservation of embryogenic tissue of a range of genotypes of sweetpotato (*Ipomea batatas* L. Lam) using an encapsulation protocol. Plant Cell Reports 16:802-806.

Blakesley D, Al-Mazrooei S, Henshaw GG. 1995. Cryopreservation of embryogenic tissue culture of sweetpotato (*Ipomea batatas*): use of sucrose and dehydration for cryoprotection. Plant Cell Reports 15:259-263.

Blakesley D, Al-Mazrooei S, Bhatti MH, Henshaw GG. 1996. Cryopreservation of non-encapsulated embryogenic tissue cultures of sweetpotato (*Ipomea batatas*): Plant Cell Reports 15:873-876.

Borges M, Ceiro W, Meneses S, Aguilera N, Vázquez J, Infante Z, Fonseca M. 2004. Regeneration and multiplication of *Dioscorea alata* germplasm maintained *in vitro*. Plant Cell Tissue and Organ Culture 76:87-90.

Bouafia S, Jelti N, Lairy G, Blanc A, Bonnel E, Dereuddre J. 1996. Cryopreservation of potato shoot tips by encapsulation-dehydration. Potato Research 39:69-78.

Bunn E, Tan B. 2004. Microbial contaminants in plant tissue culture propagation. In: Sivasithamparam K, Dixon KW, Barrett RL, editors. Microorganisms in Plant Conservation and Biodiversity. Springer, NL. pp. 307-335.

Carpentier SC, Witters E, Laukens K, Swennen R, Panis B. 2006. Proteome research in banana meristems to study cryoprotection. Cryobiology 53:422.

Carpentier SC, Witters E, Laukens K, Van Onckelen H, Swennen R, Panis B. 2007. Banana *Musa* spp. as a model to study the meristem proteome: acclimation to osmotic stress. Proteomics 7:92-105.

Cassells AC. 1991. Problems in tissue culture: culture contamination. In: Debergh PC, Zimmerman RH, editors. Micropropagation Technology and Application. Kluwer Academic Publishers, Dordrecht, NL. pp. 31-44.

Charoensub R, Phansiri S, Sakai A, Yongmanitchai W. 1999. Cryopreservation of cassava *in vitro*-grown shoot tips cooled to -196°C by vitrification. CryoLetters 20:89-94.

Charoensub R, Phansiri S, Yongmanitchai W, Sakai A. 2003. Routine cryopreservation of *in vitro*-grown axillary apices of cassava (*Manihot esculenta* Crantz) by vitrification: importance of a simple mononodal culture. Scientia Horticulturae 98:485-492.

Charoensub R, Hirai D, Sakai A. 2004. Cryopreservation of *in vitro*-grown shoot tips of cassava by encapsulation-vitrification method. CryoLetters 25:51-58.

Charoensub R, Phansiri S, Sakai A, Yongmanitchai W. 2007. Cryopreservation of *in vitro*-grown shoot tips of cassava. In: Howeler RH, editor. Cassava Research and Development in Asia: Exploring New Opportunities for an Ancient Crop. Proceedings 7th Regional Workshop; 28 October - 1 November 2002; Bangkok, TH. pp. 136-139.

Cha-um S, Kirdmanee C. 2007. Minimal growth *in vitro* culture for preservation of plant species. Fruit, Vegetable, Cereal Science and Biotechnology 1:13-25.

Chen HI, Tsai CD, Wang HT, Hwang SM. 2006. Cryovial with partial membrane sealing can prevent liquid nitrogen penetration in submerged storage. Cryobiology 53:283-287.

Chulafich L, Grubisshich D, Vuiichich R, Volkova LA, Popov AS. 1994. Somatic embryo production *in vitro* in *Dioscorea caucasica* Lipsky and *Dioscorea balcanica* Košanin and cryopreservation of their organogenic callus tissues. Russian Journal of Plant Physiology 41:821-826.

CIAT. 2007a. CIAT annual Report 1980, CIAT, Cali, CO.

CIAT. 2007b. Centro Internacional de Agricultura Tropical (CIAT). Cassava research and development in Asia: exploring new opportunities for an ancient crop. Proceedings of the 7th Regional Workshop; 28 October - 1 November 2002; Bangkok, TH.

CIAT. 2007c. Four decades: striving for excellence in agricultural research. CIAT, Calli, CO.

CIAT. 2007d. Multi-institutional distance learning course on the *ex situ* conservation of plant genetic resources. CIAT Publication No. 360. CIAT, Calli, CO.

CIP. 2006. Strengthening our assets: enhancing our impact. The Strategic Plan for Research of the International Potato Center 2006-2016. CIP, Lima, PE.

CIP. 2007. Medium-term plan 2008-2010 and financing plan for 2008. CIP, Lima, PE.

Côte FX, Goue O, Domergue R, Panis B, Jenny C. 2000. In-field behaviour of banana plants (*Musa* AA sp.) obtained after regeneration of cryopreserved embryogenic cell suspensions. CryoLetters 21:19-24.

Criel B, Panta A, Carpentier S, Renaut J, Swennen R, Panis B, Hausman JF. 2005. Cryopreservation and abiotic stress tolerance in potato: a proteomic approach. Communication Applied Biological Sciences Ghent University 70:83-86.

Danso KE, Ford-Lloyd BV. 2002. Induction of high-frequency somatic embryos of cassava for cryopreservation. Plant Cell Reports 21:226-232.

Day JG, Lorenz M, Wilding TA, Friedl T, Harding K, Pröschold T et al. 2007. The use of physical and virtual infrastructures for the validation of algal cryopreservation methods in international culture collections. CryoLetters 28:359-376.

Dixit-Sharma S, Mandal BB, Ahuja S, Srivastava PS. 2003. Genetic stability assessment of plants regenerated from cryopreserved embryogenic tissues of *Dioscorea bulbifera* L. using RAPD, biochemical and morphological analysis. CryoLetters 24:77-84.

Dixit-Sharma S, Ahuja-Ghosh A, Mandal BB, Srivastava PM. 2005. Metabolic stability of plants regenerated from the cryopreserved shoot tips of *Dioscorea deltoidea* - an endangered medicinal plant. Scientia Horticulturae 105:513-517.

Dussert S, Engelmann F, Noirot M. 2003. Development of probabilistic tools to assist in the establishment and management of cryopreserved plant germplasm collections. CryoLetters 24:149-160.

Engelmann F. 2004. Plant cryopreservation: progress and prospects. *In Vitro* Cellular and Development Biology Plant 40:427-433.

Engelmann F, Takagi H, editors. 2000. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT.

Engelmann F, Benson EE, Chabrillange N, Gonzalez-Arnao MT, Maris S, Michaux-Ferriere N et al. 1994. Cryopreservation of several tropical plant species using encapsulation-dehydration of apices. In: Terzi M, Cella R, Falavigna A, editors. Proceedings 8th International Congress on Plant Tissue and Cell Culture; 12-17 June, 1994, Firenze, IT. pp. 315-320.

Engelmann F, Gonzalez-Arnao M-T, Wu Y, Escobar R. 2008. Development of encapsulation dehydration. In: Reed BM, editor. Plant Cryopreservation: A Practical Guide. Springer, New York, USA. pp. 59-76.

Epperson E, Pachio DH, Guevara CL. 1997. A cost analysis of maintaining cassava plant genetic resources. Crop Science 37:1641-1649.

Escobar RH, Roca WM. 1997. Cryopreservation of cassava shoot tips through rapid freezing. African Journal of Root and Tuber Crops 2:214-215.

Escobar RH, Mafla G, Roca WM. 1997. A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. Plant Cell Reports 16:474-478.

Escobar RH, Debouck D, Roca WM. 2000a. Development of cassava cryopreservation. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 222-226.

Escobar RH, Mafla G, Roca WM. 2000b. Cassava cryopreservation - I. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 404-407.

Escobar RH, Palacio JD, Rangel MP, Roca WM. 2000c. Cassava cryopreservation - II. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 408-410.

Espinoza NO, Estrada R, Silva-Rodriguez D, Tovar P, Lizzaraga R, Dodds JH. 1986. The potato: a model crop plant for tissue culture. Outlook Agriculture 15:21-26.

Espinoza N, Lizárraga R, Sigueñas C, Buitrón F, Bryan J, Dodds JH, editors. 1992. Tissue Culture: Micropropagation, Conservation and Export of Potato Germplasm. CIP Research Guide 1. International Potato Center, PE.

Estrada R, Tovar P, Dodds JH. 1986. Induction of *in vitro* tubers in a broad range of potato genotypes. Plant Cell Tissue and Organ Culture 7:3-10.

Fabre J, Dereuddre J. 1990. Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot tips. CryoLetters 11:413-426.

Federovskii DN, Popov AS. 1992. Plasmalemma injuries in different strains of *Dioscorea deltoidea* during cryopreservation. Soviet Plant Physiology 39:381-385.

Frison EA. 1981. Tissue culture: a tool for improvement of international exchange of tropical root and tuber crops. International Institute Tropical Agricultural Research Briefs 2:1-4.

Gallet S, Gamiette F, Filloux D, Engelmann F. 2007. Cryopreservation of yam germplasm in Guadeloupe (FWI). Advances in Horticultural Science 21:244-246.

Golmirzaie A, Panta A. 1997a. Tissue culture methods and approaches for conservation of root and tuber crops. In: Razdan MK, Cocking EC, editors. Conservation of Plant Genetic Resources *In Vitro*. Vol. I: General Aspects. Science Publishers, Inc. Enfield, New Hampshire, USA. pp. 123-152.

Golmirzaie A, Panta A. 1997b. Advances in potato cryopreservation by vitrification. CIP Program Report. International Potato Center (CIP) PE. pp. 71-76.

Golmirzaie A, Toledo J. 1998. *In vitro* conservation of potato and sweetpotato germplasm. CIP program report 1997-98. CIP, Lima, PE. Available from URL: http://www.cipotato.org/publications/program_reports/97_98/41invitr.pdf.

Golmirzaie A, Toledo J. 1999. Non-cryogenic, long-term germplasm storage. In: Hall RD, editor. Methods in Molecular Biology. Vol. 111. Humana Press, Totowa, New Jersey, USA. pp. 95-101.

Golmirzaie A, Panta A. 2000. Advances in potato cryopreservation at CIP. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 250-254.

Golmirzaie A, Panta A, Toledo J. 1999. Biotechnological advances in the conservation of root and tuber crops. In: Benson EE, editor. Plant Conservation Biotechnology. Taylor and Francis, London, UK. pp. 165-178.

Golmirzaie A, Panta A, Delgado C. 2000a. Structural observations on potato shoot tips after thawing from liquid nitrogen. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm.

Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 388-389.

Golmirzaie A, Panta A, Diaz S. 2000b. Systematic determination of an adequate method for large-scale sweetpotato cryopreservation at CIP. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 460-462.

Gonzalez-Arnao MT, Panta A, Roca WM, Escobar RH, Engelmann F. 2008. Development of large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tissue and Organ Culture 92:1-13.

Grospietsch M, Stodulková E, Zámecnik J. 1999. Effect of osmotic stress on the dehydration tolerance of *Solanum tuberosum* shoot tips. CryoLetters 20:339-346.

Grout BWW, Henshaw GG. 1978. Freeze preservation of potato shoot tips. Annals of Botany 42:1227-1229.

Hahne G, Hoffman F. 1984. Dimethyl sulfoxide can initiate cell divisions in arrested callus protoplasts by promoting cortical microtubule assembly. Proceedings of the National Academy of Sciences, USA 81:5449-5453.

Halmagyi A, Deliu C, Coste A. 2005. Plant regrowth from potato shoot tips cryopreserved by a combined vitrification-droplet method. CryoLetters 26:313-322.

Hamill S, Wasmund K, Smith M, Eccleston K, McKay D. 2005. Endogenous bacteria isolated from banana meristems during tissue culture initiation: problems and potential. In: Benett IJ, Bunn E, Clarke H, McComb JA, editors. Contributing to a Sustainable Future. Proceedings Australian Branch IAPTC B. Perth, Western Australia, AU. pp. 101-111.

Harding K. 1991. Molecular stability of the ribosomal RNA genes in *Solanum tuberosum* plants recovered from slow growth and cryopreservation. Euphytica 55:141-146.

Harding K. 1994. The methylation status of DNA derived from potato plants recovered from slow growth. Plant Cell Tissue and Organ Culture 37:31-38.

Harding K. 1996. Approaches to assess the genetic stability of plants recovered from *in vitro* culture. In: Normah MN, Narimah MK, Clyde MM, editors. *In Vitro* Conservation of Plant Genetic Resources. Proceedings of the International Workshop on *In Vitro* Conservation of Plant Genetic Resource, 4-6 July 1995, Kuala Lumpur, MY. Malaysia Plant Biotechnology Laboratory, Faculty of Life Sciences, Universiti Kebangsaan, Kuala Lumpur, MY. pp. 135-168.

Harding K. 1997. Stability of the ribosomal RNA genes in *Solanum tuberosum* L. plants recovered from cryopreservation. CryoLetters 18:217-230.

Harding K. 2004. Genetic integrity of cryopreserved plant cells: a review. CryoLetters 25:3-22.

Harding K, Benson EE. 1994. A study of growth, flowering, and tuberisation in plants derived from cryopreserved potato shoot tips: implications for *in vitro* germplasm collections. CryoLetters 15:59-66.

Harding K, Benson EE. 2000. Analysis of nuclear and chloroplast DNA in plants regenerated from cryopreserved shoot tips of potato. CryoLetters 21:279-288.

Harding K, Benson EE. 2001. The use of microsatellite analysis in *Solanum tuberosum* L. *in vitro* plantlets derived from cryopreserved germplasm. CryoLetters 22:199-208.

Harding K, Staines H. 2001. Biometric analysis of phenotypic characters of potato shoot tips recovered from tissue culture, dimethyl sulphoxide treatment and cryopreservation. CryoLetters 22:255-262.

Harding K, Benson EE, Smith H. 1991. The effects of pre-freeze *in vitro* culture period on the recovery of cryopreserved shoot tips of *Solanum tuberosum*. CryoLetters 12:17-22.

Harding K, Johnston JW, Benson EE. 2008. Concepts in cryobionomics: A case study of *Ribes* genotype responses to cryopreservation in relation to thermal analysis oxidative stress nucleic acid methylation and transcriptional activity. In: Laamanen J, Uosukainen M, Häggman H, Nukari A, Rantala S, editors. Cryopreservation of Crop Species in Europe CryoPlanet COST Action 871. Agrifood Research Working Papers 153. MTT Agrifood Research FI. pp. 10-11.

Harding K, Johnston JW, Benson EE. 2009. Exploring the physiological basis of cryopreservation success and failure in clonally propagated *in vitro* crop plant germplasm. Agriculture and Food Science 18:3-16.

Helliot B, Panis B, Poumay Y, Swennen R, Lepoivre P, Frison E. 2002. Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana (*Musa* spp.). Plant Cell Reports 20:1117-1122.

Henshaw GG, O'Hara JF, Stamp JA. 1985. Cryopreservation of potato meristems. In: Kartha KK, editor. Cryopreservation of Plant Cells and Organs. CRC Press, Boca Raton, Florida, USA. pp. 159-170.

Hermann M, Heller J, editors. 1997. Andean roots and tubers: *Ahipa, Arracacha, Maca* and *Yacon*. Promoting the conservation and use of underutilized and neglected crops 21. Institute of Plant Genetics and Crop Plant Research, Gatersleben, IPGRI, Rome, IT.

Hirai D, Sakai A. 1999. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. Potato Research 42:153-160.

Hirai D, Sakai A. 2000. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-dehydration. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 205-211.

Hirai D, Sakai A. 2003. Simplified cryopreservation of sweetpotato [*Ipomoea batatas* (L.) Lam.] by optimizing conditions for osmoprotection. Plant Cell Reports 21:961-966.

IBPGR. 1987. Meristem culture and virus indexing of sweet potatoes. Practical Manuals for Handling Crop Germplasm *In Vitro* No. 1. IBPGR, Rome, IT.

IITA. 1980. Sweet potato tissue culture and virus indexing. Annual Report 1979. IITA, Ibadan, NG.

IITA. 1998. Project 15 Recombinant DNA, molecular diagnostics and cellular biotechnology for crop improvement. Annual Report 1997. IITA, Ibada, NG.

IITA. 2001. Delivering the African agricultural development potential: the IITA, vision; strategic plan 2001-2010. IITA, Ibadan, NG.

IITA. 2007a. Annual report 2007. IITA, Ibadan, NG.

IITA. 2007b. Cassava *in vitro* processing and gene banking. IITA, Ibadan, NG.

IITA. 2007c. Yam *in vitro* processing and gene banking. IITA, Ibadan, NG.

INIBAP. 2006. INIBAP annual report 2005. International Network for the Improvement of Banana and Plantain, Montpellier, FR.

IPGRI-CIAT. 1994. Establishment and operation of a pilot *in vitro* active genebank. Report of a CIAT-IBPGR Collaborative project using cassava *Manihot Esculenta* crantz as a model. A Joint Publication of IPGRI and CIAT, Cali, CO and IPGRI, Rome, IT.

ISBER. 2005. Best practices for repositories I: collection, storage and retrieval of human biological materials for research. Cell Preservation Technology 3:5-48.

ISBER. 2008. Best practices for repositories collection, storage, retrieval and distribution of biological materials for research. Cell Preservation Technology 6:5-57.

Janse JD, Wenneker M. 2002. Possibilities of avoidance and control of bacterial plant diseases when using pathogen-tested (certified) or treated planting material. Plant Pathology 51:523-536.

Jarret RL, Florkowski WJ. 1990. *In vitro* active vs. field genebank maintenance of sweetpotato germplasm: major costs and considerations. HortScience 25:141-145.

Jarret RL, Gawel N. 1991. Abscisic acid-induced growth inhibition of sweetpotato (*Ipomea batatas* L.) *in vitro*. Plant Cell Tissue and Organ Culture 24:13-18.

Johnston JW, Harding K, Bremner DH, Souch G, Green J, Lynch PT et al. 2005. HPLC analysis of plant DNA methylation: a study of critical methodological factors. Plant Physiology and Biochemistry 43:844-853.

Johnston JW, Harding K, Benson EE. 2007. Antioxidant status and genotypic tolerance of *Ribes in vitro* cultures to cryopreservation. Plant Science 172:524-534.

Johnston JW, Benson EE, Harding K. 2009. Cryopreservation induces temporal DNA methylation epigenetic changes and differential transcriptional activity in *Ribes* germplasm. Plant Physiology and Biochemistry 42:123-131.

Jova MC, Kosky RG, Pérez MB, Pino AS, Vega VM, Torres JL et al. 2005. Production of yam microtubers using a temporary immersion system. Plant Cell Tissue and Organ Culture 83:103-107.

Joyce SM, Cassells AC. 2002. Variation in potato microplant morphology *in vitro* and DNA methylation. Plant Cell Tissue and Organ Culture 70:125–137.

Joyce SM, Cassells AC, Jain SM. 2003. Stress and aberrant phenotypes in *in vitro* culture. Plant Cell Tissue and Organ Culture 74:103–121.

Kaczmarczyk A, Rutten T, Melzer M, Keller ERJ. 2008. Ultrastructural changes associated with cryopreservation of potato (*Solanum tuberosum* L.) CryoLetters 29:145-166.

Kartha KK. 1985. Meristem culture and germplasm preservation. In: Kartha KK, editor. Cryopreservation of Plant Cells and Organs. CRC Press Inc. Boca Raton, Florida, USA. pp. 115-134.

Kartha KK, Leung NL, Moroginski LA. 1982. *In vitro* growth and plant regeneration from cryopreserved meristems of cassava (*Manihot esculenta* Crantz.). Zeitschrift Pflanzenphysiol. Bd. 107S:133-140.

Keller ERJ. 2007. Cryopreservation for maintenance of plant germplasm in Germany. Advances in Horticultural Science 21:228-231.

Keller ERJ, Schüler K, Gäde H, Fischer M, Hammer K. 1999. Present situation in the field collections of vegetatively propagated species in the Gatersleben genebank. In: Engelmann F, editor. Management of Field and *In Vitro* Germplasm Collections, Proceedings of a Consultation Meeting; 15–20 January 1996; CIAT, Cali, CO. IPGRI, Rome, IT. pp. 21-24.

Keller ERJ, Dreiling M. 2003. Potato cryopreservation in Germany - using droplet methods for the establishment of a new large collection. Acta Horticulturae 623:193-200.

Keller ER, Grübe M, Senula A. 2005. Cryopreservation in the Gatersleben genebank - state of the art in potato, garlic and mint. Memorias Congreso Intercional Biotecnología y Agricultura (Bioveg 2005) Centre de Bioplantas, Ciego de Avila, CU. pp. 97-105.

Keller ER, Senula A, Leunufna S, Grübe M. 2006. Slow growth storage and cryopreservation-tools to facilitate germplasm maintenance of vegetatively propagated crops and living plant collections. International Journal of Refrigeration 29:411-417.

Keller ERJ, Kaczmarczyk A, Senula A. 2008a. Cryopreservation for plant genebanks: a matter between high expectations and cautious reservation. CryoLetters 29:53-62.

Keller ER, Senula A, Kaczmarczyk A. 2008b. Cryopreservation of herbaceous dicots. In: Reed BM, editor. Plant Cryopreservation: a Practical Guide. Springer, New York, USA. pp. 281-232.

Kim H-H, Yoon J-W, Park Y-E, Cho E-G, Sohn J-K, Kim T-S, Engelmann F. 2006. Cryopreservation of potato cultivated varieties and wild species: critical factors in droplet vitrification. CryoLetters 27:223-234.

Kryszczuk A, Keller J, Gübe M, Zimnoch-Guzowska E. 2006. Cryopreservation of potato (*Solanum tuberosum* L.) shoot tips using vitrification and droplet method. Journal of Food Agriculture Environment 4:196-200.

Kyesmu PM, Takagi H. 2000. Cryopreservation of shoot apices of yams (Dioscorea species) by vitrification. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 411-413.

Leunufna S. 2004. Improvement of the *in vitro* maintenance and cryopreservation of yams (*Dioscorea* spp.). PhD dissertation. Martin-Luther University, Halle-Wittenberg, DE.

Leunufna S, Keller ERJ. 2003. Investigating a new cryopreservation protocol for yams (*Dioscorea* spp.). Plant Cell Reports 21:1159-1166.

Leunufna S, Keller ERJ. 2005. Cryopreservation of yams using vitrification modified by including droplet method: effects of cold acclimation and sucrose. CryoLetters 26:93-102.

Liu T-HA, Hsu N-W, Wu R-Y. 2005. Control of leaf-tip necrosis of micropropagated ornamental statice by elimination of endophytic bacteria. *In Vitro* Cellular and Development Biology Plant 41:546-549.

Lizarraga R, Huaman Z, Dodds JH. 1989. *In vitro* conservation of potato germplasm at the International Potato Center. American Journal of Potato Research 66:253-276.

Lizarraga R, Panta A, Jayasinghe U, Dodds JH, editors. 1991. Tissue culture for the elimination of pathogens. CIP Research Guide 3. CIP, Lima, PE.

Lizarraga R, Panta A, Espinoza N, Dodds JH, editors. 1992. Tissue culture of *Ipomea batatas*: micropropagation and maintenance. CIP Research Guide 32. CIP, Lima, PE.

Lopez-Delgado H, Jimenez-Casas M, Scott IM. 1998. Storage of potato microplants *in vitro* in the presence of acetylsalicylic acid. Plant Cell Tissue and Organ Culture 54:145–152.

Lusty C, Arnaud E, Roux N, coordinators. 2006. Global conservation strategy for *Musa* (banana and plantain). Global Crop Diversity Trust, Rome, IT. Available from URL: http://www.croptrust.org/documents/web/Musa-Strategy-FINAL-30Jan07.pdf

Mafla G, Roca W, Reyes R, Roa JC, Munoz L, Baca AE et al. 1993. *In vitro* management of cassava germplasm at CIAT. In: Roca WM, Thro AM, editors. Proceedings of the 1st International Scientific Meeting of Cassava Biotechnology Network. CIAT, CO. pp. 168-174.

Mafla G, Roa JC, Guevara CL. 2000. Advances on the *in vitro* growth control of cassava using silver nitrate. In: Carvalho LJCB, Thro AM, Vilarinhos AD, editors. Proceedings IV International Scientific Meeting of the Cassava Biotechnology Network; 3-7 November 1998, Salvador, Bahia, Brazil. EMBRAPA, CENARGEN and CBN, Brasilia, BR. pp. 439-446.

Mafla G, Roa JC, Ocampo C, Gallego G, Jaramillo G, Debouck DG. 2004. Efficacy of silver nitrate for slow growth conservation of cassava (*Manihot esculenta* Crantz). Determination of viability and genetic stability. In: Abstracts of the 6th International Scientific Meeting of the Cassava Biotechnology Network (CBN); 8-14 March 2004, CIAT, Cali, CO. pp. 134.

Mafla G, Roa JC, Aranzales E, Debouck D. 2007. Manual de procedimientos para la conservación *in vitro* del germoplasma del género *Manihot*. Available from URL: www.ciat.cgiar.org/urg

Malaurie B, Trouslot M-F, Berthaud J, Bousalem J, Pinel A, Dubern J. 1998a. Medium-term and long-term *in vitro* conservation and safe international exchange of yam (*Dioscorea* spp.) germplasm. Electronic Journal of Biotechnology 1:103-117.

Malaurie B, Trouslot M-F, Engelmann F, Chabrillange N. 1998b. Effect of pretreatment conditions on the cryopreservation of *in vitro*-cultured yam (*Dioscorea alata* 'Brazo Fuerte' and *D. bulbifera* 'Nouméa Imboro') shoot apices by encapsulation-dehydration. CryoLetters 19:15-26.

Malaurie B, Trouslot M-F, Chabrillange N. Engelmann F. 2000. Survival and recovery of yam (*Dioscorea* spp.) apices after cryopreservation using encapsulation-dehydration. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 414-417.

Mandal BB. 1999. Conservation biotechnology of endemic and other economically important plant species in India. In: Benson EE, editor. Plant Conservation Biotechnology. Taylor and Francis, London, UK. pp. 211-225.

Mandal BB. 2000. Cryopreservation of yam species: a comparative study with three different techniques. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 233-237.

Mandal BB, Dixit-Sharma S. 2007. Cryopreservation of *in vitro* shoot tips of *Dioscorea deltoidea* Wall, an endangered and medicinal plant: effect of cryogenic procedure on storage duration. CryoLetters 17:461-470.

Mandal BB, Chandal KPS, Dwivedi S. 1996a. Cryopreservation of yam (*Dioscorea* spp.) shoot apices by encapsulation-dehydration. CryoLetters 17:165-174.

Mandal BB, Malik SK, Chandal KPS. 1996b. Cryopreservation of encapsulated apices of yams (*Dioscorea* spp.) ultra structural studies on recovery growth. SLTB Annual Meeting, Dundee, Scotland, Selected Abstracts CryoLetters 18:73.

Mandal BB, Ahuja-Ghosh S, Srivastava PS. 2008. Cryopreservation of *Dioscorea rotundata* Poir: a comparative study with two cryogenic procedures and assessment of true-to-type regenerants by RAPD analysis. CryoLetters 29:399-408.

Manzhulin AV. 1983 Factors affecting survival of potato shoot apices after deep freezing. Fiziologia Rastenii 31:639-645.

Manzhulin AV, Butenko RG, Popov AS. 1983. Effect of preliminary preparation of potato apices on their viability after deep-freezing. Fiziologia Rastenii 30:1188-1193.

Marin ML, Mafla G, Roca WM, Withers LA. 1990. Cryopreservation of cassava zygotic embryos and whole seeds in liquid nitrogen. CryoLetters 11:257-264.

Matsumoto T, Sakai A, Takahashi C, Yamada K. 1995. Cryopreservation of *in vitro* apical meristems of wasabi (*Wasabia japonica*) by encapsulation-vitrification method. CryoLetters 161:89-196.

Mix-Wagner G. 1999. *In vitro* collection of potato cultivars at BGRC in Germany. In: Engelmann F, editor. Management of Field and *In Vitro* Germplasm Collections, Proceedings of a Consultation Meeting; 15–20 January 1996; CIAT, Cali, CO. IPGRI, Rome, IT. pp. 99-103.

Mix-Wagner G, Schumacher HM, Cross RJ. 2003. Recovery of potato apices after several years of storage in liquid nitrogen. CryoLetters 24:33-41.

Morris GJ. 2005. The origin, ultrastructure and microbiology of the sediment accumulating in liquid nitrogen storage vessels. Cryobiology 50:231-238.

Niedz RP, Bausher MG. 2002 Control of *in vitro* contamination of explants from greenhouse and field-grown trees. *In Vitro* Cellular and Development Biology Plant 38:168-171.

Ng NQ, Daniel IO. 2000. Storage of pollens for long-term conservation of yam genetic resources. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 136-139.

Ng SYC. 1988. *In vitro* tuberisation of white yam (*Dioscorea rotunda* Poir). Plant Cell Tissue and Organ Culture 14:121-128.

Ng SYC. 1991. *In vitro* conservation and distribution of root and tuber crop germplasm. In: Ng NQ, Perrino P, Attere F, Zedan H, editors. Crop Genetic Resources of Africa. Vol. II. IITA, Ibadan, NG. pp. 95-106.

Ng SYC. 1992. Micropropagation of white yam (*Dioscorea rotundata* Poir). In: Bajaj YPS, editor. Biotechnology in Agriculture and Forestry. Vol. 19. Springer-Verlag, Berlin. Germany pp. 135-159.

Ng SYC, Hahn SK. 1985. Application of tissue culture to tuber crops at IITA. In: Proceedings of the Inter-Center Seminar on International Agricultural Research Centers (IARCs) and Biotechnology. IRRI, Los Baños, PH. pp. 29-40.

Ng SYC, Ng NQ. 1991. Reduced growth storage of germplasm. In: Dodds JH, editor. *In Vitro* Methods for Conservation of Plant Genetic Resources. Chapman and Hall, London, UK. pp. 11-39.

Ng SYC, Ng NQ. 1996. Yam *in vitro* genebank management at IITA. In: Engelmann F, editor. Management of Field and *In Vitro* Germplasm Collections, Proceedings of a Consultation Meeting; 15–20 January 1996; CIAT, Cali, CO. IPGRI, Rome, IT. pp. 16-18.

Ng SYC, Ng NQ. 1997. Germplasm conservation in food yams (*Dioscorea* spp.): constraints, application and future prospects. In: Razdan MK, Cocking EC, editors. Conservation of Plant Genetic Resources *In Vitro*. Vol. I: General Aspects. Science Publishers, Inc. Enfield, New Hampshire, USA. pp. 257-286.

Ng SYC, Ng NQ. 2000. Cryopreservation of cassava and yam shoot tips by fast freezing. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 418-420.

Ng SYC, Ng NQ. 2002. Genetic resources and conservation. In: Hillocks, RJ, Thresh JM, Belloti AC, editors. CAB International Cassava: Biology, Production and Utilization. CAB International, Egham, UK. pp. 167-177.

Ng SYC, Mantell SH, Ng NQ. 1999. Biotechnology in germplasm management of cassava and yams. In: Benson EE, editor. Plant Conservation Biotechnology. Taylor and Francis, London, UK. pp. 179-209.

Nilsson JR. 1980. Effects of dimethyl sulphoxide on ATP content and protein synthesis in *Tetrahymena*. Protoplasma 103:189-200.

OECD. 2007. OECD best practice guidelines for biological resource centres. OECD, Paris, FR.

Panis B. 2008. Cryopreservation of monocots. In: Reed BM, editor. Plant Cryopreservation: a Practical Guide. Springer, New York, USA. pp. 241-280.

Panis B. 2009. Cryopreservation of *Musa* germplasm. Technical Guidelines No. 9. (Engelmann F, Benson E, editors.) (2nd ed.). Bioversity International, Montpellier, FR.

Panis B, Thinh NT. 2001. Cryopreservation of *Musa* germplasm. INIBAP Technical Guidelines No. 5. (Escalant JV, Sharrock S, editors.) INIBAP, Montpellier, FR.

Panis BJ, Withers LA, De Langhe EAL. 1990. Cryopreservation of *Musa* suspension cultures and subsequent regeneration of plants. CryoLetters 11:337-350.

Panis B, Totté N, Van Nimmen K, Withers LA, Swennen R. 1996. Cryopreservation of banana (*Musa* spp.) meristem cultures after preculture on sucrose. Plant Science 121:95-96.

Panis B, Schoofs H, Thinh NT, Swennen R. 2000. Cryopreservation of proliferating meristems cultures of banana. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 238-244.

Panis B, Strosse H, Van Den Hende S, Swennen R. 2002. Sucrose preculture to simplify cryopreservation of banana meristem cultures. CryoLetters 23:375-384.

Panis B, Piette B, Swennen R. 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. Plant Science 168:45-55.

Panta A, Panis B, Ynouye C, Criel B, Swennen R, Roca W. 2006. Improvement of potato cryopreservation for the long-term conservation of Andean land races at the International Potato Center (CIP). Cryobiology 53:401.

Panta A, Ynouye C, Espinoza C, Roca W. 2007a. Manual of potato, sweetpotato and other Andean root and tuber *in vitro* genebank crops. Internal Protocols. CIP, Lima, PE.

Panta A, Ynouye C, Panis B, Swennen R, Roca W. 2007b. Potato cryopreservation with PVS2. CIP, Lima, PE.

Pennycooke JC, Towill LE. 2000. Cryopreservation of shoot tips from *in vitro* plants of sweet potato [*Ipomoea batatas* (L.) Lam.] by vitrification. Plant Cell Reports 19:733-737.

Pennycooke JC, Towill LE. 2001. Medium alterations improve regrowth of sweet potato [*Ipomoea batatas* (L.) Lam.] shoot tips cryopreserved by vitrification and encapsulation-dehydration. CryoLetters 22: 381-389.

Perazzo G, Panta A, Rodriguez F, Gómez R, Toledo J, Huamán Z, et al. 2000. Clonal true-to-type verification of potato accessions retrieved from *in vitro* conservation and cryopreservation. CIP program report 1999-2000. CIP, Lima, PE. Available from URL: (http://www.cipotato.org/publications/program_reports/99_00/20clonverif.pdf).

Pillai SV, Sundaresan S, Sheela MN. 2007. Biotechnology in cassava germplasm conservation and breeding in India. In: Howeler RH, editor. Cassava Research and Development in Asia: Exploring New Opportunities for an Ancient Crop. Proceedings 7th Regional Workshop; 28 October - 1 November 2002; Bangkok, TH. pp. 140-149.

Popov AS, Volkova LA, Butenko RG. 1995. Cryopreservation of germplasm of *Dioscorea deltoidea* (Medicinal Yam) In: Bajaj YPS, editor. Biotechnology in Agriculture and Forestry. Vol. 32. Cryopreservation of Plant Germplasm I. Springer-Verlag, Berlin, Heidelberg, DE. pp. 487-499.

Ramon M, Geuns JMC, Swennen R, Panis B. 2002. Polyamines and fatty acids in sucrose precultured banana meristems and correlation with survival rate after cryopreservation. CryoLetters 23:345-352.

Reed BM. 1988. Cold acclimation as a method to improve survival of cryopreserved *Rubus* meristems. CryoLetters 9:166-171.

Reed BM. editor. 2008a. Plant Cryopreservation: a Practical Guide. Springer, New York, USA.

Reed BM. 2008b. Cryopreservation - practical considerations. In: Reed BM, editor. Plant Cryopreservation: a Practical Guide. Springer, New York, USA. pp. 3-13.

Reed BM, Uchendu E. 2008. Controlled rate cooling. In: Reed BM, editor. Plant Cryopreservation: a Practical Guide. Springer, New York, USA. pp. 77-92.

Reed BM, Dumet DJ, Denoma JM, Benson EE. 2001. Validation of cryopreservation protocols for plant germplasm conservation: a pilot study using *Ribes* L. Biodiveristy and Conservation 10:939-949.

Reed BM, Kovalchuk I, Kushnarenko S, Meier-Dinkel A, Schoenweiss K, Pluta S, Straczynska K, Benson EE. 2004. Evaluation of critical points in technology transfer of cryopreservation protocols to international plant conservation laboratories. CryoLetters 25:341-352.

Roca WM. 1984. Cassava. In: Sharp WR, Evans DA, Ammirato PV, Yamada Y, editors. Handbook of Plant Cell Culture; Vol. 2: Crop Species. Macmillan, New York, USA. pp. 269-301.

Roca WM. 2007. (Coordinator), editor. Global Strategy for *ex situ* conservation of sweetpotato genetic resources. Global Crop Diversity Trust, Rome, IT.

Roca WM, Reyes R, Beltrán J. 1984. Effect of various factors on minimal growth in tissue culture storage of cassava germplasm. In: Proceedings of the 6th Symposium of the International Society for Tropical Root Crops. 20 – 25 February 1983; Lima, PE. pp. 441-446.

Roca WM, Chaves R, Marin ML, Arias DI, Mafla G, Reyes. R. 1989. *In vitro* methods of germplasm conservation. Genome 31:813-817.

Roca WM, Henry G, Angel F, Sarria R. 1992. Biotechnology research applied to cassava at the International Center of Tropical Agriculture (CIAT). AgBiotech News and Information. 14: 202N-308N.

Roca WM, Debouck DG, Escobar RH, Mafla G, Fregene M. 2000. Cryopreservation and cassava germplasm conservation at CIAT. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 273-279.

Ryan MJ, Smith D, Jeffries P. 2000. A decision-based key to determine the most appropriate protocol for the preservation of fungi. World Journal of Microbiology and Biotechnology 16:183-186.

Sahijram L, Soneji JR, Bollamma KT. 2003. Invited review: analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). *In Vitro* Cellular and Development Biology Plant 39:551-556.

Sakai A. 2000. Development of cryopreservation techniques. In: Engelmann F, Takagi H, editors. 2000. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 1-8.

Sakai A, Kobayashi S, Oiyama, I. 1990. Cryopreservation of nuclear cells of navel orange (*Citrus sinensis* Osb.var *brasiliensis* Tanaka) by vitrification. Plant Cell Reports 9:30-33.

Sakai A, Hirai D, Niino T. 2008. Development of PVS-based vitrification and encapsulation-vitrification protocols. In: Reed BM, editor. Plant Cryopreservation: a Practical Guide. Springer, New York, USA. pp. 33-57.

Sarakarn A, Limsila A, Suparhan D, Wongtiem P, Hansetasuk J, Watananonta, W. 2007. Cassava germplasm conservation and crop improvement in Thailand. In: Howeler RH, editor. Centre Internacional de Agricultura Tropical (CIAT) Cassava Research and Development in Asia: Exploring New Opportunities for an Ancient Crop. Proceedings 7th Regional Workshop, Bangkok, TH. 28 October - 1 November 2002. CIAT, Cali, CO. pp. 58-66.

Sarkar D, Naik PS. 1998a. Factors affecting minimal growth conservation of potato microplants *in vitro*. Euphytica 102:275-280.

Sarkar D, Naik PS. 1998b. Cryopreservation of shoot tips of tetraploid potato (*Solanum tuberosum* L.) clones by vitrification. Annals of Botany 82:455-461.

Sarkar D, Kuashik SK, Naik PS. 1999. Minimal growth conservation of potato microplants: silver thiosulfate reduces ethylene-induced growth abnormalities during prolonged storage *in vitro*. Plant Cell Reports 18:133-122.

Sarkar D, Chakrabarti SK, Naik PS. 2001. Slow growth conservation of potato microplants: efficacy of ancymidol for long-term storage *in vitro.* Euphytica 117:133-142.

Sarkar D, Sud KC, Chakrabarti, SK, Naik PS. 2002. Growing of potato microplants in the presence of alginate silver thiosulfate capsules reduces ethylene-induced growth abnormalities during minimal growth conservation *in vitro*. Plant Cell Tissue and Organ Culture 68:79-98.

Sarkar D, Pandey SK, Chanemougasoundharam A, Sud KC. 2005. The role of calcium nutrition in potato (*Solanum tuberosum*) microplants in relation to minimal growth over prolonged storage *in vitro*. Plant Cell Tissue and Organ Culture 81:221–227.

Schnabel-Preikstas B, Earle ED, Steponkus PL. 1992. Cryopreservation of sweet potato shoot tips by vitrification. Cryobiology 29:738-739.

Schäfer-Menuhr A. 1993. Cryopreservation of old potato cultivars. Proceedings of the 12th Conference of the European Association of Potato Research. 18-23 July 1993. Paris, FR. Poster 177.

Schäfer-Menuhr A, Müller E, Mix-Wagner G. 1996. Cryopreservation: an alternative for the long-term storage of old potato varieties. Potato Research 39:507-513.

Schäfer-Menuhr A, Schumacher HM, Mix-Wagner G. 1997. Long-term storage of old potato varieties by cryopreservation of shoot tips in liquid nitrogen. Plant Genetic Resource Newsletter 111:19-24.

Schäfer-Menuhr A, Mix-Wagner G, Keller J, Schüler K. 1998. Liquid nitrogen: a medium to store germplasm. Beiträge zur Züchtungforschung 4:157-162.

Scott GJ, Best M, Rosegrant M, Bokanga M. 2000. Roots and tubers in the global food system: a vision statement to the year 2020. In: A co-publication of the International Potato Centre, Peru (CIP), Centro Internacional de Agricultura Tropical (CIAT); International Food Policy Research Institute (IFPRI), International Institute of Tropical Agriculture (IITA) and International Plant Genetic Resources Institute (IPGRI). International Potato Centre, Lima, PE.

Scowcroft WR. 1984. Genetic variability in tissue culture: impact on germplasm conservation and utilization. International Board for Plant Genetic Resources. Report (AGPG:IBPGR/84/152), Rome, IT.

Sharma SK, Bryan GJ, Winfield MO, Millam S. 2007. Stability of potato (*Solanum tuberosum* L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds: a comparative phenotypic, cytogenetic and molecular assessment. Planta 226:1449–1458.

Sharrock S, Engels J. 1996. Complementary conservation. INIBAP Annual Report. International Network for the Improvement of Banana and Plantain, Montpellier, FR. pp. 6-9.

Smith D, Ryan MJ. 2008. The impact of OECD best practices on the validation of cryopreservation techniques for microorganisms. CryoLetters 29:63-72.

Steponkus PL, Langis R, Fujikawa S. 1992. Cryopreservation in plant tissues by vitrification. In: Steponkus PL, editor. Advances in Low Temperature Biology. Vol. 1. Jai Press Ltd., London, UK. pp. 1-61.

Stewart P, Taylor M, Mycock D. 2001. The sequence of the preparative procedures affects the success of cryostorage of cassava somatic embryos. CryoLetters 22:35-42.

Takagi H, Thinh NT, Kyesmu PM. 1998. Cryopreservation of vegetatively propagated tropical crops by vitrification. Acta Horticulturae 461:485-494.

Thieme R. 1992. An *in vitro* potato cultivar collection: microtuberization and storage of microtubers. Plant Genetic Resources Newsletter 88/89: 17-19.

Thinh NT, Takagi H, Yashima S. 1999. Cryopreservation of *in vitro*-grown shoot tips of banana (*Musa* spp.) by vitrification method. CryoLetters 20:163-174.

Thomas P, Swarna GK, Roy PK, Patil P. 2008. Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv Grand Naine. Plant Cell Tissue and Organ Culture 93:55-63.

Thro AM, Roca WM, Restrepo J, Caballero H, Paots S, Escobar R, Mafla G, Hernandez C. 1999. Can *in vitro* biology have a farm-level impact for small-scale cassava farmers in Latin America? *In Vitro* Cellular and Development Biology Plant 35:382-387.

Towill LE. 1981a. *Solanum etuberosum*: a model for studying the cryobiology of shoot tips in the tuber-bearing *Solanum* species. Plant Science Letters 20:315-324.

Towill LE. 1981b. Survival at low temperatures of shoot tips from cultivars of *Solanum tuberosum*: CryoLetters 2:373-382.

Towill LE. 1982. Low temperature storage (-196°C) storage of true seed from tuber-bearing *Solanum* species. American Potato Journal. 59:141-147.

Towill LE. 1983. Improved survival after cryogenic exposure of shoot tips derived from *in vitro* plantlets of potato. Cryobiology 20:567-573.

Towill LE. 1984. Survival at ultra-low temperatures of shoot tips from *Solanum tuberosum*, groups of *Andigena, Phureja, Stenotomum, tuberosum* and other tuber-bearing *Solanum* species. CryoLetters 5:319-326.

Towill LE, Jarret RL. 1992. Cryopreservation of sweet potato (*Ipomea batata* [L.] Lam.) shoot tips by vitrification. Plant Cell Reports 11:175-178.

Ulrich K, Stauber T, Ewald D. 2008. *Paenibacillus* - a predominant endophytic bacterium colonising tissue cultures of woody plants. Plant Cell Tissue and Organ Culture 93:347-351.

Unnikrishnan M, Easwari Amma CS, Sreekumari MT, Sheela MN, Mohan C. 2007. Cassava germplasm conservation and improvement in India. In: Howeler RH, editor. Centre Internacional de Agricultura Tropical (CIAT) Cassava Research and Development in Asia: Exploring New Opportunities for an Ancient Crop. Proceedings 7th Regional Workshop Bangkok, TH. 28 October - 1 November 2002. CIAT, Cali, CO. pp. 87-100.

Van den houwe I, Swennen R. 2000. Characterization and control of bacterial contaminants in *in vitro* cultivars of banana (*Musa* spp.) Acta Horticulturae 530:69-79.

Van den houwe I, De Smet K, Tezenas du Montcel H, Swennen R. 1995. Variability in storage potential of banana shoot cultures under medium term storage conditions. Plant Cell Tissue and Organ Culture. 42:269-274.

Van den houwe I, Gun J, Swennen R. 1998. Bacterial contamination in *Musa* shoot tip cultures. Acta Horticulturae 490:485-492.

Van den houwe I, Panis B, Swennen R. 2000. The *in vitro* germplasm collection at the *Musa* INIBAP International Transit Centre and importance of cryopreservation. In: Engelmann F, Takagi H, editors. Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. Japan International Research Centre for Agricultural Sciences, Tsukuba, JP, IPGRI, Rome, IT. pp. 255-260.

Van Soest L. 2006. Coordinator. Global conservation strategy for potato. Global Crop Diversity Trust, Rome, IT. Available from URL: http://www.croptrust.org/documents/web/Potato-Strategy-FINAL-30Jan07.pdf

Viana AM, Mantell SH. 1989. Callus induction and plant regeneration from excised zygotic embryos of the seed-propagated yams *Dioscorea composita* Hemsl. and *D. cayenensis* Lam. Plant Cell Tissue and Organ Culture. 16:113-122.

Villafranca MJ, Veramendi J, Sota V, Mingo-Castel AM. 1998. Effect of physiological age of mother tuber and number of subcultures on *in vitro* tuberization of potato (*Solanum tuberosum* L.). Plant Cell Reports 17:787-790.

Villalobos VM, Abdelnour A. 1992. Cryoconservation of *Musa* spp and its potential for long-term storage of other tropical crops. In: Adams RP, Adams JE, editors. Conservation of Plant Genes: DNA Banking and *In Vitro* Biotechnology. Academic Press Inc, London, UK. pp. 197-210.

Volk GM, Walters C. 2003. The preservation of genetic resources in the national plant germplasm collections. Plant Breeding Reviews 23:291-344.

Volk GM, Walters C. 2006. Plant vitrification solution 2 lowers water content and alters freezing behaviour in shoot tips during cryoprotection. Cryobiology 52:48-61.

Vuylsteke DR. 1989. Shoot tip culture for the propagation, conservation and exchange of *Musa* germplasm. Practical Manuals for Handling Crop Germplasm *In Vitro* 2. IBPGR, Rome, IT.

Ward ACW, Benson EE, Blackhall NW, Cooper-Bland S, Powell W, Power JB, Davey MR. 1993. Flow cytometric assessments of ploidy stability in cryopreserved dihaploid *Solanum tuberosum* and wild *Solanum* species. CryoLetters 14:145-152.

Westcott RJ. 1981a. Tissue culture storage of potato germplasm 1. Minimal growth storage. Potato Research 24: 331-342.

Westcott RJ. 1981b. Tissue culture storage of potato germplasm 2. Use of growth retardants. Potato Research 24:331-342.

Westcott RJ, Henshaw GG, Roca WM. 1977. Tissue culture storage of potato germplasm: culture initiation and plant regeneration. Plant Science Letters 9:309-315.

Williams AC, Barry BW. 2004. Penetration enhancers. Advanced Drug Delivery Reviews 565:603-618.

Withers LA. 1985. Minimum requirements for receiving and maintaining tissue culture propagation material. FAO, UN, Rome, IT.

Yoon Ju-W, Kim H-H, Ko H-C, Hwang H-S, Hong E-S, Cho E-G, Engelmann F. 2006. Cryopreservation of cultivated and wild potato varieties by droplet vitrification: effect of subculture of mother-plants and preculture of shoot tips. CryoLetters 27:211-222.

Zámečník J, Faltus M, Bilavčík A. 2007. Cryoprotocols used for cryopreservation of vegetatively propagated plants in the Czech Republic cryobank. Special issue on technology, application and validation of plant cryopreservation. Advances in Horticulture Science 21:247-250.

Zhao MA, Xhu YZ, Dhital SP, Khu DM, Song YS, Wang MY, Lim HT. 2005. An efficient cryopreservation procedure for potato (*Solanum tuberosum* L.) utilizing the new ice blocking agent, Supercool X1000. Plant Cell Reports 24:477-481.

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