

**TECHNICAL GUIDELINES FOR THE MANAGEMENT OF
FIELD AND *IN VITRO* GERMPLASM COLLECTIONS**

Barbara M. Reed, Florent Engelmann, Ehsan Dullo and Johannes M.M. Engels
(eds.).

IPGRI/FAO/SGRP, Rome, Italy

LOGOS OF IPGRI, FAO AND SGRP

+ BLURB OF IPGRI & SGRP ON THE INSIDE COVER

95 Pages

I GENERAL CONSIDERATIONS FOR THE ESTABLISHMENT AND MANAGEMENT OF GERMPLASM COLLECTIONS 2

I.1 ESTABLISHMENT OF THE COLLECTION	2
Assess current status and seek information:	2
Determine the present and future germplasm needs for each crop:	3
I.2 ACQUISITION AND ENTRY OF PLANTS INTO THE COLLECTION	4
Assemble comprehensive / representative diversity:	4
Setup labeling/numbering and record keeping system: (Appendices A and B)	5
Establish procedures for log-in of new plant materials:	5
Be aware of quarantine regulations:	6
Consider access and benefit sharing arrangement:	6
I.3 GERMPLASM HEALTH	7
Provide isolation procedures for samples entering the germplasm collection:	7
Particular problems produced by virus infection:	8
Timing of virus indexing:	8
Virus elimination methods:	8
I.4 CONSERVATION METHODS AVAILABLE TO GERMPLASM COLLECTIONS	9
Importance of complementary methods:	1
Level of duplication needed:	1
Safety duplication methods:	2
How to determine which complementary methods to use:	3
Cost comparison of field and <i>in vitro</i> storage:	4
I.5 COLLECTION MANAGEMENT	5
Correctly identify new plant material: (Appendix C)	5
Rationalize collection size:	7
Create a facilities operations manual:	8
Distribute germplasm: (Appendices D, E, and F)	9

II PROCEDURES FOR ESTABLISHING AND MAINTAINING FIELD AND *IN VITRO* COLLECTIONS 11

II.1 FIELD PROCEDURES	11
II.1.1 ESTABLISH AND MAINTAIN VIGOROUS, HEALTHY PLANTS	11
Select appropriate propagation methods:	11
Select appropriate planting sites:	12

Select appropriate planting procedures:	13
Use proper cultivation practices:	13
Manage disease and pest problems:	13
Harvest and store propagules properly:	14
II.1.2 MAINTAIN COLLECTION SECURITY	15
Monitor genetic stability:	15
Prevent incorrect labeling of plants:	16
II.1.3 DISTRIBUTE PLANT GERMPLASM	16
II.1.4 RESEARCH NEEDS IN THE FIELD COLLECTION	17
II.2 <i>IN VITRO</i> PROCEDURES	21
II.2.1. LABORATORY FACILITIES	21
Laboratory:	21
Culture growth rooms:	22
II.2.2 CULTURE STORAGE FACILITIES	25
II.2.3 OPERATIONS	25
Laboratory upkeep:	26
Staffing:	26
Develop a procedures manual:	27
II.2.4 ESTABLISH A TISSUE CULTURE SYSTEM	27
Research techniques:	27
Develop protocols: (Appendices G and H)	28
Design a record-keeping system: (Appendix I)	28
II.2.5 INTRODUCE PLANT MATERIAL INTO TISSUE CULTURE:	29
Explants: (Appendices J and K)	29
Multiplication:	33
II.2.6 CULTURE STORAGE	34
Slow-growth storage: (Appendix L)	34
Safety duplication:	37
Monitor genetic stability:	37
II.2.7 CRYOPRESERVATION	39
Facility requirements:	39
Techniques:	39
Storage Procedures:	41
Monitor genetic stability:	42
II.2.8 DISTRIBUTION	43
Plant materials:	43
Procedures for recipients of dispatched cultures:	44
Phytosanitary and quarantine considerations: (Appendix M)	44
Packaging and shipping:	44
II.2.9 RESEARCH NEEDS IN THE <i>IN VITRO</i> COLLECTION	45
Optimizing slow growth protocols:	45
Germplasm health:	45

	4
Monitoring genetic stability:	45
Cryopreservation:	46
III REFERENCES	46
IV APPENDICES	57
Appendix A. Example of a record keeping system for a germplasm collection (NCGR).	57
Appendix B. Accession inventory database information for NCGR (Foxpro database).	61
Appendix C. Basic CEC apple descriptors, passport data (IBPGR).	63
Appendix D. Example of a plant distribution policy statement.	64
Appendix E. Plant distribution schedule from USDA/ARS Operations Manual (Hummer, 2003).	66
Appendix F. Distribution procedures for plant germplasm (Hummer, 2003).	67
Order Processing	67
Order Preparation	67
Shipping	68
Paperwork Completion	68
Appendix G. Sample plant tissue culture medium recipe sheets.	69
Example of tissue culture medium sheet from NCGR (Hummer, 2003).	69
Example of tissue culture medium sheet from CARDI (Bateson, personal communication).	69
Example of stock solution sheets from CARDI (Bateson, personal communication).	71
Appendix H. Protocols for plant tissue culture of <i>Fragaria</i> (Hummer, 2003).	72
Appendix I. Record keeping system for <i>in vitro</i> storage at NCGR (Reed, personal communication).	73
Appendix J. Contamination and explant indexing for <i>in vitro</i> cultures.	74
Contamination detection for explants and multiplying plantlets at NCGR (Hummer, 1996).	74
Detection of endophytic bacteria in banana tissue cultures at INIBAP (Van den houwe, personal communication).	74
Appendix K. Antibiotic treatments for <i>in vitro</i> cultures (Reed et al., 1995).	75
Appendix L. Inventory data taken for <i>in vitro</i> -stored cultures.	76

	5
Data taken for cassava and yam at CARDI (Bateson, personal communication).	76
Data taken for <i>Corylus</i>, <i>Fragaria</i>, <i>Humulus</i>, <i>Mentha</i>, <i>Pyrus</i>, <i>Ribes</i>, <i>Rubus</i>, and <i>Vaccinium</i> at NCGR (Reed, personal communication).	77
Appendix M: INIBAP germplasm health statement.	78

ACKNOWLEDGEMENTS

The editors wish to thank FAO and SGRP for their financial contribution for the publication of this volume.

PREFACE

Plant species which are vegetatively propagated, which have long life cycles and/or produce non-orthodox seeds are traditionally maintained in field collections. Maintaining plants in the field is costly and carries high risks of loss, therefore the strategies and procedures employed to establish and maintain field collections need to be practical, rational and economic in addition to being scientifically sound. Experience in cost-effective management of field collections lies with individual curators and is not readily available to guide others. Furthermore, there are increasing opportunities for using *in vitro* methods for the conservation of crops normally conserved in the field, and there is a need to develop the strategies and procedures for managing *in vitro* collections as routine and integral part of the overall conservation strategy of a crop gene pool or collection.

IPGRI, together with the System-wide Genetic Resources Programme (SGRP), the Food and Agriculture Organization of the United Nations (FAO) and the International Centre of Tropical Agriculture (CIAT) organized in 1996 a technical consultation meeting held CIAT, Cali, Colombia, to examine the role of field and *in vitro* germplasm collections in strategies to conserve and use clonally propagated crops, problems and constraints in their management, and the complementarity of field and *in vitro* germplasm collections. One of the recommendations of the meeting was to develop general guidelines for the management of field and *in vitro* germplasm collections, which should identify key issues and provide genebank managers with decision criteria and options in establishing, maintaining and using field and *in vitro* germplasm collections.

The first draft of these Guidelines has been produced by Dr. Barbara M. Reed, Plant Physiologist at the USDA/ARS National Clonal Germplasm Repository, Corvallis, Oregon (USA). It has been further developed and updated by a consultant and several IPGRI staff members have provided significant inputs in this document.

These Guidelines are divided in two main sections. The first section presents general considerations for the establishment and management of germplasm collections. The topics addressed concern the establishment of the collection, the acquisition and entry of plants into the collection, germplasm health issues, a presentation of the various conservation methods available to germplasm collections and collection management procedures. The second section focuses on the procedures for the establishment and maintenance of field and *in vitro* collections, which are dealt with in two sub-sections. The first sub-section, which concerns field collections, presents and discusses procedures for establishing and maintaining vigorous and healthy plants, for maintaining the security of the collections and for distributing plant germplasm. Finally, research needs related to the management of field collections are identified. The second sub-section deals with *in vitro* collections. The laboratory and storage facilities requested for the establishment and maintenance of *in vitro* collections are presented and the need for the establishment of detailed standard operational procedures highlighted. *In vitro* culture and conservation procedures are then presented and analyzed, including the establishment of a tissue culture system, the introduction of plant material *in vitro*, slow growth storage, cryopreservation and distribution of

plant material. Finally, research needs aiming at improving *in vitro* conservation of plant germplasm collections are identified.

Boxes are included throughout the document to illustrate various points addressed in the text through concrete examples of procedures developed by genebank curators, or to provide detailed information on specific subjects. Similarly, a total of 13 Appendices are provided as examples of documents employed in the management of field and *in vitro* collections (e.g. record keeping systems for field and *in vitro* collections) and of particular procedures (e.g. detection of contaminants in *in vitro* cultures).

It is hoped that these Guidelines will contribute to improving the conservation of vegetatively propagated crops through a more rational, complementary and cost-effective use of field and *in vitro* conservation techniques.

Jan Engels
Group Director
Genetic Resources Science and Technology

Florent Engelmann
Honorary Research Fellow
In Vitro Conservation and Cryopreservation

I GENERAL CONSIDERATIONS FOR THE ESTABLISHMENT AND MANAGEMENT OF GERMPLASM COLLECTIONS

I.1 ESTABLISHMENT OF THE GERMPLASM COLLECTION

Assess current status and seek information:

This section discusses the need for long-term collecting and maintenance strategies and for defining the present and future germplasm needs for a clonally propagated crop.

Need for a long-term collecting and maintenance strategy:

“Before we begin to estimate the completeness of a given collection we should establish clear criteria for doing so.” (Westwood 1989, p. 118).

Long-term funding must be available for collecting, characterization, and maintenance when genebanks are mandated. Valuable genes, clones, or plants can be lost if funds are allocated for collecting but not for characterization or maintenance. Diverse or inaccessible genes could be lost when a collection is reduced based on the evaluation of only a single trait.

Appropriate collecting strategy: Appropriate collecting strategies should be designed specifically for each crop (Guarino *et al.* 1995). Collecting for field collections calls for a judicious collecting strategy because of space limitation and expenses involved in maintaining such collections. A germplasm collection needs not include every known cultivar, especially with regard to seed propagated F1 hybrids. However, individual landraces and old cultivars, especially of cross-pollinated crops should be collected and preserved (e.g. Shallot); specific genotypes should be selected to represent specific traits. A representative of every named variety from every locality is also not needed in a national collection (Eastwood and Steele 1975). A single genotype might vary in phenotypic response from farm to farm due to environmental or microclimatic conditions. This phenotypic variation may further confuse collectors when cultivars are renamed as they pass to different countries or areas with other languages. When two accessions are suspected to be the same it may be necessary to research the passport information, and further characterization may be required. With some crops, several years of field evaluation are needed to consider environmental effects on genotype response (e.g. bulb crops which may bolt infrequently or respond differently to cold induction or dry spring weather).

Acquisition policy: The type and number of accessions in a collection will depend upon the institute’s mandate, personnel, storage capacity, funding and additional responsibilities such as research or propagation. The mandate may limit collections to specific crops and their wild relatives, certain genotypes, active collections only, etc. A curator may choose to obtain all available material and characterize accessions later to identify duplicates, but the drawback of this approach is that the low-level of funding often prohibits future characterization. Ideally, collecting and characterization should be planned simultaneously, whenever possible. Clones

could then be characterized side by side in the field when collected. Historical and cultural information obtained from farmers, botanists, horticulturalists, or native people during collecting expeditions usually provides valuable information. Local knowledge about the origin of a clone and disease and insect resistance can decrease characterization costs and limit duplication. It is important to properly document such information on the accessions during collecting as well as characterization information.

Other considerations: Political and economic considerations affect germplasm collecting and maintenance. Many issues determine where and how large a collection will be. Some of the questions that one needs to ask are, for example:

- Are there enough resources and land at the site to accept the proposed germplasm?
- Can the proposed germplasm be preserved without adding personnel?
- Is the site's climate and ecology conducive to maintenance?
- Might the genus be handled on a restricted or reduced scale, relative to the other crop genera? (National Plant Germplasm Committee 1986)
- Is there an urgent and immediate danger to the existence of specific ecotypes that should result in immediate collecting (e.g. major construction works, diversion of rivers, dams)?

Determine the present and future germplasm needs for each crop:

When establishing a collection, adding new genera, or adding new plants, the following may be considered:

- the current status of the crop's vulnerability (genetic base),
- the germplasm's potential to agriculture and breeding programs,
- the potential range of genetic diversity available elsewhere, and
- the availability of the proposed germplasm.

Completeness of the collection: Estimation of the collection's completeness should be based upon the crop's genetic base. It is advisable to establish a committee of plant breeders, horticulturalists, plant pathologists, and entomologists to discuss and analyze the collection and lay out a clear plan for conservation of a long-term collection. The genetic potential of new germplasm in breeding for disease resistance, yield increase and quality improvement must also be considered. The vulnerability of native species or cultivated landraces should be considered as collections are organized. Taxa in danger of loss and not preserved in other countries, or those with high economic value have the highest collecting priority.

Including the range of genetic diversity: Commercial crops generally contain less than 10% of the total genetic diversity available to a genus (Westwood 1989). Most genetic diversity is found in the wild species. A complete collection should include representation from three parts: the primary genepool, consisting of all cultivated races and the wild races with which they freely cross; the secondary pool, including all species that can be, with difficulty, crossed to the primary genepool and produce at least some fertility; and the tertiary gene pool, containing the

related species which will not cross with the primary genepool under normal conditions, but may be utilized with extreme measures, e.g. embryo rescue, chromosome doubling (Harlan and de Wet, 1971).

Genetic vulnerability: A “statement of genetic vulnerability” for the crop will provide administrators with economic evidence to justify germplasm preservation. This statement should include statistics on the main cultivars, including: production, handling, post-harvest storage, and marketing problems; disease, insect, pest, and soil problems; environmental limitations; and yield and quality data. The range of genetic diversity present in the available germplasm should be noted and the benefits of using this germplasm to solve major problems should be discussed. The economic benefits of using the germplasm conserved in the collection should be stressed whenever applicable (*i.e.* reduced fertilizer and pesticide use, improved quality, longer storage times, shipping quality, etc.) (Chang *et al.* 1989; Westwood 1989). If collections already exist for the crop, gaps in the local collection should be described and a plan for filling them should be developed.

I.2 ACQUISITION AND ENTRY OF PLANTS INTO THE COLLECTION

This section discusses appropriate sampling strategies; the procedures needed in germplasm collections for record keeping, labeling, and registering of accessions; quarantine regulations and intellectual property rights which must be considered when collecting samples or exchanging plants with other facilities.

Assemble comprehensive / representative diversity:

Develop appropriate sampling strategies: Information about population and reproductive biology, phenology, ethnobotany, taxonomy and accessibility must be considered when designing a collecting strategy (Guarino *et al.* 1995). Each crop type will vary in how its genetic diversity is distributed among cultivars, landraces, and wild species; some may require collecting related species or genera. Plant material for field genebanks may be obtained from existing research and breeders’ collections; landraces and cultivated forms grown by native people and farmers; and from plant expeditions to collect wild species. A geographic region may be surveyed for genetic diversity to define a collecting strategy and existing collections or herbaria accessed to determine likely collecting sites. Plans to collect information such as passport data, morphological descriptors and herbarium samples should be included as well (Chang *et al.* 1989; Westwood 1989; Guarino *et al.* 1995).

(1) Wild species: A list of the total number of wild species known and a list of those that are already in the collection should be compiled. Guidelines are available for collecting wild species from populations of both widely-distributed and restricted-range species (Hawkes 1980; National Plant Germplasm Committee 1986). Herbarium specimens should be collected when wild species are collected to provide a base for taxonomic identification. Seed or pollen of fertile species may be collected. Storage of pollen requires little space and could be useful as a supplement for a base collection of clonal lines, for example, from species with recalcitrant seed.

Species with recalcitrant seed do not necessarily have desiccation-sensitive pollen. Pollen alone, however, is not a satisfactory base collection because some cytoplasmic genes may be lost during transmission. Information is also needed on stability and longevity at subzero temperatures and on the development of handling systems before practical storage is achieved (Towill and Roos 1989).

(2) Cultivars and rootstocks: Clones should represent known diversity, not all known cultivars. For domestic clonal cultivars IPGRI has suggested that 250 to 500 cultivars should be enough of each crop to retain the desired genetic diversity. This seems a reasonable guideline. The curator should look to the appropriate “Crop Advisory Council” for guidance on how many cultivars and rootstocks are needed. For the wild species and rootstocks, however, IBPGR has not made suggestions. All wild species and valid subspecies or botanical varieties must be represented in addition to the cultivars, in order to achieve complete genetic diversity (for the genus) (Westwood 1989). A collection’s deficiencies should be noted according to established criteria and collecting prioritized accordingly.

Set-up labeling/numbering and record keeping system: (Appendices A and B)

Record keeping should include plant catalogues, images (photographs, drawings), characterization and evaluation data, planting dates, harvest dates, and accession records with related information. Data for an accession should be available to those who use the collection. Data should be duplicated at regular intervals and stored at a remote site on a regular schedule to guard against loss from fire, computer failure, and tampering. The frequency of back-up should depend on the regularity of updating of the primary database. Many mainframe data bases are backed up daily, but those not frequently used could be backed up as new data are added.

Correctly and clearly written labels are extremely important in germplasm collections. Computer produced labels from verified computer records reduce transcription errors in names and numbers. Errors can also be reduced by identifying plants with a mixture of letters and numbers, and by using more than one identifier, such as name/accession number or accession number/field location. Field maps are also essential and provide a backup to field labels which are easily lost or destroyed.

Establish procedures for registering new plant materials:

Incoming plant materials, including whole plants, scions, tissue culture material, cuttings, seed, and pollen, should be assigned an accession number. This number should link with the accession data, i.e. collecting data, characterization, evaluations, type of propagule received, etc. Each accession number should be unique and should never be re-assigned in case the accession is lost.

To facilitate data continuity, completeness and accuracy, it is recommended that one staff member be primarily responsible to register accessions and assigning sequential numbers. A second staff member, who could act as a backup or in a transitional role, should also know these

procedures. Missing passport data from exchanged materials should be requested when the material is registered, otherwise it may be forgotten and unavailable at a later date. Passport data should include: country of origin, location of collection site, species name, local names, and other base information (**Appendices A, B, C**). Options exist for registering new accessions: 1) register all plants as new accessions and verify identities, etc. later; 2) use temporary registration numbers for questionable accessions until they are verified.

Be aware of quarantine regulations:

Germplasm collection managers and collectors must be aware of and comply with any restrictions applying to the movement of plants from one country to another. National and/or local quarantine regulations may apply to imported or exported plant material. Generally these will be specified by both the donating and the receiving country. If there are no restrictions, it is wise to have an in-house policy (see section III.3) to limit the entry of pathogens or pests into the collection or the surrounding areas. Selection of plant material without apparent disease symptoms is recommended. Discarding diseased accessions may affect the diversity represented. Newly introduced plants should be observed closely throughout the first one or two crop cycles in the collection. Many pathogens can be eliminated by proven treatment methods. IPGRI has produced jointly with FAO technical guidelines for the safe movement of germplasm for a number of crop species (see Box 9 on page XX).

Consider access and benefit sharing arrangements:

International collecting must be conducted in accordance with the requirements of the Convention on Biological Diversity (CBD) (legally-binding) (Convention on Biological Diversity 1992), the legally binding International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) (FAO, 2002) and the International Code of Conduct for Plant Germplasm Collection and Transfer (FAO, 1994) (non-binding). Both CBD and ITPGRFA reaffirm national sovereignty over genetic resources and the authority of national governments to regulate access to these resources. Article 15 of the CBD “Access to genetic resources” calls for access on mutually agreed terms with prior informed consent (unless waived) and for benefit-sharing between the recipient and the source country. Article 10.2 of ITPGRFA establishes a multilateral system which is efficient, effective and transparent both to facilitate access to plant genetic resources for food and agriculture and to share in a fair and equitable way, the benefits arising from the utilization of these resources, on a complementary and mutually reinforcing basis. Additional information on intellectual property rights in relation to the conservation and use of plant genetic resources can be found notably in publications by Leskien and Flitner 1999, IPGRI 1999 and Libreros 2002.

Summary

This section explored the ways of determining what to include in the genebank and how to properly represent the genetic diversity. These topics require research, consultation with crop experts, and possible cooperation from other genebanks, either national or international. Initial intake of plant material in an orderly manner facilitates the remaining functions of the genebank. Labeling, numbering, record keeping systems, and standard log-in procedures allow curators to easily track all accessions and link them with passport data.

I.3 GERMPLASM HEALTH

This section discusses requirements to maintain germplasm health in a germplasm collection. Procedures for excluding foreign pathogens and for detecting and eliminating virus diseases are discussed. Numerous publications detail pathogen identification procedures (see e.g. Diekmann and Putter 1996; Sheppard and Cockerell 1996; Sutherland *et al.* 2002; Mathur and Kongsdal 2003).

Provide isolation procedures for samples entering the collection:

Materials entering the collection should be inspected by an entomologist and plant pathologist to avoid introducing insects, nematodes, bacteria, fungi, and viruses into the collection. In some cases new planting materials should be propagated from the original cutting, tuber, etc. and the original destroyed. Pathogen identification and indexing work require the skills of a trained plant pathologist. For small facilities without resources to hire a pathologist, other options are available: establishing linkages with university laboratories may provide some needed professional assistance; commercial laboratories may also be available to analyze samples for a fee; virus-free plants may be available from research facilities and could replace the virus-infected sample. General recommendations for transferring various types of vegetatively propagated materials may be found in a series of Technical Guidelines for the Safe Movement of Germplasm published jointly between FAO and IBPGR/IPGRI (see complete list page XX). Rabinowitch and Orna (1984) also describe the procedures for handling new accessions at the Field Gene Bank for Vegetatively Propagated Short Day Alliums at Rehovot, Israel. The procedure for handling new accessions at the International Potato Centre (CIP), is that newly acquired potato tubers or sweet potato storage roots or stem cuttings are first inspected, then planted in pots in an isolation screenhouse. When new growth is produced, the new cuttings are established in isolation, the original plant is incinerated and substrate sterilized and discarded. Once the new plants are fully grown they are inspected by the virologist (George 1993, 1996).

Consideration of the diseases of a crop is important in deciding whether to place an accession in a field plot, a screened enclosure, or under *in vitro* culture. Generally the most serious pathogen threat to a germplasm collection will be viruses; however, viroids, phytoplasmas, bacteria, fungi and nematodes may also infect plants systemically and may be difficult to detect. Vegetative material is often infested with arthropod pests, and mites, thrips, and mealybugs may be difficult to detect and act as virus vectors.

Accessions which are very susceptible to pathogens may be lost if they are placed in the field. Virus-free accessions are best kept virus-free either in screened enclosures or *in vitro*. Field collections of accessions with sap-transmitted virus diseases may be somewhat protected by placing them in isolated fields and eliminating weed hosts. When virus indexing capabilities are unavailable, curators should refrain from distributing materials which are known to have come from virus-infected areas. However, threatened plants (endangered species or cultivars) should be added to the collection whatever their virus status, provided that it can be ensured that there is no risk of virus spread. They must be kept physically separate from the main collection, until virus testing and elimination are done when necessary resources become available.

Particular problems produced by virus infection:

Clonally propagated plants can accumulate virus diseases which may impair plant vigor, hardiness, graft compatibility or other characteristics. Some viruses produce obvious symptoms while others are latent or symptomless. It is important to use virus-negative plants for research studies or when evaluating plant characteristics to obtain consistent results. Identification of viruses in garlic (*Allium sativum*) and related species has been described (Barg *et al.* 1994) and a review of viruses in *Allium* crops is also available (Walkey 1990).

Timing of virus indexing:

Initial indexing and virus elimination may be done when propagules are first received and before *in vitro* culture, or materials may be placed into *in vitro* culture for safekeeping while testing and elimination are taking place. Virus testing and elimination could be done at any point after *in vitro* culture, as time and personnel permit, but no plants should be distributed until testing is completed.

The amount of virus in the plant varies. In field-grown plants the amount varies throughout the growing season, while in greenhouse plants the amount of virus present can depend upon temperature. Reliability of some detection methods may vary with the virus concentration. Serological techniques or molecular probes are highly sensitive and are not as seasonally dependent as some traditional methods, but they are not available for many viruses. Sap inoculation or graft inoculation of indicator plants may be required for the detection of many viruses. Indicator plants should generally be inoculated in early spring, or the beginning of the growing season, when viruses are more easily detected. Laboratory techniques may be used to test *in vitro* plants but generally *in vitro* plants do not provide adequate inoculum for inoculation assays. A few viruses may be identified by visually examining the plants. However, this is generally unreliable, especially with *in vitro* plants. Examples of protocols for detecting *Musa* viruses followed by INIBAP's Virus Indexing Centers can be found in Diekmann and Putter (1996) and those for detection of potato viruses in Salazar (1996).

Virus elimination methods:

Thermotherapy: Heat therapy followed by apical meristem culture has been used to successfully

eliminate many viruses from a variety of plant species (George 1993). The heat treatment may be done either *in vitro* or *in vivo*. Meristem culture alone may successfully eliminate some viruses, but is usually combined with heat therapy for better results. Since virus elimination procedures are not 100% successful, all plants generated by these techniques must be retested to verify their virus status. Ideally, perennial plants should be retested after going through a normal dormant or winter season. Some viruses can be eliminated with cold treatment.

Chemotherapy: Chemotherapy, either alone or in combination with other techniques, is becoming increasingly available as a virus elimination tool (George 1993). Anti-viral chemicals may be either sprayed onto a plant or incorporated into tissue culture media. Often a chemical therapy is followed by meristem culture. The chemical concentration, treatment durations, and possible adverse effects have not been investigated for most crop plants (Smith 1980).

Summary

Germplasm health procedures are important in maintaining a healthy collection and providing pathogen-free plants to requesters. A major difficulty is often the lack of techniques or anti-serum for specific viruses. Curators should consider the health status and susceptibility of the plant when deciding how to conserve it.

I.4 CONSERVATION METHODS AVAILABLE TO GERMPLASM COLLECTIONS

This section discusses the use of field, *in vitro* and cryopreservation as complementary methods for managing clonal plant collections. The costs, availability for pathogen eradication, distribution, collecting, and safety duplication are also considered.

Box 1 : Comparison of conservation options for coconuts (from Dulloo *et al* 2004)

	<i>In situ</i> on farm /Home Gardens/natural habitats	Botanic Gardens (Living plants in gardens/ greenhouses)	“Conventional” Genebanks (seed banks, field genebanks)	Slow growth conditions (short-term)	Cryopreservation liquid N (long-term)
Mature plants	<input checked="" type="checkbox"/> Coconuts conserved on farm widely and in Home gardens and natural stands exist on small isolated islands and atolls	<input checked="" type="checkbox"/> Occurs in botanic gardens but limited scope for conserving genetic diversity	<input checked="" type="checkbox"/> Field genebank most widely used conservation method so far. National and international Coconut field genebanks exist	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable
Seeds and zygotic embryos	<input checked="" type="checkbox"/> Not feasible- seeds are recalcitrant, no natural soil seed banks	<input checked="" type="checkbox"/> Not feasible	<input checked="" type="checkbox"/> Seeds are recalcitrant and too large; seed conservation not feasible	<input checked="" type="checkbox"/> Field collecting protocol established for zygotic embryos; In vitro culture functional	<input checked="" type="checkbox"/> Cryopreservation protocol has been established for Zygotic embryos; suitable for long term conservation
Somatic embryos	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Mass propagation problematic. Not applicable	<input checked="" type="checkbox"/> not applicable
Pollen	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Possible, for short term conservation (2-6 moths)	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Coconut pollen can be cryopreserved and could be suitable for long term conservation
Apices	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> cryopreservation protocol established; relatively low survival and regeneration of plants very difficult
DNA	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> Storage as DNA libraries exists – value not known	<input checked="" type="checkbox"/> Not applicable	<input checked="" type="checkbox"/> long-term storage possible (LN or –80°C freezer). Use of stored DNA questionable.

Importance of complementary methods:

It is now well recognised that an appropriate conservation strategy for a particular plant gene pool requires a holistic approach, combining the different *ex situ* and *in situ* conservation techniques available in a complementary manner (Engelmann and Engels 2002). *In situ* and *ex situ* methods, including a range of techniques for the latter, are options available for the different gene pool elements (*i.e.*, cultivated species, including landraces and modern varieties, wild relatives, weedy types, etc.). Selection of the appropriate method or methods should be based on a range of criteria, including the biological nature of the species in question, practicality and feasibility of the particular method chosen (which depends on the availability of the necessary infrastructure) as well as the cost-effectiveness and security afforded by its application (Maxted *et al.*, 1997). For example, Dulloo *et al.* (2004) discuss the considerations and provides a framework for developing a complementary conservation strategy specifically for coconuts (see Box 1).

Improved security for germplasm collections is needed. Curators may minimize losses in many ways, but material which is not duplicated in another form or at another location is still risk prone. Field collections of many plant species currently act as both active (for distribution) and base (for conservation) collections, and frequently no duplicate is available (Jarret and Florkowski 1990). Frequent handling of propagules of annual, biennial and perennial plants that require some form of harvesting and replanting increases the possibility of loss, damage, or mislabeling. Reinfection of propagules in the field with virus diseases is also problematic. Insect and nematode borne viruses are quickly transmitted to virus-free material once it is in the field. Simultaneous conservation of accessions *in vitro* provides protection from pests, pathogens, and climatic hazards, and increases their availability for distribution if the materials are maintained virus free.

Level of duplication needed:

The level of duplication in field collections is critically important because of the vulnerability of the collection to erosion in the field (Dulloo *et al.*, 2001). Sufficient replicates of accessions should be kept in each duplicate collection (*i.e.* field and *in vitro*; three field plots; one field plot and one set *in vitro*; or one field or *in vitro* and one set cryopreserved) so that an accession is never lost (see Box 2). Occasional losses could be tolerated if they are easily replaced from the other collection or replicate. If genebanks are maintained under minimal conditions they will require more backups to improve the security of conservation. Loss of an entire collection is possible for any storage method, so duplicate collections are very important, no matter how many replicates are in the active collection. Germplasm maintained in field genebanks may be lost to disease epidemics and *in vitro* collections may be lost due to mite infestations or microbial infections). The level of duplication may also depend on the holdings of other genebanks. Accessions available at another genebank may require fewer duplicates than unique samples.

Box 2: NUMBER OF REPLICATES OF AN ACCESSION

In genebanks around the world, the number of replicates in field genebanks varies among collections from 22 to 100 for grasses, five to 10 for cassava, 10-12 for sweet potato, two to three for garlics, trees, and shrubs, six to 10 for herbaceous plants, and three to 20 for bananas. Space and labor are the limiting factors. Plant characterization requires fewer plants, agronomic evaluation requires many more. Germplasm distribution from the genebank may require keeping more plants as well. For *in vitro* collections the number of replicates varies from three to 20, depending on the particular crop.

Safety duplication methods:

Duplication using field plots is best done at non-contiguous sites or at locations other than the main collection. The form and location of duplicate collections will be dictated by the crop type and cultural methods. Three duplicate field plots would provide protection against different soil types, disease exposure or other environmental factors. *In vitro* storage is excellent for medium-term storage and is used as the active collection in some genebanks. It may be a duplicate for field collections, or the main collection with a second culture collection or a field collection as the duplicates. Box 3 on the complementary methods used at International Potato Centre (CIP) illustrates the safety duplication principle very well. *In vitro* collections are not as useful for evaluation or characterization, so those data should be available before any action is taken in regard to eliminating any parts of the field collection. Some fast-growing vegetative crops can be replanted in the field for evaluation or characterization as needed within a short time. Long-term storage of clonal materials should be in cryopreservation if possible, with *in vitro* and/or field collections kept for active use. Seed of species material can be held in cryopreservation or cold storage to extend the life of the collection. How duplication of a collection is to be done should be considered as early in the planning process as possible.

Box 3: COMPLEMENTARY METHODS USED AT CIP

“The overall strategy for conservation of genetic resources maintained at CIP has several components. First, in addition to the maintenance of native cultivars of these crops in the field genebanks, backup duplicate sets of tubers or roots of each accession in the collections are stored at more than one location for security reasons. Thus, tubers and storage roots are maintained in cold-storage rooms at La Molina, Peru, as well as at each site where these collections are grown in Peru. These materials can be used to restore those accessions that can be lost because of frost, hailstorms or other adverse conditions. Second, the most diverse accessions are also maintained in *in vitro* culture as a backup to the field genebank, for the cleaning of pathogens of selected genotypes and for their international distribution. The production of *in vitro* tuberlets or mini-storage roots is also an alternative for medium-term storage and distribution. Third, seed lots obtained from nonsterile accessions are conserved both for distribution and to secure their long-term conservation. For safety reasons, the *in vitro* potato collection is also stored at the Instituto Nacional de Investigaciones Agropecuarias (INIAP) in Quito, Ecuador. The *in vitro* sweet potato collection is duplicated at the Centro IDEA in Caracas, Venezuela. In addition, accessions are restored to replace lost ones or to increase genetic diversity in national or institutional collections in countries where these genetic resources were collected.”(Huaman, 1999)

How to determine which complementary methods to use:

There are several factors to consider when establishing plant collections (Box 4). The best storage method or combination of methods will vary with the crop type or perhaps with the genotype in question. It is important to maintain field collections for evaluation, characterization, identification, and sometimes distribution purposes. In the case of crops with few insect-borne virus problems, field collections are preferred if they are in the proper climatic zone for good growth. Field collections may be preferred for genotypes which commonly produce variants since they are more easily identified and rogued in the field than *in vitro*.

Box 4 ADVANTAGES OF GERMPLASM CONSERVATION METHODS

(+= applicable; - = not applicable)

Character	Slow growth		
	Field	<i>In vitro</i>	Cryo
Medium term	+	+	-
Long term	-	-	+
Characterize	+	-	-
Evaluate	+	-	-
Virus elimination	-	+	-
Distribution	+	+	+/-
Base collection	+	+	-
Core collection	+	+/-	-
Safety duplication	+	+	+

Curators of collections with a heavy virus load, or those at risk of loss in the field from biotic or abiotic factors, should consider the advantages of *in vitro* storage. Crops with difficult quarantine problems may require distribution as *in vitro* plants. Perennial or tree crop collections may reduce the number of field replicates and thus land costs by duplicating the collection and storing it in tissue culture. Any germplasm curator with interest in long-term storage of crop germplasm should consider cryopreservation for a base collection, especially for irreplaceable accessions such as those that are endangered, extinct elsewhere, habitat destroyed, etc.

If cryopreservation is available, it can provide a secure backup at little ongoing operational cost. Initial costs appear high, but cryopreservation procedures fit well with an *in vitro* facility and require few additional expenditures other than labor. A cryopreserved duplicate should not be considered as the only form for a collection, rather it should be a base collection as insurance against loss of field or *in vitro* stored accessions.

When available, cryopreservation is the preferred form for long-term (base collection) conservation, with *in vitro* storage as the second choice and field collections as the third. For study and utilization (active collections), field collections are preferred, with *in vitro* collections the second choice. Safety duplication in order of priority can be provided by: cryopreservation, *in vitro* storage, field, and black box (storage at a remote facility with no maintenance).

Cost comparison of field and *in vitro* storage:

The cost of field and *in vitro* storage are similar in many cases, however, the larger the collection, the more economical is the *in vitro* option. The cassava field collection at CIAT, which has 6000 accessions, costs (US\$) \$5/year/accession to maintain while the *in vitro* collection costs \$4.20 per accession (Roca pers. comm.). The sweet potato field (virus infected) and *in vitro* (virus free) collections in the USA have almost identical costs for 1000 accessions, about \$28 versus \$22/accession /year (Jarret and Florkowski 1990). *Musa* field genebanks cost \$12/accession/year for 1000 accessions while the *in vitro* collection costs \$133/accession, but the cost of labor in Belgium for the *in vitro* genebank is higher than it is for the field collection in Nigeria. In addition, 5-10% of accessions in the *Musa* field collections die each year, while typically none die in the *in vitro* stored collection (R. Swennen and B. Panis, pers. comm.). Cryopreservation is not in general practice at this time, but, in the case of the collection of temperate fruit trees maintained at the USDA-ARS National Clonal Germplasm Repository (Corvallis, Oregon, USA), costs are estimated at \$50-75 per accession for initial transfer to storage (not including labour costs) with small annual upkeep costs (around \$1 for liquid nitrogen for the storage dewar) (Hummer and Reed 1999). Similar annual upkeep costs (around \$1 per accession) have been calculated for the cryopreserved cassava collection which is being established at CIAT (Escobar *et al.* 2000; Roca *et al.* 2000).

Summary

The safety of germplasm collections requires duplication and/or additional collections at other sites. Duplication can be any useful combination of field plantings, *in vitro*

culture, or cryopreservation storage.

I.5 COLLECTION MANAGEMENT

This section contains information on identifying, and characterizing accessions, rationalizing collections and designating accessions as part of a core collection. Additionally, the development of a facilities operation manual and general distribution policies are discussed. Readers are encouraged to consult two recent publications entitled ‘Management of Field and *In vitro* Germplasm Collections’ (Engelmann 1999) and ‘A Guide to Effective Management of Germplasm Collections’ (Engels and Visser 2003), where numerous managerial issues are addressed in detail.

Correctly identify new plant material: (Appendix C)

Plants entering a collection must be correctly identified at the species, cultivar, and/or clone level. This is one of the major problems associated with new materials coming into genebanks.

Verify identity: Plant accession identity must be verified. The identification of an accession provided by the collector or donor should not be assumed correct until it is verified at the repository. This verification should be done by crop experts or taxonomists. Access to knowledgeable crop experts is very important. Plants may be compared to published descriptions or identified by genetic or molecular methods for verification. Identity verification is one of the most difficult tasks at a genebank. Verification is an ongoing process, not just a one-time procedure. Horticultural and botanical taxonomy of each clonal accession should be validated and updated every 3-5 years, as not only mislabeling and mix-ups can occur, but also as a result of nomenclature changes. The names of all taxa must be validated by checking against appropriate international nomenclature codes, as for example GRIN taxonomy, Mansfeld, International Plant Names Index (IPNI) etc. (see Box 5). If the identity of an accession is questionable, replacement material should be requested or recollected from the source; anyone who has received the incorrect plant material should be notified and offered replacement material when available. Notes on verification history should be included in the database.

Box 5: VALIDATION OF TAXON NAMES

Links to sites where single taxa can be checked:

GRIN taxonomy: http://www.ars-grin.gov/cgi-bin/npgs/html/tax_search.pl?

Mansfeld catalogue: <http://mansfeld.ipk-gatersleben.de/mansfeld/Query.htm>

IPNI: http://www.ipni.org/ipni/query_ipni.html

Links to the taxonomic nomenclature checker, a tool that allows to check whole lists of names against the GRIN taxonomy and the Mansfeld catalogue:

<http://pgrdoc.ipgri.cgiar.org/taxcheck/grin/index.html>

<http://pgrdoc.ipgri.cgiar.org/taxcheck/mansfeld/index.html>

Characterize accessions: (**Appendix C**) Each accession in the collection should be characterized using standard descriptors. IPGRI has produced a series of descriptor lists (see Box 6), which provide an international format and a universally understood 'language' for plant genetic resources data. IPGRI and FAO have also published a List of Multi-Crop Passport Descriptors (Alercia *et al.* 2001) which is a reference tool to provide international standards to facilitate germplasm passport information exchange across crops. These descriptors aim to be compatible with IPGRI crop descriptor lists and with the descriptors used for the FAO World Information and Early Warning System (WIEWS) on plant genetic resources (PGR). More recently (de Vicente *et al.* 2004), IPGRI has developed a list of descriptors for genetic markers technologies in an effort to attempt to define standards for documenting information about genetic markers.

Bananas, are characterized into major groups using 15 characters (Stover and Simmonds 1987). Characterization will help identify and eliminate duplicate accessions, will make the collection more useful to plant breeders, and may improve plant maintenance. In some cases, chromosome counts are helpful or required. For example, potato germplasm ranges in ploidy level from 2x to 5x; diploid species generally require more care than tetraploids. Cooperative research with nearby universities may provide evaluation data. Important genetic evaluations include: 1) disease resistance 2) insect and mite resistance 3) soil pest resistance and tolerance to soil environments 4) plant hardiness and tolerance to other environmental stresses 5) plant size, shape and productivity 6) crop quality and nutritional factors.

Box 6: DESCRIPTOR LISTS AVAILABLE FROM IPGRI

<http://www.ipgri.cgiar.org/system/page.asp?frame=publications/indexpub.htm>

for the following clonally propagated crops:

Allium	Grape	Plum
Almond	Litchi	Potato variety
Apple	Mango	Strawberry
Apricot	Mangosteen	Sweet potato
Avocado	Oca	Taro
Banana	Papaya	Tropical fruit
Cherry	Peach	Ulluco
Citrus	Pear	Walnut
Colocasia	Pineapple	Xanthosoma
Cultivated potato	<i>Pistacia</i> spp.	Yam
Fig	Pistachio (<i>Pistacia vera</i> L.)	

Make characterization data accessible: Characterization data should be available to plant breeders, agronomists and horticulturalists for selecting breeding material and improving crop types. Data that is widely available will make the collection more useful and, therefore, more valuable. Data may be provided as published book chapters or journal articles, as in-house publications, by request to the facility, or by electronic media.

Rationalize collection size:

Germplasm collections must maximize genetic diversity while limiting costs. This requires that duplicate accessions in the collection be identified, samples validated, core collections established, and the number of accessions and replications be kept as low as possible.

Identify duplicate accessions: Duplicate accessions should be identified as early as possible to lessen the demand on staff, reduce collection size, decrease maintenance costs, and facilitate data collection. Duplicate accessions may be identified with the aid of a crop specialist or using molecular markers, if available. Details on possible procedures to follow are presented in Sackville Hamilton *et al.* (2002). CIP used the following strategy for identifying duplicates in its potato collection. The potato collection assembled at CIP was reduced from more than 15,000 accessions to about 3,500 different cultivars. Morphologically similar accessions were sorted out from a computerized database containing data on key morphological potato characters. These potentially duplicate accessions were then further compared for all visible characters under the same environment. Those that were morphologically identical were also compared by electrophoretic analysis of their total proteins and esterases extracted from their tubers. With this methodology, duplicate identification in the sweet potato field genebank has so far reduced the number of Peruvian accessions that are clonally maintained in the field from 1,939 to 1,161. (Huaman, 1989). Concerning the use of molecular markers, a recent publication entitled "Using Molecular Marker Technology in Studies on Plant Genetic Diversity" (de Vicente and Fulton 2003) discusses the fundamental principles of genetic diversity, the qualities of the markers used to measure it and the most widely used technologies, including those based on proteins, DNA and the polymerase chain reaction.

New acquisitions should be compared to others from similar locations to determine duplication or uniqueness to warrant inclusion. The determination of morphological descriptors may be difficult for plants growing at different sites. According to Peeters and Williams (1984), "The effect of the environmental conditions on phenotypic expression is a well known problem for curators when documenting collections." Some morphological descriptors are valuable for detecting phenotypically similar accessions in the same collection, but may not be useful for identifying cultivars at different sites. Accurate comparisons among accessions can only be made with plants growing together at the same site. In Colombia, similar cassava accessions, as identified from well-characterized descriptors, were planted together and visually observed. Visual evaluation was followed by biochemical and molecular marker comparisons. The visual assessments agreed with the biochemical tests for 80% of the accessions in question. *In vitro* collections must be planted out, as there are no descriptors developed for *in vitro* plants.

Identify core collection: Core collections are a subset of the entire collection representing the

range of genetic diversity. The accessions chosen will be based on factors important to that crop. What is the most important criterion for one crop may be less important for others. Origin, morphology, unique characteristics, agronomic importance may be of use in these decisions (Hodgkin *et al.*, 1995; Johnson and Hodgkin 1999). The core collection should represent the collection's diversity, but not be totally inclusive of all genotypes. Core collections should be designated for intensive and special study. The data acquired and made available to users can be applied to the genetic improvement of the crop. Accessions in the core collection are not of higher value than the other accessions, but constitute a unit to be studied and characterized. The need for a core collection will depend upon the collection's size. Very small collections may not contain enough accessions to require selection of a core, or depending upon their composition may be as diverse as a typical core, or not diverse enough to be considered representative. An *in vitro* collection may be planted out and evaluated as part of the core collection. IPGRI has published a technical bulletin (Van Hintum *et al.* 2000) which sets out the procedures that can be used to establish, manage and use a core collection, drawing on the accumulated experience so far. In the case of cassava, origin of accessions was the most important criteria for developing a core collection of cassava. Priority was assigned based on the country's importance as a center of diversity, representation in collection and ecosystem diversity. Morphological and biochemical diversity was used to select some clones and others were included because they are widely grown landraces (IPGRI, 1994).

Create a facilities operations manual:

Each genebank should have standard procedures for all work done. A facilities operation manual containing standard work procedures is useful as a training document, reference manual, and as documentation of procedures to provide continuity during changes in staff. The manual should document all the steps which are carried out in the facilities and also provide all the protocols and procedures for each of these steps, including training to safety procedures and harvest schedules. Protocols should be written by the staff members who actually perform the work and should be quite detailed so new workers can easily make use of them.

Develop protocols: Staff members can detail the work that they do, including step by step procedures for complicated tasks. Specific protocols can aid in providing continuity with staff turnover and are useful as training and reference manuals if properly written.

Develop a well organized documentation system: Data from collecting expeditions, field management, characterization, etc. must be documented and be easily accessible to the genebank staff and interested researchers. It is very important to create a documentation system even before the first accession arrives on site.

Keep location maps: Field maps should be developed before planting and kept up to date regularly. Old maps should be retained and dated for reference. Maps should be used during planting, harvest, and evaluation. Culture room or storage room maps may also be needed. Storage location of cryopreserved samples should be carefully documented. Many of these functions can now be computerized.

Develop labeling and numbering system: Computer generated labels from authenticated lists of species and cultivars will avoid spelling and numbering errors. Each accession should be

identified separately, even though it may be a duplicate cultivar from a different source. Separate types of labels may be required for field and *in vitro* accessions, but the same accession number should be used.

Train staff: Well trained staff are especially important to maintain the health of the plants, detect diseases, and perform standard characterizations. The type and level of training will vary with the crop, the facility and the evaluations involved. Often expertise is needed in agronomy/horticulture of the crops, pathology, field maintenance, nursery and *in vitro* culture.

Develop safety procedures: Standard operating procedures should be established for all hazardous practices. A facility chemical hygiene plan should be developed and followed to provide a safe workplace (Box 7).

Box 7: CHEMICAL HYGIENE PLAN

CIP developed a Chemical Hygiene Plan to create and maintain a safe working environment and limit the exposure of personnel to hazardous chemicals/situations in the laboratory, greenhouses, screenhouses and fields. Specific Safe Standard Operating Procedures Included (SOP)

Field, Screenhouses and Greenhouses	Laboratory Safety Checklist
Application of Pesticides	Autoclave Operation
Calibration of Orchard Sprayer	DMSO use
Respirator Use	Medium for Tissue Culture
Mixing of Fertilizers	Microtechnique for stains and dyes in cells
Application of Sodium Monoborate	Sterile Transfer Procedures
Transport of Soil	Virus Testing Procedures
Soil Pasteurization	Cryopreservation Protocols
Power Equipment Operation	Hazardous Waste Permit
(Hummer, 1996)	

Distribute germplasm: (Appendices D, E, and F)

This section discusses items related to a policy on distribution of accessions, permits and agreements, and information exchange.

Policy statement: A distribution policy should be decided upon and communicated to the user community. This policy should clearly establish guidelines on eligible recipients, number of accessions that can be sent to a requester, number of propagules allowed per accession, availability of propagules (*i.e.* time of year, type of propagule), disease status allowed for distribution, and valid justification for the request.

Provide necessary permits and agreements: International shipments require import permits from the requesting country and phytosanitary certificates from the plant source. Quarantine regulations should be kept on reference and checked when the initial request is made.

Requirements which can not be met will require cancellation of the order. Waiting to check regulations until the shipment is ready to send will result in a waste of staff time and plant materials. Accessions with patents or breeders rights require the permission of the right owner before the material is distributed. Most owners allow ready access to other breeders and require payment only from those propagating the plant for subsequent sale. **Distribution of protected plants may require conforming with the International Convention for the Protection of New Varieties of Plants (UPOV) and each sovereign country's regulations.**

Conformity to International Treaty on Plant Genetic Resources for Food and Agriculture: In addition to permits and agreements, holders of collections must also comply with the provisions of the newly adopted International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA), which was adopted by the 31st session of the conference of Food and Agricultural Organisation of the United Nations in November 2001 and entered into force on 29 June 2004. This treaty establishes a multilateral system for facilitated access and benefit sharing on 35 crops and crop complexes and a number of forages listed in Annex I of the treaty (FAO, 2002). The conditions for access and benefit sharing will be contained in a yet-to-be-finalised Material Transfer Agreement consistent with provisions provided in the treaty. Article 15 of the Treaty specifically addresses the collections held 'in trust' by the Future Harvest Centers of the CGIAR under the auspices of FAO, as per the agreement signed between Centres and FAO in 1994. With the coming in force of the Treaty Centres will be invited to enter into an agreement with Governing Body of the Treaty. Pending this, an interim MTA has been prepared, endorsed and approved by CG Centres and FAO for system wide use (SGRP, 2003).

Solicit information on plant evaluation and characterization from users: The users of the germplasm normally will collect some new information on the genetic traits of accessions and should send such information back as analyzed data to be added to the computerized database. This is the best way to add relevant information to the database on a limited budget. Additional data may be gleaned from the research literature, but often basic data of this type is not published.

Maintain distribution records: A record of the date of request, plants requested, plant form, requester's name and address, shipment date, and shipping cost should be kept on file. Distributed plant material may become back-ups in case of a catastrophic loss.

Information provided with plants distributed: Associated information about an accession adds to its value to a plant breeder or scientist. Accurate identity, pedigree, evaluation, and characterization data and other information should be provided when ever possible.

Provide information on the collection's genetic diversity: General information on the collection's genetic diversity makes the collection more valuable to breeders. This information may include geographic distribution; variation in expression of important traits such as disease resistance, fruit quality, and size; and species composition of the genus.

Summary

This section summarized general topics of genebank management which may apply to

both field and *in vitro* genebanks. Information is included on accession identification and characterization, collection rationalization and the designation of core collections. Additionally the development of a facilities operation manual and general distribution policies are discussed.

II PROCEDURES FOR ESTABLISHING AND MAINTAINING FIELD AND *IN VITRO* COLLECTIONS

II.1 FIELD PROCEDURES

This section describes activities required to establish and maintain plants in the field, including the choice of propagation methods, planting sites, spacing, disease and pest management, harvest and storage of propagules. The objectives of field genebanks are to maintain healthy, vigorous plants and to minimize risk of loss or genetic change.

II.1.1 ESTABLISH AND MAINTAIN VIGOROUS, HEALTHY PLANTS

Select appropriate propagation methods:

Propagation is necessary for establishing and utilizing clonal germplasm, but due to the wide diversity of genotypes, it is often a difficult part of the field genebank operation.

Establish methods for propagation and care of accessions: Accessions may be grouped by the propagation method they require with general procedures established for each group. Research should be carried out for genotypes that do not respond well to these general methods. Curators working with crops also held in other countries may wish to contact those facilities to obtain additional information on propagation of specific genotypes. Woody perennials may require budding, grafting to rootstock, layering, or rooting stem cuttings; they may grow best on standard rootstocks to avoid problems with seedling rootstocks or soil-response. If cold tolerance is limited and the possibility of freezing exists, self-rooted trees may be required so root sprouts will replace those which are killed in the winter. Other plants may be propagated from runners, crowns, axillary shoots, rhizomes, stolons, root divisions, root suckers, or corms. The specific conditions and species at each repository will determine the methods to use. Potted accessions may have specific requirements for soil type, pH, pot size and type, and moisture.

Establish methods for storage of propagules (tubers, storage roots, etc.): Variation in the vegetative and resting period of different accessions requires special attention. If all accessions are harvested at the same time, there are remarkable differences in size of, maturity of, and insect damage to underground parts. It is important to group accessions according to vegetative period and plant them in such a way as to favor sequential harvests. Tubers and storage roots also have different resting periods and some have none at all and must be replanted immediately.

Select appropriate planting sites:

Field collections will be healthier and safer if planted under appropriate ecological conditions. Locations should be chosen to be suitable for all accessions and to minimize environmental stresses such as weather, disease, and natural pests. The location will be influenced by crop type and use. For example, outcrossed species such as grasses which are grown for seed as well as maintained as plants must be isolated from potential pollinators. Crops susceptible to root rots should be planted on well drained soil. The presence of nearby crops that may harbor disease and insect pests should be considered as well. The site should have space reserved for expansion as collections grow, especially in the case of perennial species, and should be easily accessible for monitoring. Land should also be available for necessary crop rotations for annual crops to control disease and manage soil fertility. A land-use and cropping history which includes fertilizer, chemical use, and disease information is helpful.

Plant health considerations: Proper site selection is an important factor in maintaining the health of the collection. To ensure the security of large, diverse collections, attention must be paid to protection from the worst diseases, insects and pests. Such protection, when necessary, is provided best under screenhouse (SH) culture. The need for a SH depends on whether field growing would expose the plants to lethal or debilitating diseases, pests or temperatures. This question must be answered for each different crop collection. Proper site selection would tend to minimize these potential hazards. For crop species susceptible to viruses that are transmitted by insect vectors or pollinators, the screenhouse offers essential protection when used properly (National Plant Germplasm Committee, 1986). Disease management, good cultivation practices, appropriate propagation methods are all necessary for plant health. It is important to identify the accessions most susceptible to disease and pests and treat them as needed. Since many accessions enter genebanks without evaluation data, it is often difficult for curators to know this information in advance. If the problems are soil borne, move susceptible plants to fumigated plots. If disease affects foliage, treat susceptible accessions with pesticides more frequently. After harvest, disinfect and treat propagules to prevent storage losses.

Protect plants from environmental loss: Site selection is important to not exposing the collection to extreme conditions. Appropriate climatic and growing conditions for the crops; land safe from floods, drought, wind, freezing; and adequate year-round water or appropriate irrigation are needed for the collection. Cultivars from different eco-geographical origins are usually planted in one location despite the fact that these diverse genotypes may not all survive under these conditions. Careful attention by the field staff to transfer struggling accessions to possible alternative sites, the greenhouse, or *in vitro* culture is very important to avoid genetic loss. Freeze, heat, or shade protection for tender accessions should be planned when the accession is acquired.

Ensure physical safety of collection: Germplasm may be lost due to vandals, theft, wars, volcanoes, hurricanes, floods, earthquakes, animals, and wild fires. Fencing may exclude vandals and large herbivores. Fire breaks may be established if bush fires are a common threat.

Natural disasters such as volcanoes, floods, and earthquakes may be addressed by siting the facility in a safe location or establishing a duplicate collection at a remote site. A secondary remote site for storage of the duplicate collection may be needed in case of war, theft, and famine conditions. Complementary storage systems should be considered to take these special circumstances into consideration. Special caging may be required for bird and small mammal damage.

Select appropriate planting procedures:

Field preparation and spacing will vary with the crop and cultivar. The adult size growth habit of the plants and the number of replicates per accession must be taken into consideration. Plants which readily spread by rhizomes or runners may require wider spacing between plots to prevent clones from mixing. Accessions with different morphologies may be planted in adjacent plots when creeping or spreading is a problem. Particularly invasive clones may require planting in cans, pots, or boxes to reduce mixing or competition with less vigorous accessions. Competition between plants may also affect field evaluation data. Accessions may need to be planted in groups according to vigor, height, branching habit, or lodging tendencies. Crops that must be harvested on a regular basis should be planted in groups by harvest dates or time to maturity. Replicates of perennial crops may be planted together to allow for easy identification. Avoid volunteer plants by using adequate crop rotation system and by watering the field after field preparation and removing volunteers before planting new materials.

Use proper cultivation practices:

Cultivation practices will depend on the crop and the intended uses of the collection (conservation, evaluation, distribution). Research may be needed to improve cultural practices for difficult to grow accessions. Crop rotation schedules should be planned and space allocated in advance. Weed control is necessary to limit competition and reduce weedborne pathogens and insects. Soil fertility should be monitored and adjusted as needed. Collections used for characterization or evaluation may be grown differently than those maintained for distribution. For example, evaluation may require a greater number of plants or a special field layout suitable for replicated experimental designs. Genebanks mandated to propagate disease-free planting stock for farmers may have additional specific requirements related to distribution.

Manage disease and pest problems:

Field genebanks should manage collections to limit diseases and pests that place the collection at risk. Diseases and insect pests cannot be entirely eliminated, nor is it economical or necessary. Accessions with special vulnerability to particular diseases or pests may require special treatment such as being placed in screen or greenhouses or being treated for those diseases on a specific schedule.

Screenhouses should be constructed and managed to prevent disease-carrying insects from entering. Managers should regularly monitor for insects. Workers and visitors should not enter the screenhouses after visiting field plots. The entryway into the screenhouses should have a set of two doors. The outer door should be closed before the inner door is opened to reduce the

entry of insects. Screens and structures should be checked periodically to assure they remain insect proof.

A well designed integrated pest management (IPM) program will decrease chemical pesticide use and protect workers and the environment. IPM involves practices such as monitoring insect populations; applying biological controls, trapping insects, using pheromone traps, using clean cultural practices, and applying pesticides at appropriate times. IPM practices should be used in both the field and greenhouses/screenhouses to decrease pesticide use.

Elimination methods for virus and other diseases vary with crop type. Rouging diseased plants from fields can decrease the spread of certain pathogens. Insect-borne viruses can be contained only in screened enclosures. Virus elimination procedures are discussed in an earlier section. Soil fumigation may be required for some crops.

Harvest and store propagules properly:

Crops which require annual harvests must be properly cured, labeled and stored for the next planting season. Tubers and roots may need to be disinfected after harvest to eliminate insects and disease organisms which can destroy the accession during storage. Special attention must be paid to labeling, as harvest and storage are occasions during which accessions can be mixed. A well defined harvest and storage procedure is needed, as well as well-trained, experienced, and conscientious workers. Propagules need to be checked regularly during storage to avoid loss, and research on improved storage methods may be needed.

As an example, IITA adopted miniset techniques, using planting setts of sizes ranging from 30 to 45 g, to propagate yam tubers on ridges for yam germplasm conservation. Each germplasm accession is planted on a ridge in two rows of three meter long. Planting space is 20 cm both within and between rows. Space between accessions or ridges is 80 cm. Yam tubers produced from this method are small, which are easier to handle and less prone to physical damage during harvesting, transportation and storage. To reduce the risk of genetic erosion during rejuvenation or growing out, planting setts are treated with fungicide such as Benlate or Demosan, and an insecticide such as Perfekthion before planting. During the growing stage, foliar sprays of fungicide are applied to prevent fungal diseases and of insecticides as and when necessary to prevent insect pest damage. Planting in nematode-free soil and the use of systemic nematocides (such as Basamid and Furadan) are important measures to control nematodes. Nematode infection affects both the establishment and survival of plants during growing season and the storability of yam tubers after harvest.

Germplasm losses during tuber storage, between the time when the tubers are harvested and before they are planted in the next growing season, are as great as, if not greater than those during the growing stage in the field. The main factors that are responsible for losses during tuber storage are bacterial and fungal infections. Bacteria and fungi invade the tissues of the tubers through wounds caused by insect pests or physical damage during harvest and transportation, and following primary infection by nematodes. Storage beetles could also pose some problems. Although water loss and sprouting are major causes of yam tuber deterioration,

they are less significant in germplasm conservation than in storage for commercial purpose. Storage of tubers at a lower temperature can inhibit sprouting. To reduce the risk of tuber rotting during storage, extreme care (including cleanliness) is taken during harvest and transportation to avoid physical damage to the tubers. In addition, tubers are treated with both fungicide and insecticide before storage in a traditional yam barn or in a store room conditioned at about 18°C and 50-60% relative humidity. During storage, regular checking is essential to ensure that tubers are in good conditions. Any rotten tubers are removed to prevent them from infecting other healthy tubers, are necessary to ensure that tubers are in good conditions. Using the above described procedures, a total of two thousand and five hundred accessions of different yam germplasm are successfully maintained at IITA since 1990. (Ng and Ng 1999)

II.1.2 MAINTAIN COLLECTION SECURITY

Monitor genetic stability:

Genetic shifts in the collection can arise from genetic instability of accessions due to chromosomal changes and gene mutations causing morphological or biochemical variation (Box 8). These genetic instabilities are manifested as somaclonal variation in tissue culture.

Box 8: GENETIC STABILITY

“Clonal stability depends on the method of preservation, the length of time, and the inherent genetic stability of the clone. Mutations can occur as a result of background gamma- and x-ray irradiation, exposure to mutagenic chemicals, and other environmental influences. Each clone has its own natural rate of mutation, so generalizations cannot be made.” (Westwood, 1989).

Avoid propagating off types: Individuals of a clone should be inspected regularly for off types. If the accession appears to be a mixture of genotypes, characterization data should be used to determine which is the correct propagule. Some crops, such as potato and sweet potato, have high levels of somatic mutations and require careful scrutiny during propagation. Collections containing the entire range of genetic diversity will usually have some members which are variable. These should be noted in the database and carefully monitored. Herbarium specimens or photographs of accessions may also be useful for verifying the identity of questionable specimens.

Accessions from which seeds are collected for propagation (grasses and legumes) can lose genetic information over time if too few individuals of an accession are maintained and self-pollination occurs in a percentage of cases. This problem is lessened in accessions which primarily produce seed apomictically. Species which intercross easily lose superior genotypes when cross-pollination is not controlled and seed are used. Studies on seed physiology and reproductive biology would identify grasses which could be stored as bulked seed and would

reduce the size of field collections. Crops of this type need to establish protocols for maintaining accessions according to breeding behavior (Sackville Hamilton and Chorlton, 1997). Additional aspects on the management of germplasm at the accession level are presented in Sackville Hamilton, Engels and van Hintum (2002).

Evaluate genetic stability: Monitoring collections for genetic stability is not an easy task. Each crop has specific descriptors which can be used to characterize the accessions. A well identified collection may be used to develop RNA or DNA fingerprinting which may be used for future evaluations. The relevance of the existing techniques is discussed in a recent publication entitled “Using Molecular Marker Technology in Studies on Plant Genetic Diversity” (de Vicente and Fulton 2003). Most collections will depend on vigilant staff members to identify problems.

Prevent incorrect labeling of plants:

Avoid mixing propagules: Mixing can easily occur in fields where roots, stolons, rhizomes, runners can invade adjacent plots. Repotting or planting errors and growth of propagules such as runners into adjacent pots may occur in screenhouses and greenhouses. Wide spacing of pots can be used to limit growth of stolons or runners into adjacent pots. Careful attention by staff members will minimize planting errors.

Minimize labeling and handling mistakes: More than one identification number (*i.e.* plot number and accession number) should be used when planting or harvesting fields. A field map should be available showing the sequence of planting, and, if possible, labels should be printed from computer files, or carefully checked to minimize the problem. Labels should be indelible and as indestructible as possible. Advance planning before any planting or repotting effort will minimize errors.

II.1.3 DISTRIBUTE PLANT GERMPLASM

Techniques for the distribution of germplasm are specific to each crop. Some general guidelines apply to distribution of accessions from a field collection. Some specific guidelines for the safe movement of germplasm of specific crops are available (see Box 9) (See also Distribution Policy, Section II.5).

Ensure a supply of plant parts: Most clonal genebanks contain both base and the working/active collection material. Accessions must be managed to provide propagules as well as to maintain healthy collections. Accessions which are often requested may need to be replicated in greater numbers than other accessions. Planting stocks probably should be produced separately from the germplasm collection.

Ensure movement of healthy material: Propagules should be taken from healthy stock and inspected for disease and insect pests prior to shipment. Indexing for difficult to detect pathogens, such as viruses, is important for limiting their spread. Distribution of materials from greenhouses or screenhouses may be necessary for crops with insect- or mite-borne viruses and *in vitro* cultures may be required.

Packaging and shipping: The type of shipping container, packing materials and the choice of shipping company will depend greatly on the plant part to be distributed. Dormant or storage organs require fewer precautions and may spend a longer time in transit without damage than actively growing propagules. Timing shipments to avoid severe weather (either hot or cold) and notifying the recipient or customs official prior to the plant's arrival will improve the likelihood the plants will arrive in good condition. Fragile propagules may require express delivery services. International shipments are facilitated if necessary papers are attached to the outside of the container for easy access by officials without disturbing the plants, with copies inside for the recipient.

Box 9: DISTRIBUTION GUIDELINES

Most germplasm distribution is based on the exchange of pathogen-free propagules. Careful indexing for bacterial, fungal and viral pathogens is required. Guidelines for the safe movement of germplasm are available for the following crops.

Acacia:	Old <i>et al.</i> 2003
Allium:	Diekmann, 1996
Cassava:	Frison and Feliu, 1991.
Citrus:	Frison and Taher, 1991
Cocoa:	Frison and Feliu, 1989
Coconut:	Frison <i>et al.</i> 1993
Edible aroids:	Zettler <i>et al.</i> , 1989
Eucalyptus:	Ciesla <i>et al.</i> , 1996
Grapevine:	Frison and Ikin, 1991.
<i>Musa</i> :	Frison and Putter, 1989; Diekmann and Putter, 1996.
Pinus spp.:	Diekmann <i>et al.</i> 2003
Potato :	Jeffries 1998
Small fruits:	Diekmann <i>et al.</i> , 1994.
Stone fruits:	Diekmann and Putter, 1996
Sugarcane:	Frison and Putter, 1993.
Sweet potato:	Moyer <i>et al.</i> , 1989
Vanilla:	Pearson <i>et al.</i> , 1991.
Yam:	Brunt <i>et al.</i> , 1989.

II.1.4 RESEARCH NEEDS IN THE FIELD GENE BANK

As discussed before, field genebanks are very costly to maintain and are in general very susceptible in losing accessions for a variety of reasons (Maxted *et al.*, 1997, Engelmann and Engels, 2002; Dulloo *et al.* 2001). Field genebanks have many constraints (see below) which require careful management and research to overcome them. Most of these constraints are related to agronomic problems, but in the context of germplasm conservation in field genebank, where we are dealing with diverse materials, often with little biological and ecological information, specific research is

required to help in their propagation, establishment and maintenance. In 1995 a Field Genebank Management Training Workshop, organized by IPGRI, identified a number of research needs including: causes of genetic instability; cryopreservation and other alternative conservation techniques (see Box 10); causes of seed recalcitrance; low-input maintenance strategies; improved storage facilities for annual crops; improved crop descriptors; extension of storage time of planting material; and improved disease indexing (IPGRI/UNEP, 1995).

In order to bring down costs and reduce the amount of germplasm losses, research needs to be promoted in the following areas which reflect the main constraints which field genebanks face.

a. Germplasm health

Pest and diseases are the major problems affecting field genebanks. Accessions introduced from different locations may be infected with diverse arrays of pathogens. Infected accessions can become major sources of pathogens that can be transmitted to other accessions. Research is needed to develop optimal health procedures during collecting and introduction in field genebanks. Also new ways of screening new accessions and disease indexing that are less laborious and time consuming, are required.

b. Adaptation to environment

Genebank materials include accessions from a wide variety of origins which are adapted to environmental conditions, different from those found at the field genebank location. Research is needed to study and understand the specific environmental requirements of different accessions in order to better manage them in field genebank.

c. Taxonomic research

The proper identification of taxa is another problem often experienced in genebanks. In particular, the taxonomic identity of accessions of many unknown wild relatives poses problems and taxonomic research is needed for their proper description and management. For certain species such as potato, which has polyploids from 2x to 5x, this is of extreme importance for their proper management; indeed, diploid species generally require more care than tetraploids (Huaman, 1999).

d. Reproductive biology

Lack of information on breeding systems of certain taxa renders the proper management of accessions in the field collection difficult. Studies on reproductive biology (e.g. outcrossing rates, sexuality/apomixis) would help identify accessions or species that are suited to storage as seeds.

Box 10: AN EXAMPLE OF RESEARCH DONE AT FIELD GENE BANKS

Coffee collection in CATIE, Costa Rica.

CATIE manages one of the largest field genebanks of *C. arabica* in the world. A total of 8,590 coffee trees representing 1,997 accessions are conserved under the shade of the leguminous tree species *Erythrina poeppigiana* which is pruned twice annually. The conserved material is suffering from three main problems: the age of the trees, the climatic conditions and the cultivation method. Most of the accessions (57.5%) were introduced before 1970 and are now at least 30 years old. The localisation of the field genebank at 602 m above sea level in a humid zone of Costa Rica does not supply optimal conditions of cultivation neither for *C. arabica* nor for the other coffee species. The cultivation method has been similar to the method of commercial plantations and the same for wild and cultivated accessions. It is obvious that the lack of knowledge about the value of the conserved genetic resources for breeding has played a crucial role and limited the funds allocated to the maintenance. A detailed analysis of the remaining coffee trees collected in Ethiopia by FAO reveals that the genetic losses have not been serious despite the problems described previously. A total of 2,523 plants representing 442 collection numbers were introduced in 1965. Four to eight individuals were planted per accession. Thirty-five years later, only eight accessions have been lost and 1,533 trees (60.8%) are still living in the genebank. However 10.1% and 20.3% of the remaining accessions are only represented by one or two individuals, respectively. As *C. arabica* is self-fertile at approximately 90%, the remaining plants can be considered as representative of the mother trees that were harvested in Ethiopia.

In the year 2000, CATIE started a project for rejuvenating the conserved genetic resources and ensuring their preservation. The genetic resources are being rejuvenated by grafting on vigorous rootstocks *C. canephora*, resistant to several root knot nematode species (*Meloidogyne* spp.). The wild material of *C. arabica* and other species will be conserved separately from the cultivars, mutants and introgression lines, which will constitute the working collection. This will allow for giving specific and adapted care to the wild material, such as permanent shade. The wild genotypes will be duplicated and each genotype will be represented by two clonal plants. The number of individuals will be reduced in the working collection to four individuals per accession in case of heterozygotic material (e.g. cv. Mundo Novo, introgression lines) and eight individuals chosen in all remaining accessions in case of homozygotic material (e.g. Typica- and Bourbon-derived cultivars, mutants). Such strategy of rationalisation based on passport and evaluation data will reduce by half the area of conservation and thus the cost of maintenance. (Dulloo *et al.* 2001)

e. Genetic diversity

The principle aim of any field genebanks is to conserve the maximum diversity of germplasm using a minimum sample size and number. Research on genetic diversity can help to define core collections for field collections, thereby reducing collection size and costs. Such research can also help to better characterize the field collection and identify gaps in such collections. Genetic studies

can also help determining the causes for genetic instability, including mutations, drifts and shifts in field collections. Better means for a reliable identification (fingerprinting) of accessions, which are relatively easy and cheap to run (kits), are also needed.

f. Genebank management

Research is needed to improve management and maintenance of field collection. For certain collections, research is required to find best ways for propagation. For example at IPK, Germany about one third of *Allium* accessions are propagated vegetatively because many of the wild species are difficult to be propagated by seeds and needs research (Keller *et al.*, 1999). Research on cultural practices may also help in reducing contamination and labour costs. Protocols need to be established for maintaining an accession according to its breeding behaviour and the principal objective for conserving it in the germplasm collection. Research is also needed in the recovery of fertility in herbaceous plants, as well as improved storage facilities for annual crops and the extension of storage time of planting material before planting out.

g. Economics

The development of low input management strategies can help reduce cost of maintaining field collections. Research on alternative methods for conservation (e.g. seed, *in vitro*, cryopreservation or *in situ* conservation) should be promoted. Studies on seed physiology may help defining conditions to allow conserving seeds rather than whole plants and thus reduce necessity of maintaining field collections.

Summary

General procedures for field collections include: Propagation methods, planting sites, planting procedures, cultivation practices, disease and pest management, and harvest and storage of propagules. Monitoring the genetic stability of a crop requires careful vigilance on the part of the curator and the field staff. Careful rouging, labeling, and protecting plants from biotic and abiotic dangers are important to the safety of the germplasm. Distribution of plants from field genebanks requires planning to provide propagules, inspection or indexing for diseases, and proper packaging for successful transport. Additional factors are discussed in the General Genebank Management section.

II.2 IN VITRO PROCEDURES

II.2.1. LABORATORY FACILITIES

In vitro culture and storage facilities are quite variable. There are many ways to achieve the desired level of asepsis, and each laboratory will need to do what is necessary for the security of their cultures. This section highlights important points to consider and provides examples from *in vitro* genebanks.

Laboratory:

A basic laboratory requires a clean, tightly constructed room in a building with adequate lighting, electricity, heat/cooling system, water, and ventilation. Many references are available for setting up and running tissue culture facilities (see e.g. Biondi and Thorpe, 1981; Debergh and Zimmerman, 1991; George 1993, 1993/1996; Kyte and Kleyn, 1996, 2001).

Minimal facilities: It is possible to develop a working tissue culture facility with only minimal resources. Equipment need only to be functional and maintained in a clean working environment.

A laboratory can be housed in a university laboratory or a room in any building. Temperature and light requirements can be met in many ways. Usually a temperature control unit is required (air conditioning). Any closet can be converted to a growth room with a few light fixtures attached to shelves. Shelves and other equipment can be manufactured or purchased locally as needed. Shelf units must be well painted to avoid rust and fungal growth in the growth room. Once painted, these surfaces can be more easily disinfected.

Sterile transfer facilities: Several options are available for sterile transfer. All facilities should be designed to minimize foot traffic and outside airflow in the room where transfers are done. A laminar flow cabinet is necessary for any sterile work. Culture hoods should be checked regularly for leaks with testing equipment (smoke) or with open bacteriological plates such as those described later for contaminant screening. Bacteriological plates can be placed throughout the hood and contamination patterns noted. Tissue culture medium should not be used to detect leaks as it is a poor medium for growth of microorganisms. Whenever laminar flow hoods are moved they should be checked for leaks.

Equipment: Equipment needed includes laminar flow benches, pH meters, balances, sterilization equipment (either autoclave or pressure cookers), hot plates or stove, magnetic stirrer, appropriate chemicals, analytic and culture glassware, refrigerator, and freezer. Protective clothing, gloves and safety devices such as showers, eyewash, and fire extinguishers are also recommended. Arrangement of the equipment and supplies in a logical fashion will allow for the most efficient use of time and should minimize mistakes. A flow chart of lab activities might be used to organize lab equipment and supplies. For cryopreservation, a reliable source of liquid nitrogen (LN), tanks, dewar, vials, and specific chemicals will be needed in addition to tissue culture facilities.

Instruments and glassware: Long lightweight forceps, fine short forceps, scalpels with

replaceable blades; alcohol, gas burners, or other tool sterilization equipment; and sterile racks or dishes for holding sterile tools are needed in most instances. The need for additional instruments (e.g. stereo microscope for dissection of apices and embryos) will depend on the culture vessels used and the plant materials cultured. Cleanliness of glassware and other items is of prime importance to the health of the *in vitro* collection. A high-quality dishwasher with a distilled or deionized water rinse, or very careful workers are needed. Glassware should be dedicated to tissue culture only and not mixed with other laboratory glassware. It is important to thoroughly rinse all glassware. A source of good quality water (single or double distilled, deionized, filtered, etc.) is needed for media preparation as well. The required treatment will vary with the purity of the local water supply.

Many types of autoclavable containers are suitable for tissue culture. Baby food jars, jam jars or other readily available glass containers may be obtained at low cost or free. Metal lids may rust, but autoclavable plastic or brown paper secured with rubber bands may also be used. Autoclave times may vary with the type autoclave, type of container, and the type of closure. Most larger containers (20-50 ml medium) require a minimum of 20 min at the recommended temperature and pressure for adequate sterilization. Some laboratories autoclave containers after washing and before adding medium to decrease contamination risks. It is important to use heat indicators to assure that sterilization has been accomplished.

Culture growth rooms:

Culture growth rooms with temperature control, lighting, and shelving, as well as culture storage rooms are needed. An interior room may reduce contamination from external sources. Lights with ballasts located externally may be needed to control heat buildup in the culture room. Ideally, humidity is 40-50%, but in most laboratories it is not possible to regulate it closely. High humidity increases fungal growth, while low humidity dries cultures and creates dust problems. An isolation growth room is advisable for *in vitro* explants of materials taken directly from the field to allow time to detect insect infestations and prevent their spread to other cultures.

Light requirements: Light requirements range from 10 to 1000 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ but most plant cultures require 50-200 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ (see Box 11 for units of light measurement). Some research may be necessary to determine the appropriate levels for the genera in question. Bleaching of leaves indicates too intense light, while long internodes and small leaves often signal suboptimal light levels. Light from different sources will vary in intensity and spectra. Either warm or cool fluorescent bulbs produce proper spectra for plant growth, although spectral requirements may vary with genotype. Daylength requirements also vary among genera. A time clock to control day length is useful. If many lights are used, several time clocks with staggered “on” times are needed to avoid overloading the electrical system or generator. Areas with frequent power outages should take that into consideration. Most cultures can stand a relatively long period in the dark without further adverse effects. Natural lighting may be used in areas with stable daylengths. Indirect, ambient lighting can supplement part or all of the light requirements, but may increase heat in the growth room.

Temperature: Ventilation systems or air conditioning units

Box 11: UNITS OF LIGHT MEASUREMENT

There are a number of ways to measure light and some are better suited to more accurately describe the light regimes experienced by plants (for additional details see George 1993 pp. 215-217).

Illuminance - intercepted visible light per unit area

- lumens per m^2 or lux.

- foot candles (old terminology)

1 foot candle = 1 lumen per square foot.

= 10.764 lux or lumen m^{-2}

Irradiance - intercepted radiant energy per unit time per unit area. Measured in terms of energy;

- watts per square metre (W m^{-2})

- joules per square metre per second ($\text{J m}^{-2} \text{s}^{-1}$)

- ergs per square centimetre per second ($\text{erg cm}^{-2} \text{s}^{-1}$)

or in terms of quantum units;

moles of photons per square metre per second ($\text{mol m}^{-2} \text{s}^{-1}$)

microeinsteins per square metre per second ($\text{mE m}^{-2} \text{s}^{-1}$)

$1 \text{ erg cm}^{-2} \text{s}^{-1} = 1 \times 10^{-3} \text{ W m}^{-2}$

$1 \text{ mmol m}^{-2} \text{s}^{-1} = 1 \text{ mE m}^{-2} \text{s}^{-1}$

= $0.215 \text{ W m}^{-2} = 0.215 \text{ J m}^{-2} \text{s}^{-1}$

Quantum units are particularly suitable for measuring light where its energy may be utilised for photochemical reactions, such as photosynthesis.

As the energy of a microeinstein varies inversely with the wavelength of light, exact conversions to units of illuminance are not possible for multiple band light sources. However, the following approximate conversion factors can be used for three fluorescent light sources commonly used in growth rooms.

lux @ $\text{mE m}^{-2} \text{s}^{-1}$ for ‘Warm white’ divide by 83

lux @ $\text{mE m}^{-2} \text{s}^{-1}$ for ‘Cool white’ divide by 80

lux @ $\text{mE m}^{-2} \text{s}^{-1}$ for ‘Grolux’ divide by 59

Daylight may provide irradiance of ca. 200 - 700 W m^{-2} (equivalent to an illuminance of ca. 50,000 - 150,000 lux) but plant cultures are typically grown in flux densities which are about 10 times less than daylight.

Small hand-held lux meters can be obtained from camera shops. Larger meters measuring photons can be obtained from suppliers of scientific equipment.

For further information see George, 1993.

are needed to regulate temperature, but the air should not flow directly onto the cultures. Common growth room temperatures range from 22 to 28°C, but will depend upon the requirements of the genera. Each group's requirements will need to be determined either from the literature or by experimentation. Temperatures will normally stratify from ceiling to floor and these differences may be used to accommodate disparate genera. Monitoring devices such as thermographs or alarms are useful to detect power outages or equipment failures. Over-temperature cut-off switches should be incorporated to avoid overheating the culture room during hours when it can not be monitored.

Air Filtering Systems: In most cases, it is unnecessary to use HEPA filtered air (high efficiency particle-removal air filters) in the growth room, but they may be essential in tropical laboratories. Routinely changing filters on the building ventilation system should be adequate unless unusual circumstances apply. If culture contamination increases, it may indicate that the building ventilation system filters need cleaning or changing. If high levels of contaminants enter through the ventilation system, ultraviolet lights in flow hoods or air vents may be required. The ambient relative humidity and season will influence the amount of contaminants in the air supply. Frequent vacuuming and mopping decrease airborne contaminants. Monitoring of airborne contaminants using bacteriological plates provides indications that filters should be changed.

Box 12: SOME BASIC REFERENCES FOR SETTING UP

A TISSUE CULTURE LABORATORY

- Debergh, P.C. and R.H. Zimmerman (eds.). 1991. Micropropagation Technology and Application. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- George, E. F. 1993. Plant propagation by tissue culture - Part 1. Exergetics Limited, U.K.
- George, E.F. 1996. Plant propagation by tissue culture - Part 2. Exergetics Limited, U.K.
- Kyte, L.A. and J. Kleyn. 2001. Plants from Test Tubes. An Introduction to Micropropagation, 3rd Edition. Timber Press, Portland, Oregon. USA
- Thorpe, T.A. (ed.). 1981. Plant Tissue Culture Methods and Applications in Agriculture, 1st ed. Academic Press, New York. USA

Summary

This section described the physical requirements for a plant tissue culture laboratory. Each *in vitro* genebank should strive to attain the highest standard possible for their situation. Minimal facilities can usually be provided to allow for sterile culture, no matter what the circumstances.

II.2.2 CULTURE STORAGE FACILITIES

The storage of *in vitro* cultures, whether in warm or cool conditions, requires certain precautions. This section highlights the main areas for consideration for the *in vitro* storage methods.

Cold storage: Cold storage facilities require reliable equipment and alarm systems. Whether the storage is at 15 °C or 0°C, a malfunction can overheat or freeze valuable cultures. A fail-safe system which shuts off the unit and sounds an alarm if the temperature varies by a set amount will prevent cultures from freezing or over heating; cultures would then only warm to ambient temperature. Wiring the alarm into an existing security system is highly recommended. Condensation on walls and shelves may occur in tropical climates or in the summer. This may cause fungus problems and may require a dehumidifier or regular monitoring and clean-up. Storage temperatures employed in laboratories may vary for a particular genus.

Warm storage: Cultures that are stored at growth room temperature require the same monitoring as those in the cold room. It is important to see that the temperature remains within the preferred limits. Storage temperatures vary from genus to genus and may also vary among laboratories (Ashmore 1997). Sweet potato genotypes, for example, will survive several months at temperatures ranging from 15°C to 35°C, but results are genotype dependent (Jarret and Florkowski, 1990).

Cryogenic storage: Storage in liquid nitrogen requires a reliable, vented holding tank; a readily available liquid nitrogen source; and an alarm system to warn of tank failure or low nitrogen levels. Properly vented dewars must be used when handling and carrying liquid nitrogen. Safety equipment, such as gloves and safety glasses, for personal protection should be available and used properly. A special shipping dewar may be needed to transport frozen plant materials long distances by mail or courier services. Additional information is in the section on cryopreservation.

Summary

Storage may be as *in vitro* cultures in warm or cool conditions, or as meristems, pollen, or dormant buds in liquid nitrogen. The selection of the method or methods will depend on the plant genotype and the available techniques. Storage *in vitro* decreases the labor, cost, and chance for contamination of cultures. The longer plants can be stored without transfer or subculture, the lower the cost, and the more secure the accessions. The important goal is the recovery of a healthy plant with good propagation potential.

II.2.3 OPERATIONS

General operation of an *in vitro* genebank requires more than just the technical aspects of culture manipulation. Care of the physical plant and personnel functions are of great importance to the success of the genebank.

Laboratory upkeep:

Many problems which arise in tissue culture laboratories can be eliminated through careful maintenance of the facilities and equipment. Cleanliness is extremely important. Floors should be routinely wet mopped with disinfectant; dust and insects, especially mites, controlled; and the filters in the laminar flow hoods and building's ventilation system changed or cleaned regularly. Contaminated cultures should be autoclaved before they are washed, or should be removed from the lab to a separate washing area. Field and greenhouse personnel should change their shoes and clothing before entering the lab and growth rooms. In tropical areas, contamination problems are compounded by high humidity and require stricter attention to cleanliness. Fresh plant material should not enter the lab until surface disinfection has begun. This will decrease the introduction of insects into the laboratory or growth room. New explants should be held in a separate room or the lids wrapped with tape or plastic wrap until the possibility of insects is ruled out. Cultures introduced from other laboratories should be wiped with 70% alcohol or bleach and isolated from the main culture room to avoid transferring mite infestations.

Staffing:

The education and experience level of the tissue culture staff will affect the quality and efficiency of the laboratory operations. Researchers with advanced degrees in plant physiology and knowledgeable in micropropagation are needed to develop techniques and maintain optimal facility function. Laboratory technicians should have a background in plant science and micropropagation/tissue culture; advanced degrees are optional, depending upon their required level of independence. Laboratory assistants with training in basic botany and/or horticulture and tissue culture techniques are preferred, however, in practice, intelligent persons without an extensive educational background can be trained on-site.

If tasks are repetitive in nature there will be a high turnover in laboratory assistants. This may be avoided by rotating work assignments as much as possible or assigning special projects. Each assistant should be trained to make medium, wash dishes, transfer cultures, check cultures for contaminants, do basic record keeping, and other required laboratory tasks. This flexibility will keep the job from becoming boring and will aid in employee retention. Educating workers on the mission of the facility may also provide a morale boost and establishing a research-oriented approach to work might help as well.

Because many techniques and procedures will be developed at the facility, on-site training is needed for each new staff member, even those with plant science and micropropagation backgrounds. The head laboratory technician or lead scientist should be prepared to spend one to two weeks intensively training each new worker to use standardized laboratory protocols developed within the laboratory. This training should be followed by close supervision for as long as needed. This will assure consistent results among workers and produce reliable research results and healthier collections.

The number of personnel required will depend upon the collection's size and diversity, the amount of research conducted, and the results desired. Germplasm collections usually hold highly diverse genotypes and, therefore, *in vitro* propagation methods are not routine for all genotypes. The more genera involved and the more complete the collection, the greater the

number of staff required for optimal functioning. Any facility which wishes to have a tissue culture backup or distribution collection will require at least one highly qualified technician loosely supervised by the facility director. An active collection with research and development needs, however, requires a staffing level that includes one scientist in charge of planning, research and analysis; one technician in charge of daily operation of the laboratory; and laboratory assistants as needed for routine media preparation, cultures transfers and dish washing.

Develop a procedures manual:

All procedures and recipes should be written out in detail and placed in a procedures manual as a reference guide for workers. A manual will aid in standardizing techniques and protocols for the entire staff and can be used as a training handbook for new workers. The manual should be updated yearly or any time major procedure changes are instituted.

Summary

This section described laboratory upkeep, staffing and procedures. The development of a procedures manual is important for providing standardization for the genebank. A manual serves as a training manual for staff, a reference guide for administrators and provides for continuity of procedures. Well trained, stable laboratory staff are of prime importance to the continuity and success of any *in vitro* genebank.

II.2.4 ESTABLISH A TISSUE CULTURE SYSTEM

The wide diversity of genetic resources available in genebanks require a similar diversity of tissue culture media and growing conditions. Standard methods may not be applicable to all accessions of a genus, or even all cultivars of a species. A thorough literature review and library research before beginning culture is extremely important for determining a starting point. Modifications to published techniques will be required in most cases and new techniques must be developed for other genera. This section provides information on the steps to take when starting with a new taxon.

Research techniques:

A great deal of information seeking is required prior to actually bringing plant material into culture. To begin to develop media recipes and protocols it is necessary to compile literature on the *in vitro* techniques pertinent to the genera to be collected and grown in culture. A reference library of pertinent literature and reference books is important for developing and improving protocols (see Box 13).

Box 13: REFERENCE SOURCES FOR *IN VITRO* PROTOCOL DEVELOPMENT

Scientific journals where many tissue culture articles are found:

Acta Horticulturae

HortScience

***In vitro* Cellular and Developmental Biology-Plant**

Journal of Horticultural Science

Journal of the International Association of Plant Tissue Culture and Biotechnology

Plant Cell Reports

Plant Cell Tissue and Organ Culture

Scientia Horticulturae

Books:

George, E.F. 1993/1996. Plant Propagation by Tissue Culture - Part 2. Exegetics Limited, UK.

Withers, L.A. and P.G. Alderson. (Eds.) 1986. Plant Tissue Culture and its Agricultural applications. Butterworths, London.

Develop protocols: (Appendices G and H)

Protocols and media for initiating, multiplying, rooting, and storing plants from each species or group of accessions must be developed. Standardized stock solution recipes are essential for repeatable media. A standardized medium sheet should be designed for each medium recipe and stock solution. Well-designed media sheets can minimize errors by serving as worksheets during medium preparation. The sheets should be easy to use and have a convenient method for checking off each ingredient as it is added. The more precise the worksheets, the fewer mistakes will be made. Standard protocols for storage and repropagation should be written down and dated. A new medium sheet should be developed whenever a recipe is changed, but the old one should be filed and retained for reference.

Design a record-keeping system: (Appendix I)

A well-designed record-keeping system will allow researchers to follow each accession from acquisition through culture and storage. Develop a record-keeping system that includes and links acquisition information (origin and passport data), field data (growth conditions, disease status, location) and *in vitro* records for each accession. Image data may also be useful. An easily used and widely accessible computer database is most desirable. Computerized data should be backed

up regularly and stored in a safe location. Data should be stored at more than one location.

Data: The *in vitro* database should track each accession's explanting date, source, initiation medium, multiplication medium, rooting medium, growth information, experimental data, length of subculture, etc. Identifying plants by the same numbering or labeling system as the field genebank allows the plants to be traced back to the mother plant when necessary. If the numbering systems are different, a database should be created to coordinate the numbering so it can be easily traced.

Labeling: All possible sources of labeling errors should be identified and minimized. Ideally, labels should be computer printed. This will avoid labeling mistakes such as transposition of numbers which are common on hand written labels. One label should follow an explant through the entire process. If a system for producing computerized labels is not available, it is important to use clear, indelible ink for writing labels and to write clearly. Using a mixture of letters and numbers will decrease the possibility of transposing numbers.

Summary

This section discussed the development of tissue culture systems, research techniques, procedures and record keeping. These important functions must be established for the genebank as a whole with additional tissue culture systems added as new plants are acquired.

II.2.5 INTRODUCE PLANT MATERIAL INTO TISSUE CULTURE:

Often one of the most difficult parts of *in vitro* culture is the initial stage. This section outlines problems and techniques typically found when placing plants in culture.

Explants: (Appendices J and K)

Origin of material: All plant material selected for tissue culture should be collected from vigorous, healthy mother plants, tubers, corms, etc. Plant tissue culture vigor directly reflects the state of the mother plant. Explants should be taken only from mother plants that are typical for the genotype desired in culture. Plants kept in greenhouses or screenhouses, when they are available, harbor fewer bacterial, fungal and insect contaminants than field material. Dormant field material is usually cut, washed and forced or grafted onto rootstocks in the greenhouse. Directly collected material is often contaminated, however old leaves may be stripped off, stems washed and new growth forced as with dormant material. Healthy roots, tubers, corms should be washed and sprouted under relatively clean conditions to provide material for explants. The appropriate plant part, growth stage, and physiological age for a particular genus must be determined from the literature or experimentation.

In vitro collecting may be useful in some cases. This technique involves directly placing materials into sterile tubes for transport to a distant laboratory. Preliminary surface sterilization

and medium with fungicides and antibiotics may be needed (Withers, 1995, Taylor, 1996). A Technical Bulletin describing the application of *in vitro* collecting to a range of species has been published recently by IPGRI (Pence *et al.* 2002). Box 14 provides references on *in vitro* collecting for specific plants.

Box 14: EXAMPLES OF *IN VITRO* COLLECTING:

Cocos nucifera (Coconut): Assy Bah, B., T. Durand-Gasselien and C. Pannetier, 1987.; Engelmann F. 2002.

Colocasia esculenta var. *esculenta* (Taro): Taylor, M. 2002.

Gossypium (Cotton): Altman, D.W., Fryxell, S.D., Koch, S.D., and Howell, C.R., 1990.

Forage grasses: Ruredzo, T.J., 1989; 1991.

Theobroma cacao (Cacao): Yidana, J.A., Withers, L.A. and Ivins, J.D., 1987.

Miscellaneous species: Pence, V. 1996

General references: Withers, L.A. 1995; Pence *et al.* 2002

Number of explants: Each facility will discover through experience the number of explants it must collect to obtain the number of plantlets needed for establishment, multiplication and storage. That number may vary with season, climate, propagation rate, crop, genetic stability, demand or distribution rate, facility, stress, and storage situation. If internal or external contaminants are a problem, more explants may be needed.

Choice of materials: It is important to use properly identified material with passport data and, when it is available, plant health and other characterization data. Information on quarantine regulations should be available and consulted. Any material to be introduced into tissue culture should come from plants that are disease free, if they are available. If possible, virus-tested, virus-free plants should be used. If testing is not possible, indicate that the material is untested in the database records. Virus elimination procedures could be initiated. Virus-free materials may be available from other institutes, and it may be useful to obtain them as replacement accessions if virus testing or elimination are not available on-site.

Surface disinfection (bacteria, fungi, thrips): Chlorine bleach with a surfactant added is the most common and safest disinfectant for laboratory workers. Common dish washing detergent (liquid) may be used as a surfactant if materials such as Tween 20 are not available. *Mercury based compounds should be avoided* due to their toxicity to workers and the environment. Concentrations of NaOCl ranging from 0.5% to 1% are commonly used. Commercial bleach solutions may also be used at concentrations of 10 to 15%. Fresh commercial bleach should be used as it loses chlorine as it ages. Soaking explants in a fungicide may also be useful if fungal contaminants are likely. Pence and Sandoval (2002) have written a chapter solely dedicated to

the control of contamination during the introduction *in vitro* of plant material, which should be consulted for additional details on the existing procedures.

Plant response to surface disinfestation varies greatly and the concentrations of sterilents used must be determined individually. Short periods of sonication (10-15 min. in NaOCl) may be useful for woody plants. Over-sterilization results in browning, spotting, or death, while under-sterilization results in contaminants. If necessary, double disinfection may be considered (Ng, 1992). Field materials may have insect eggs attached which can withstand surface sterilization, so some additional treatments may be needed to eliminate the insects. Treatment with oil sprays or dips; treating the mother plant with insecticide before explants are taken; or both may be required. Each explant should be placed in an individual tube to decrease the possibility of cross contamination. Field explants should be carefully examined for mites, thrips and other arthropods. Wrapping the cultures in plastic wrap will help to decrease the spread of field organisms. Continual fungal contaminations, especially those that occur in “tracks” across the container are often a sign of thrips or mites even though the organisms can not be seen. Small brown spots on the older leaves and eggs on the stems of plants are other indications of thrips. Explants should be grouped into one container only after indexing for contaminants is negative (see below).

Medium requirements for culture initiation: Media requirements for initiating growth in culture may be different than those required for standard growth, proliferation, and storage. This should be determined before initiating cultures of rare materials. Experimentation may be necessary at this stage. Optimal growth of an accession is not required, just moderate growth and multiplication. For some genera, such as *Dioscorea* spp. (yam), frequent transfer to fresh medium may be required during the initiation period due to the exudation of phenolic compounds into the medium (Ng, 1994).

Data on plant growth response/media/conditions: It is important to document the variation present in the *in vitro* collection. Data should be collected at all stages of propagation to use as base descriptors of the plant response *in vitro*. The database set up for the *in vitro* collection should provide categories for growth observations. Additional data including measurements of growth, descriptions of morphology and response to various media, growth regulators, and environmental conditions should be collected when possible.

Standard procedures for each genus: A genus may require one and sometimes two different media. Each genus will respond differently to surface sterilization and handling procedures. Individual differences make it difficult to generalize; however, laboratories need to develop standard procedures that adequately maintain the majority of the genotypes in a genus and minimize their genetic instability. It is not the goal of a genebank to develop optimal conditions for all accessions. An adequate medium which meets the growth requirements for all accessions is required.

Contamination indexing: (Appendix J) Explants should be placed on a medium which favors microorganism growth (Reed *et al.*, 1995; Kane, 1996). This will allow microorganisms and contamination problems to be detected early in the culture process and minimize time spent on cultures that will need to be discarded later. Contaminants are more easily seen in clear medium. If bacterial contaminants are common, Gelrite (gelatin gum), a clear gelling agent, rather than agar

should be used to solidify the initiation medium to assist detection.

New cultures should be checked for contamination every two to four days for at least one week. Slow growing bacterial contaminants require 10 or more days to appear, or months if on tissue culture media. Contaminated cultures should be removed and autoclaved. Care should be taken to avoid spreading any contaminants to clean cultures. Flaming tools with 70% ethanol will decrease contaminant spread. A 70% dip followed by a 95% dip for flaming or a soapy water dip prior to a 70% alcohol dip may also be used. Spores of some *Bacillus* species may contaminate alcohol, so care should be taken to adequately flame instruments. Alcohol lamps may not be hot enough to kill spores. New hot bead sterilizers are effective and eliminate the risk of fires in the transfer hood. Transfer tools should be sterilized carefully and alcohol dips changed regularly. Instruments should be autoclaved daily and spares should be available for replacement at midday. It may be necessary to retain infected cultures if they are the only representatives of the germplasm. Infected cultures, along with potentially contaminated cultures, should be transferred at the end of the day to minimize the spread of microorganisms to other cultures.

Antibiotic treatments: (Appendix K) It is not advisable to routinely use antibiotics in the medium as it may cause development of antibiotic-resistant bacteria. Antibiotic treatment is best done after initial testing against the contaminant involved and tests for phytotoxicity for the plant. The effective treatment for isolated bacteria is usually two to four times lower than the effective treatment in the plant. Short (10 days) treatments in effective antibiotics are preferred over longer treatments in the growth medium (Reed *et al.* 1995). Fungal contamination may be reduced with fungicide treatments or high-osmotic media (Obeidy and Smith 1990).

Screening and security checks for bacteria and insects/mites: It is important to detect and prevent the spread of contaminants before and during each culture stage. All cultures should be screened for latent contaminants on a regular basis. Screening at initiation, during subculture, and before storage are good points to eliminate contaminants. Many bacterial contaminants that appear during storage are probably latent bacteria which multiply under storage conditions.

If mites and insects are common contaminants, a detection system should be set up and monitoring should occur at regular intervals. The cultures, storage rooms, and growth room should all be routinely monitored. The use of a pyrethrum based spray in culture rooms will control mites in the room and decrease the chance of culture infestations (Plant tissue culture discussion group, Internet, [<http://plant-tc.coafes.umn.edu/listserv/>] 1996).

Precautions should be taken to protect staff members from pesticide exposure, for example by spraying during week-ends when no one will be in the growth room for several days. The staff should be trained to be observant of unusual growth or symptoms in the cultures. Mite and thrips symptoms are more obvious on older leaves, so explants should be trimmed minimally and the older leaves left intact for observation (Box 15). Thrips leave small black feeding spots on older leaves and yellow egg masses may be visible on the stems or shoot tips (Reed, unpublished observations).

If an isolation area is not available, new explants from the field should be wrapped and observed for mites and thrips for several subcultures before placing them with the rest of the collection. Each facility is different, so it is necessary to analyze each system and look for possible contamination points. Reviews of sources and prevention of contaminants are available (Cassells 1991; Leifert and Waites 1994; Reed and Tanprasert 1995).

Box 15: CONTROL OF MITE AND THRIPS INFESTATIONS

- 1) Destroy by autoclaving all infested, and potentially infested, cultures.
- 2) Remove the remaining culture vessels from the growth room and wipe with 70% alcohol and seal the containers with film.
- 3) Spray all shelves, walls and floor in the growth room with an acaricide.
- 4) Return cultures to the room and monitor closely for surviving mites.

Continuous use of vaporizing strip insecticides are inadvisable as resistant insect strains could occur. (George 1993)

Multiplication:

Growth of cultures: Once explants are established in culture and have been screened for contamination, plants must be propagated to adequate numbers to meet distribution and storage requirements. Many of the considerations during initiation also apply during propagation. All the plants in the growth room should be regularly checked and contaminated cultures removed. Cultures should be checked for build-up of phenols, metabolites, and browning in the medium and transferred onto new medium if there is a problem. Frequency of reculturing during propagation may vary from several weeks to months. Medium requirements may change during propagation. Hormone concentrations in the medium will affect shoot development and growth rates, and high concentrations, especially of cytokinins, may affect genetic stability. Growth room conditions vary among genera and must be determined from the literature or by experiment.

Most micropropagation methods are developed with a small number of genotypes. These methods may not be totally suitable for diverse collections. Genebanks with diverse genotypes usually require additional research to determine techniques suitable for a range of genotypes (Druart 1985; Parfitt and Almehdi 1986; Reed 1990; Reed and Abdelnour-Esquivel 1991; Viterbo *et al.* 1992).

Characterizing plant traits in vitro: Data collected on explants should include initiation date, medium, growth conditions, hormones, and parent plant health. Morphological characterization may include shoot multiplication rate, callus formation, rooting, and shoot length. This data should be obtained at a standard time after initiation (i.e. 4th-6th subculture) under standard conditions, on a standard medium and recorded into the *in vitro* database.

Summary

The introduction of plants into culture and their continued multiplication are of prime importance to an in vitro genebank. Screening for internal contaminants will reduce the spread of microorganisms as well as improve the growth of the cultures. Characterization of plants will provide baseline data for evaluation of stored cultures at a later date.

II.2.6 CULTURE STORAGE

This section discusses options for storage of cultures and procedures for determining storage conditions.

Slow-growth storage: (Appendix L)

Slow-growth storage lowers the risk of losing germplasm through handling errors, such as contamination and media errors; decreases mislabeling; decreases the risks of genetic instability; cuts down on labor; and reduces the overall cost of maintaining the germplasm.

Research on storage methods (slow growth): An extensive literature review should be done on slow-growth techniques. A compilation of slow-growth research was done by IPGRI (Ashmore, 1997). Much germplasm storage research is not published in refereed journals and may be difficult to find. Useful information may be obtained from IPGRI/FAO newsletters, IAPTC journals, e-mail discussion groups, and crop or regional networks. Initial storage programs can be based on published research on similar plants. Most genera tolerate a range of storage temperatures.

Storage conditions: The available storage options will vary from facility to facility. Temperate crops may be stored at 4°C or less while tropical genotypes require 15-28°C. Light conditions may be darkness or a 12-16 hr photoperiod. Light intensities vary with crop type and have not been widely studied. Each genus will have specific requirements but often standard techniques for related plant types can be successfully instituted with little modification (Reed, et al., 2003). Two or more storage conditions may be required to fill the needs of the genera involved. For example, the majority of the collection may be stored under one set of conditions, but a second set of conditions may be required for less resilient genotypes.

Differences in storage requirements among accessions of coffee were evident in a group of 32

diversity groups conserved under slow growth for 3 years. In some groups all the non-adapted genotypes were lost during the first subculture while in others few or none were lost for the entire experiment. Survival was not related to geographical origin. The 32 original groups could be subgrouped into four survival groups, and three of the four stored adequately under the conditions used. The fourth group required additional study to provide adequate slow growth conditions (Dussert, *et al.*, 1996). Similar results are reported for cassava (Roca *et al.*, 1989), *Musa* (Van den Houwe *et al.*, 1995), *Xanthosoma* (Zandvoort *et al.*, 1994) and yam (Malaurie *et al.*, 1993).

To determine an appropriate storage temperature for a genus or species, link information from the research literature with practical knowledge of the species. Take advantage of natural dormant periods or seasons of slow growth for the plants in question (i.e. cold related dormancy in temperate plants; low moisture related slow growth in coffee). Individual laboratories may vary in their treatment of a single genus. In sweet potato, storage temperatures vary from 21-28°C (Jarret, 1989; Zamora and Paet, 1996). For *Rubus*, temperate accessions are held at 4°C while those from tropical locations are stored at 25°C (Reed, 1993).

Not all replicates of an accession need to survive the storage period, but enough must remain for successful repropagation. Some laboratories (INIBAP) repropagate when half of the stored replicates remain while others (NCGR) use inventory data on plant condition and the number of living replicates to determine repropagation times. The most economical solution may be to create minimal conditions which all the genera will survive at a desired level. As each genus will respond differently to storage conditions, it will be necessary to carefully observe accessions and note how they decline during storage. Such characteristics as loss of color, defoliation, browning, or tip necrosis may be indications of lack of viability.

Pretreatments are often useful for improving storage duration. Cold acclimatization (one week at 8 h 22°C days/16 h -1°C nights) significantly improves the length of storage for some temperate plants (Reed, 1992; 2002). Two weeks of growth on the storage medium in the growth room before placing in cold storage conditions improves the length of storage of most of the temperate genera tested (Marino, 1995).

Number of cultures stored: There are no specific rules on the number of replicates that should be held in storage (Box 16). Storage replication depends on the number of plants obtained on a subculture, the purpose of the genebank (active distribution/medium term storage), and the risk of loss. A genus or species which produces few plants per subculture and is used for active distribution would require more stored propagules than the same genus or species used as a backup collection. To determine the number of replicates to store, it is necessary to store samples and determine the number which are lost during a given time period. This number will vary with accession as well as with the genus. At CIAT 100 accessions were stored and monitored for three years. During that time period one to three of five replicates were lost for each accession. Based on this data they recommend three to five replicates per accession (CIAT/IPGRI, 1994).

Box 16: NUMBER OF REPLICATES STORED *IN VITRO* PER ACCESSION

Institute	crop	number and container
CIAT	<i>Manihot</i>	5 individual tubes
INIBAP	<i>Musa</i>	20 individual tubes
NCGR	<i>Corylus, Fragaria, Mentha,</i> <i>Pyrus, Ribes, Rubus, Vaccinium</i>	10 individual sections of plastic bags
CARDI	<i>Dioscorea, Manihot</i>	10 individual tubes

Subculture regime: Subculture of stored material can be handled in several ways. Two suggested methods are: one or a few are removed and subcultured; or half of the replicates are subcultured at one time and the remainder are held until it is known that the first group is healthy and growing. These methods minimize the risk of loss through contamination or medium errors.

As cultures are returned to storage, it is best to choose the individuals to be stored from several cultures, not just one, in order to minimize the chance of selecting a variant plant. The detailed repropagation procedures will depend upon the type of storage; for example, if storage is under cold conditions, the repropagated cultures would be recultured at normal growth room temperatures until the cultures are re-established and then they would be returned to their storage temperature.

Inventory of stored plants: (**Appendix L**) The plants stored under slow-growth conditions will require routine monitoring to assess their viability and need for reculturing. The type of assessment and the type of data collected will vary with genus and species. Visual assessment may be adequate for some, while others will require actual regrowth to determine their viability. The needs of each genus or group should be determined and a standard procedure established before large scale storage is begun. Initially, visual inspections may need to be supplemented with actual regrowth tests to determine their validity. Visual assessment of microtubers, or shoots which become dormant, may be difficult.

Inventory schedule: Storage length varies greatly within most genera and species. Curators should determine the shortest period between which reculture is needed within the genus, then schedule inventories to match that time frame. For example, if three months is the shortest storage time for any genotype in that group, schedule the group's inventory for three month intervals. This schedule will circumvent the loss of that accession. If accessions are placed into storage throughout the year, a shorter standard inventory time will be necessary.

Data collection: (**Appendix L**) Standardizing what data are taken and how the inventories for each genus or species are conducted will provide valuable information on the collection and assist in improving storage techniques. The kinds of data which might provide useful information include the number stored, alive, dead, and contaminated; an evaluation of the plants' condition (good, fair, poor); color; defoliation; elongation; rooting; multiplication; callus;

and medium discoloration.

Safety duplication:

Duplicating the collection for safety: If the *in vitro* collection is the only source of plant material, duplicates should be stored in at least two places for safety. The replicates may be stored on- or off-site. If the duplicate collection is stored on-site, the two collections should be located in separate storage rooms. Replicates in off-site safety collections may be kept as a “black box” collection (a duplicate held at another location but not actively maintained) or in active storage at another facility. If cryopreserved material is available, it may also serve as a duplicate collection. For example CIP in Peru has formal agreements with other laboratories for black box storage of *in vitro* cultures (Huaman, 1999). Oil palm *in vitro* collections are informally exchanged between laboratories to duplicate collections.

Link to other conservation methods (field, seeds): Linkage with other conservation methods may influence the number of replicates, the number of duplicate collections, and other factors. The need for virus-free stock material may make *in vitro* the storage and distribution form of choice and the field collection the evaluation form. Some accessions may be more suited for storage as seed. Cryopreservation should be used, when available, as the base collection. The Institute of Crop Science of the Federal Agricultural Research Centre in Braunschweig-Völkenrode, Germany holds a collection of 778 old potato varieties, from which 519 are currently cryopreserved (Mix-Wagner *et al.* 2003). This is an excellent example of how cryopreservation can be used successfully as a duplicate collection. The cryopreserved collection is used as a base collection, while the *in vitro* collection can still be used for distribution (Schäfer-Menuhr *et al.* 1996). CATIE (Tropical Agricultural Research and Higher Education Center, Costa Rica) holds one of the largest coffee germplasm field collections worldwide, which includes 1850 accessions. In an effort to increase the security of the collection through the implementation of complementary conservation strategies, seeds of 67 accessions constituting the core collection have been cryopreserved, thereby establishing the first cryopreserved collection maintained in a tropical country (Dussert *et al.* 2000; Vasquez *et al.* 2004).

Monitor genetic stability:

Somaclonal variation, while a problem with plants regenerated from single cells, callus, or adventitious buds, is not common in plants micropropagated from axillary buds. Genera which display variability under field conditions should also be closely monitored *in vitro*. *Musa* spp. is a classic example of a variable genus, both in the field and *in vitro*. In the case of at-risk genotypes, the increased chance of loss must be balanced with possible genetic instability (*i.e.* is it better to save the genotype even though it may be unstable, or let it die out in its pure form). Sweet potato is variable in field culture and many new cultivars are selected from those variants. Mutations are no more likely *in vitro* than in field collections (Jarret and Florkowski, 1990).

Standard assessments: If conditions permit, a standard assessment of genetic stability should be made at regular intervals. Since the chance of variability is low for most genera, assessment may be of low priority when establishing an *in vitro* collection. It is probably the most realistic to identify genotypes which are variable in the field and check them on a regular basis, rather than

screening all genotypes. If instability is common in a genus, then more rigorous checks would be required.

Random samples taken from the cassava storage collection every two years for ten years were evaluated in the field at CIAT and showed no change. There were a few variations in the first growth cycle, but plants were normal in the second year showing physiological differences, but not genetic changes (CIAT/IPGRI, 1994). Similar results were seen with strawberry genotypes at NCGR (Kumar, 1995). Analysis of potato *in vitro* cultures stored on medium with mannitol showed that some regenerated plants produced RFLP markers different from the controls, indicating some genetic change (Harding, 1994).

In vitro monitoring of genetic stability is difficult. Initial characterization of cultures may be useful for comparison, but the effects of various medium components may mask or cause phenotypic changes in the cultures. If abnormal growth is seen *in vitro*, then the culture should be discarded or planted out to check the integrity of the accession. Some abnormal growth may be due to physiological conditions imposed by the culture system and the plant may be normal when planted out.

Monitoring systems: There is a need for a simple genetic variability monitoring system using techniques available to the facility: 1) Visually examine tissue culture plants; 2) Observe tissue culture plants in the field or greenhouse through an entire growth cycle to observe for any changes in morphology, then either perform more frequent testing of those genotypes which are observed to be prone to somaclonal variation, or randomly select a sample for testing; 3) Use isozyme analysis, being aware that field and tissue culture samples will have different results; 4) Test using DNA techniques such as RFLP, RAPD, or AFLP, again realizing these techniques may show different banding patterns between field and tissue culture samples. Sugarcane field collections studied from several field sites showed genetic variability for a number of clones. Variation also occurred in *in vitro* selections, with possible loss of genetic material (Glaszmann *et al.*, 1996).

CIAT found that isozyme analysis was not useful for comparing cassava *in vitro* material with field plants due to differences in physiological/environmental states. DNA fingerprinting, when it is available, may be more useful for this type of analysis. At present morphological data from field-grown plants is most useful.

Summary:

This section described storage conditions and requirements for *in vitro* cultures. Determining storage conditions, providing for inventory, evaluation of viability, and verifying genetic stability are all important components for the genebank.

II.2.7 CRYOPRESERVATION

Long-term storage of clonally propagated plants requires the use of ultra-cold storage methods. This section highlights the requirements for laboratory facilities and the basic techniques now available for use.

Facility requirements:

Laboratory: A well-equipped tissue culture laboratory needs only a few additions in order to establish a cryopreservation facility. These include a reliable liquid nitrogen (LN) source, storage dewars, and vented handling containers. A controlled freezer may be useful, but is not required. Liquid nitrogen is usually supplied by specialized companies. Alternatively, liquid nitrogen supplies may be available from local hospitals, industry, or artificial insemination centers. Small LN manufacturing plants may be purchased which produce from 100 to 900 liters per month.

Safety: Special safety considerations apply when liquid nitrogen is in use. LN must be used in a well-ventilated room to decrease the risk of suffocation; handling and storage dewars must be vented to prevent explosions; skin and eyes must be protected with cold-resistant gloves and safety glasses, closed top shoes (so LN can not enter and be trapped on the foot). LN safety considerations should be included in training of all new staff. Local hospitals may be able to provide advice on the safe use of LN.

Techniques:

Many techniques are still at the research phase, however others are currently used to store genetic resources (Reed, 2001). The use of cryopreservation in a laboratory will require some research and screening of plant materials before a storage regime can be implemented. There is still much variability among genotypes, so survival percentages can vary greatly.

Research: A thorough review of the literature is important (see Box 17 for list of key references). Direct, mail or email discussions with cryopreservation researchers is encouraged. Initial research is required to determine which cryopreservation methods are best suited to the genus to be frozen. Several techniques are available. The technique selected may be chosen for its ease, labor and time requirements; costs; the availability of equipment; or the level of technical skill needed. For some crops, only one method will work. Once a technique has been selected, the collection should be screened to confirm that the method is suitable for the range of genotypes in question. After screening, a detailed protocol for routine storage can be developed and written into the procedures manual.

Recovery of cryopreserved plants can be improved by modifying plant culture, pretreatments, cryoprotectant type, cryoprotectant exposure time, freezing or thawing rates, and recovery medium. Methods to improve the health of the cultures will also improve survival rates since plants in poor condition seldom survive cryopreservation. Most seed are stored in the vapor phase, but most meristems are stored in the liquid phase.

For the *encapsulation-dehydration* method, meristems are encased in alginate beads, pretreated in sucrose solutions, and dried to predetermined moisture contents. Dried beads are directly immersed in LN. Vials are thawed by removing them to room temperature (Fabre and Dereuddre, 1990).

Numerous modifications of each technique are available in the literature. The choice of technique will depend on the plant part preserved, the available facilities, and the type of plant (Box 18).

Cold-hardy temperate woody plants may be cryopreserved using *dormant buds*. This technique is applicable to species which can be budded or grafted after thawing. Cold-hardened branches from field trees are dehydrated to 30% moisture content, then frozen at 1°C/h to -30°C, held for 24 h and stored in the vapor phase of LN (Forsline *et al.*, 1993).

Storage Procedures:

General considerations: Several factors may influence the health of the cryopreserved collection. **Once the**

samples are frozen, they can not be warmed and refrozen. Any time frozen material is transferred from one LN container to another, it must be moved very quickly (in seconds, not minutes). Storage containers often require two persons to safely transfer materials. It is best to have the samples in LN and the tray to be placed in the dewar, also submerged in LN. The samples are transferred to the tray, then the tray is quickly replaced in the rack and dropped in the storage dewar. In contrast, pollen samples may be thawed and refrozen many times without harm.

Storage containers: Most storage containers that are easy to access lose LN at a faster rate than those which are not as convenient (i.e. wide mouth containers vs. narrow necks). If LN is not easily available, it is best to get a dewar with a long holding period. Keep in mind, each time samples are added to the dewar LN will be lost to the atmosphere, and that will decrease the holding period. Having two dewars, one for holding samples as they are processed, and one for long-term storage is probably a good strategy. All samples could be transferred to the storage dewar at one time, the dewar refilled with LN and the lid kept in place for a long time. Alarm systems to indicate emergency low LN levels are advised; these can be electric with battery backups. They are especially important if the dewars are not being actively used (checked or added to) and may be forgotten by lab staff until it is too late. Regular checks should be made and dewars filled on a regular basis. Storage divided between two dewars would provide an additional safeguard.

Replicates: The number of replicates stored will depend on the survival rates achieved, crop

Box 18: CRYOPRESERVATION OF PLANTS

<u>Part Stored</u>	<u>Use</u>
Apical meristems	retain clonal stability
Dormant buds	hardy woody plants
Somatic embryos	fast clonal propagation
Zygotic embryos	store recalcitrant seeds

type, speed of propagation, stability in culture, ease of propagation, and material available for storage (see examples in Box 19). Once thawed, meristems cannot be refrozen, so dividing the frozen meristems among several tubes increases the length of time they will remain in storage. If studies on storage life are desired, more meristems should be stored with fewer per tube. At least one control vial should be thawed within a week of freezing the storage sample; its regrowth rate can be used to estimate the expected recovery of the stored material. Control data should be recorded in the collection database. Cryopreserved storage should be considered a base collection for a field collection and should not be the only form for conserving an accession.

A simple method, based on the binomial distribution, has been developed recently to calculate the probability of recovering at least one (or any other fixed number of) plant(s) from a cryobank sample using four given parameters: the percentage of plant recovery observed from a control sample, p_{obs} , the number of propagules used for this control, n_1 , the number of propagules in the cryobank sample, n_2 , a chosen risk for the calculation of a confidence interval for the observed plant recovery (Dussert *et al.* 2003). Using this method, it is possible to assess the number of propagules which should be rewarmed immediately after freezing in order to estimate the plant recovery percentage as a function of the total number of propagules available. It also allows the calculation of the minimum plant recovery percentage to ensure that the probability to recover at least one (or A , with $A > 1$) plant(s) is higher than a fixed probability level, as a function of the control and the cryobank sample sizes. Reciprocally, once the plant recovery percentage has been estimated, it is possible to assess the minimum size of the cryobank sample to obtain a probability to recover at least one (or A , with $A > 1$) plant(s) higher than some fixed level.

Inventory: A database should be set up to include: storage date, location in dewar, number of vials, number of meristems per vial, technique used, thawing technique required, recovery medium, and other important procedures. When thawing, it is important to know how the plant material was frozen, since thawing techniques are specific to the method used when the plants were frozen, and must be followed carefully for good results.

Monitor genetic stability:

Field observation for morphological traits can be done at most genebanks. If facilities are available, molecular techniques should be used to compare the field mother plant, tissue culture mother plant, and stored materials. Basic data should be collected when the meristems are initially stored. Comparisons of tissue culture plants with field plants require both to be planted in the field or greenhouse. Stability testing is a low priority for cryopreserved plants, compared to monitoring of the field and *in vitro* collections, as molecules are very stable at LN temperatures.

Box 19: CRYOPRESERVED STORAGE

Two approaches cryopreserved storage:

1. Potato collection cryopreserved at the Institute of Crop Science of the Federal Agricultural Research Centre in Braunschweig-Volkenrode, Germany. Each of the 519 accessions cryopreserved is represented by 30 vials containing 12 shoot tips each, which are frozen in three independent experiments. One vial from each experiment is used to assess regrowth. An average regeneration rate of 40% was achieved and all cultivars could be regenerated. Those with a low regeneration rate require thawing more than one vial for recovery of the cultivar. More vials may be stored for accessions with low viability.
2. Pear collection cryopreserved at the U.S. Dept. of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository in Corvallis, Oregon. Cultivars are tested for regeneration after slow freezing. Those cultivars with regeneration greater than 40% are stored. Six vials, each with 25 meristems, are cryopreserved. One vial is thawed immediately as a control, the remainder are shipped in liquid nitrogen to the National Center for Genetic Resources Preservation, Fort Collins, Colorado for permanent storage. After storage at NCGRP, one vial is thawed as a control, leaving four vials stored (100 meristems). Cultivars with low survival are studied to improve regrowth before storage.

Summary

Cryopreservation may be used as base collection storage for clonal crops. Three main techniques are currently in use and modifications of those techniques allow storage of many genotypes. The technique used will depend greatly on the crop to be stored and the laboratory staff and facilities available. With actual storage, transfer of samples from one dewar to the next requires extra care and the use of controls to check the effects of transfer.

II.2.8 DISTRIBUTION

Another important function of *in vitro* genebanks is the distribution of plants to researchers. This section discusses aspects of distribution important to genebanks in general, but specifically for *in vitro* genebanks. Additional distribution information is presented in section II.6.

Plant materials:

Organizing multiplication of plant material for distribution: Distribution pressures vary with the type of collection. Collections providing virus-free planting stock will have very different requirements from those providing minimal propagules to plant breeders. Timing of

multiplication for virus-free stock may include storage of the propagules until the time of distribution. Distribution to breeders could occur whenever the plant material is available. Accessions under heavy demand should be stored in greater numbers than other accessions so they can be distributed without a long repropagation time.

Correct stage of growth for safe distribution: Distribution form will vary with the plant genotype involved. Established shoot cultures, microtubers, or rooted shoots may be sent, depending on the requirements of the recipient. Growth stage will vary with the crop and the purpose of the genebank. Virus-free planting stock may require rooted shoots, or even plantlets acclimated to soil, and time of supply may depend upon the planting season, while distribution to a scientist working with *in vitro* plants will require only a shoot culture.

Procedures for recipients of dispatched cultures:

Medium instructions, handling instructions, culture conditions, and historical information should be provided to requesters. At the time of request it is useful to determine that the requester is able to culture or acclimate the *in vitro* plants (has facilities, knowledge) so that plant materials are not wasted. Assistance may be offered to recipients in some cases or a different form of plant may be sent that is more easily used. Written protocols should be distributed. An evaluation/information form should be included with shipments to aid improvements to the shipping procedures. Questions may be asked concerning the plants arrival time, plant condition (good, fair, poor, dead), if poor or dead, possible reason (broken package, frozen in transit, etc.), growth of plants either *in vitro* or acclimation rate, and other comments.

Phytosanitary and quarantine considerations: (Appendix M)

Phytosanitary certification and import permits are usually required for international shipments. Quarantine considerations may also be in effect for diverse regions within one country (i.e. *Corylus* from the eastern USA can not be transported to the western states due to the presence of Eastern Filbert Blight). Additional information on tests and treatments of the germplasm may be given in a GERMPASM HEALTH STATEMENT as shown in Appendix M (Diekmann and Putter, 1996).

Packaging and shipping:

The amount of time required for a shipment to reach its destination must be considered. Choice of shipper, notification of the recipient of pending arrival, and correct packing procedures will improve the plants' condition on arrival. The temperature enroute and at the destination should be considered, since packages may freeze or overheat while sitting out on loading docks. Packing materials may need to include ice packs or insulation, and shipping containers should resist compression. Appropriate labels for the exterior of the package may improve handling by the shipper, for example, plant material, perishable, do not freeze, etc.

Documentation and record keeping: Records should be kept of plants requested, plants

distributed, recipient information, and shipping costs.

Summary

Distribution is an important function of the *in vitro* genebank. Careful documentation of the plants requested, shipped, costs, and procedures will provide information to administrators and budget planners.

II.2.9 RESEARCH NEEDS IN THE *IN VITRO* GENE BANK

Although tissue culture, storage, and cryopreservation methods have been determined for many plants, these systems are not available or fully operational for many clonally propagated plants. This section includes areas of research needed for improved storage in clonal genebanks.

Optimizing slow growth protocols:

Slow-growth storage would benefit from investigations on the effects of plant growth regulators and growth retardants. Useful information would also be gained from determining the effects of light quality and quantity, temperature and light interactions, and propagule size and growth stage.

Laboratories should design and implement storage tests with representative samples of their germplasm and use enough replicates for statistical evaluation. The treatments tested may involve: light quality and duration, storage temperature, propagule size, type of propagule (microtubers, bulbs, rooted plantlets, unrooted shoots), medium, growth regulators, pregrowth or acclimation period to temperature or media, storage container, etc. Methods should be developed which minimize the use of plant growth regulators and growth retardants since these substances can continue to affect the plantlets when repropagated on regular media (Jarret and Gawel, 1991). Morphological characterization of stored plants may also be useful. Publication of results will aid other germplasm curators.

Germplasm health:

Virus surveys and virus therapy are needed along with virus indexing techniques with a possible focus on techniques for effective virus testing *in vitro* and whether viruses can be transmitted *in vitro*. Investigations on and development of indexing techniques for latent endogenous bacteria are needed for many crops.

Monitoring genetic stability:

Studies are needed on selection pressures *in vitro*; variation in the field compared to *in vitro*; using field evaluation on material with known instabilities; development of molecular markers or other molecular techniques to monitor genetic stability.

Cryopreservation:

The applicability of cryopreservation methods to a wide range of species and genotypes requires further study. Methods should be developed which are applicable at several localities.

The potential of cryopreservation for eliminating viruses from infected plants, as a substitute or in complement to classical virus eradication techniques such as meristem culture and cryotherapy should be further explored. Preliminary experiments performed with plum shoot tips (Brison *et al.* 1997), banana meristematic cultures (Helliot *et al.* 2002) and grape shoot tips (Wang *et al.* 2003) have shown various degrees of elimination of different viruses after freezing of infected viruses.

Summary

Immediate research needs are the development of tissue culture and cryopreservation systems. Continuing development of techniques to evaluate genetic stability will make it more feasible to determine genetic change. Improved plant health will improve culture, storage, and exchange of germplasm.

III REFERENCES

- Alercia A., S. Diulgerhoff and T. Metz. 2001. Multicrop Passport Descriptors. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.
- Altman, D.W., S.D. Fryxell, S.D. Koch, and, C.R. Howell 1990. *Gossypium* germplasm conservation augmented by tissue culture techniques for field collecting. *Economic Botany* 44:106-113.
- Altman, A. and H.D. Rabinowitch. 1985. Actual need and possible techniques for *in vitro* storage of *Allium* genepool. A Status Report. IBPGR, Rome.
- Ashmore, S.E. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome, Italy.
- Assy Bah, B., T. Durand-Gasselin and C. Pannetier. 1987. Use of zygotic embryo culture to collect germplasm of coconut (*Cocos nucifera* L.) FAO/IBPGR Plant Genetic Resources Newsletter 71:4-10.
- Astley, D. 1990. Conservation of genetic resources. In: H.D. Rabinowitch and J.L. Brewster (eds.) Onions and Allied Crops. I. Botany, Physiology and Genetics. CRC Press, Inc., Boca Raton, Florida.
- Bajaj, Y.P.S. 1995. Cryopreservation of plant cell, tissue and organ culture for the conservation of germplasm and biodiversity. Pp. 3-18 in Y.P.S. Bajaj (ed.). *Biotechnology in Agriculture and*

Forestry. Cryopreservation of Plant Germplasm I, Vol. 32. Springer-Verlag, New York.

Barg, E., Lesemann, D.-E., Vetten, H.J. and Green, S.K. 1994. Identification, partial characterization, and distribution of viruses infecting *Allium* crops in South and South-East Asia. *Acta Hort. (ISHS)* **358**:251-258

Benson, E.E. 1999. *Plant Conservation Biotechnology* pp. 139-154. Taylor and Francis, London

Biondi, S. and T.A. Thorpe. 1981. Requirements for a Tissue Culture Facility. Pp. 1-20 in T.A. Thorpe (ed.). *Plant Tissue Culture Methods and Applications in Agriculture*. Academic Press, New York.

Brison M., M.T. de Boucaud, A. Pierronnet and F. Dosba. 1997. Effect of cryopreservation on the sanitary state of a cv. of *Prunus* rootstock experimentally infected with Plum Pox Potyvirus. *Plant Sci.* **123**:189-196.

Brunt, A.A., Jackson, G.V.H. and Frison, E.A. (eds.). 1989. *FAO/IBPGR Technical Guidelines for the Safe Movement of Yam Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Cassells, A.C. 1991. Problems in tissue culture: culture contamination P p. 31-44 in P.C. Debergh and R.H. Zimmerman (eds.). *Micropropagation Technology and Application*. Kluwer Academic Publishers, Dordrecht, Netherlands.

Ciesla, W.M., M. Diekmann and C.A.J. Putter (eds.). 1996. *FAO/IPGRI Technical Guidelines for the Safe Movement of Eucalyptus spp. Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Plant Genetic Resources Institute, Rome.

Chang, T-T., S.M. Dietz and M.N. Westwood. 1989. Management and use of plant germplasm collections. Pp. 127-160 in L. Knutson and A.K. Stoner (eds.). *Biotic Diversity and Germplasm Preservation, Global Imperatives*. Beltsville Symposia in Agricultural Research # 13. Kluwer, Dordrecht.

Chang Y. and B. M. Reed. 2000. Extended Alternating-Temperature Cold Acclimation and Culture Duration Improve Pear Shoot Cryopreservation *Cryobiology* **40**:311-322.

De Vicente C. and T. Fulton. 2003. *Using Molecular Marker Technology in Studies on Plant Genetic Diversity*. International Plant Genetic Resources Institute, Rome.

De Vicente C., T. Metz and A. Alercia. 2004. *Descriptors for Genetic Marker Technologies*. International Plant Genetic Resources Institute, Rome.

Debergh, P.C. and R.H. Zimmerman (eds.). 1991. *Micropropagation Technology and Application*. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Dereuddre, J., C. Scottez, Y. Arnaud, and M. Duron. 1990. Resistance of alginate-coated axillary shoot tips of pear tree (*Pyrus communis* L. cv. Beurré Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen. *C. R. Acad. Sci. Paris* **310**:317-323.

Diekmann, M. (ed.). 1996. *FAO/IPGRI Technical Guidelines for the Safe Movement of Allium Germplasm*. Food and Agriculture Organization of the United Nations, Rome/ International Plant Genetic Resources Institute, Rome, Italy.

Diekmann, M. and C.A.J. Putter (eds.). 1996. FAO/IPGRI Technical Guidelines for the Safe Movement of Stone Fruits Germplasm. Food and Agriculture Organization of the United Nations, Rome/ International Plant Genetic Resources Institute, Rome, Italy.

Diekmann, M. and C.A.J. Putter (eds.). 1996. FAO/IPGRI Technical Guidelines for the Safe Movement of *Musa* Germplasm. 2nd edition. Food and Agriculture Organization of the United Nations, Rome/ International Plant Genetic Resources Institute, Rome, Italy.

Diekmann, M., Frison, E.A. and Putter, T. (Eds.) 1994. FAO/IPGRI Technical Guidelines for the Safe Movement of Small fruit Germplasm. Food and Agriculture Organization of the United Nations, Rome/ International Plant Genetic Resources Institute, Rome, Italy.

Diekmann, M., J.R. Sutherland, D.C. Nowell, F.J. Morales and G. Allard. (eds.). 2003. FAO/IPGRI Technical Guidelines for the Safe Movement of *Pinus* spp. Germplasm. Food and Agriculture Organization of the United Nations, Rome/ International Plant Genetic Resources Institute, Rome, Italy.

Druart, P. 1985. *In vitro* germplasm preservation techniques for fruit trees. Pp. 167-171 in A. Schäfer-Menuhr (ed.). *In vitro* Techniques. Propagation and Long Term Storage. Martinus Nijhoff, Dordrecht.

Dulloo, M.E. V. Ramanatha Rao, F. Engelmann and J.M.M. Engels, 2004. Complementary Conservation strategy for coconuts. In P. Batugal and V. Ramanatha Rao (eds.) Coconuts genetic resources. IPGRI (in press).

Dulloo M.E., A. Charrier, S. Dussert, F. Anthony, S. Tesfaye, J.J. Rakotomalala, C. Agwanda and N. Legnate (2001). Conservation of coffee genetic resources – constraints and opportunities. Proceedings of 19th World Colloquium on Coffee Science, ASIC, Trieste, Italy, 14-18 May 2001

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

Dussert S., N. Chabrillange, N. Vasquez, F. Engelmann, F. Anthony, A. Guyot & S. Hamon, 2000. Beneficial effect of post-thawing osmoconditioning on the recovery of cryopreserved coffee (*Coffea arabica* L.) seeds. *CryoLetters* **21**: 47-52.

Eastwood, R.B. and Steele, W.M. 1978. The conservation of yam germplasm in West Africa. *Plant Foods for Man* **2**:153-158.

Engelmann F. 1999. Management of Field and *In vitro* Germplasm Collections, Proceedings of a Consultation Meeting - 15-20 January, 1996, CIAT, Cali, Colombia, International Plant Genetic Resources Institute, Rome.

Engelmann F. 2002. Coconut. Pp. 68-71 in *In vitro* collecting techniques for germplasm conservation. Pence V.C., J. Sandoval, V. Villalobos and F. Engelmann (eds.). IPGRI Technical Bulletin N°7. International Plant Genetic Resources Institute, Rome.

Engelmann F. and J.M.M. Engels. 2002. Technologies and strategies for *ex situ* conservation. Pp. 89-104 in *Managing Plant Genetic Diversity*, J.H.H. Engels, V.R. Rao, A.H.D. Brown & M.T. Jackson (eds.), CABI, Wallingford and IPGRI, Rome.

Engelmann F. and H. Takagi, 2000 (eds.). *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*. International Plant Genetic Resources Institute, Rome.

Engels J.M.M. and B. Visser. 2003. *A Guide to Effective management of Germplasm Collections*. Handbooks for Genebanks N° 6. International Plant Genetic Resources Institute, Rome.

Escobar R.H., D. Debouck and W.M. Roca. 2000. Development of cassava cryopreservation. Pp. 222-226 in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*. F. Engelmann and H. Takagi, 2000 (eds.), International Plant Genetic Resources Institute, Rome.

FAO, 2002. *The International Treaty on Plant Genetic Resources for Food and Agriculture*. Food and Agriculture Organisation of the United Nations. 45pp.

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

Forsline, P.L., C. Stushnoff, L.E. Towill, J. Waddell, and W. Lamboy. 1993. Pilot project to cryopreserve dormant apple (*Malus* sp.) buds. *HortScience* 28:118.

Frison, E.A. and Feliu, E. (Eds.). 1989. *FAO/IBPGR Technical Guidelines for the Safe Movement of Cocoa Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Frison, E.A. and Feliu, E. (Eds.). 1991. *FAO/IBPGR Technical Guidelines for the Safe Movement of Cassava Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Frison, E.A. and Ikin, R. (Eds.). 1991. *FAO/IBPGR Technical Guidelines for the Safe Movement of Grapevine Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Frison, E.A. and Putter, C.A.J. (Eds.). 1989. *FAO/IBPGR Technical Guidelines for the Safe Movement of Musa Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Frison, E.A. and Putter, C.A.J. (Eds.). 1993. *FAO/IBPGR Technical Guidelines for the Safe Movement of Sugarcane Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Frison, E.A. , Putter, C.A.J. and Diekmann, M. (Eds.). 1993. *FAO/IBPGR Technical Guidelines for the Safe Movement of Coconut Germplasm*. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Frison, E.A. and Taher. M.M. (Eds.). 1991. *FAO/IBPGR Technical Guidelines for the Safe Movement of Citrus Germplasm*. Food and Agriculture Organization of the United Nations,

Rome/International Board for Plant Genetic Resources, Rome.

George, E. F. 1993. Plant propagation by tissue culture - Part 1. Exegetics Limited, U.K.

George, E. F. 1996. Plant propagation by tissue culture - Part 2. Exegetics Limited, U.K.

Glaszmann, J.C., P. Rott, and F. Engelmann. 1996. Role of *in-vitro* maintenance of sugarcane for germplasm conservation and exchange. Pp. 67-70 in Croft, B.J., Piggins, C.M., Wallis, E.S., and Hogarth, D.M. (eds.). Sugarcane Germplasm Conservation and Exchange. Australian Center for International Agricultural Research Proceedings No. 67.

Guarino, L., V.R. Rao, and R. Reid (eds.). 1995. Collecting Plant Genetic Diversity. Technical Guidelines. CAB International, Wallingford, UK.

Harding, K. 1994. Methylation status of DNA derived from potato plants recovered from slow growth and cryopreservation. *Euphytica*. 55: 141-146.

Harlan, J.R. and J.M.J. de Wet. 1971. Toward a rational classification of cultivated plants. *Taxon* 20: 509-517.

Hawkes, J. G. 1980. Crop Genetic Resources Field Collection Manual. International Board for Plant Genetic Resources, Rome.

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

Hodgkin, T., A.H.D. Brown, Th.J.L. van Hintum and E.A.V. Morales. 1995. Core Collections of Plant Genetic Resources. John Wiley & Sons, Chichester, UK.

Huaman, Z. 1999. Management of potato and sweet potato field genebanks. Pp. 38-42 in *Management of field and in vitro germplasm collections*, Proceedings of a consultation meeting - 15-20 January, 1996, CIAT, Cali, Colombia, F. Engelmann (ed.). International Plant Genetic Resources Institute, Rome.

Huaman Z. And Stegemann, H. 1989. Use of electrophoretic analyses to verify morphologically identical clones in a potato collection. *Plant Varieties and Seeds* 2:155-160.

Huaman, Z., De la Puente, F., and Arbizu, C. 1995. Collecting vegetatively propagated crops (especially roots and tubers). Pp. 457-466 in L. Guarino, V. Ramanantha Rao and R. Reid (eds.). Collecting Plant Genetic Diversity. Technical Guidelines. CAB International, Wallingford, U.K.

Hummer, K. (ed.) 2003. Operations Manual. USDA-ARS National Clonal Germplasm Repository. Corvallis, Oregon, USA.

Hummer K. and B.M. Reed. 1999. Establishment and operation of a temperate clonal field genebank. Pp. 29-31 in *Management of field and in vitro germplasm collections*, Proceedings of a consultation meeting - 15-20 January, 1996, CIAT, Cali, Colombia, F. Engelmann (ed.). International Plant Genetic Resources Institute, Rome.

IBPGR. 1986. IBPGR Advisory Committee on *in vitro* storage. Planning and Operation of *in vitro* Genebanks. International Board for Plant Genetic Resources, Rome.

- IBPGR. 1988. IBPGR Advisory Committee on *in vitro* storage. Conservation and Movement of Vegetatively Propagated Germplasm: *In vitro* Culture and Disease Aspects. International Board for Plant Genetic Resources, Rome.
- IPGRI. 1994. Proceedings of the International Network for Cassava Genetic Resources. International Plant Genetic Resources Institute, Rome.
- IPGRI/UNEP 1995. Capacity building for training in plant genetic resources conservation and use in developing countries. University of Puerto Rico, Mayaguez, Puerto Rico.
- IPGRI. 1996. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. International Plant Genetic Resources Institute, Rome.
- IPGRI. 1999. Key Questions for Decision-Makers - Protection of Plant Varieties under WTO Agreement on Trade-Related Aspects of Intellectual Property Rights. International Plant Genetic Resources Institute, Rome.
- 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, Rome and CIAT, Cali, Colombia.
- Jarret, R.L. 1989. A repository for sweet potato germplasm. *HortScience* 24:886.
- Jarret, R.L. and Gawel, N. 1991. Chemical and environmental growth regulation of sweet potato (*Ipomea batatas* (L) Lam.) *in vitro*. *Plant Cell, Tissue Organ Culture*. 25:152-159.
- Jarret, R.L. and W.J. Florkowski. 1990. *In vitro* active vs. field genebank maintenance of sweet potato germplasm: Major costs and considerations. *HortScience*, 25:141-146.
- Jeffries C.J. (ed.). 1998. FAO/IPGRI Technical Guidelines for the Safe Movement of Potato Germplasm. Food and Agriculture Organization of the United Nations, Rome/ International Plant Genetic Resources Institute, Rome, Italy.
- Johnson RC and T. Hodgkin. 1999. Core Collections for Today and Tomorrow. International Plant Genetic Resources Institute, Rome.
- Kane, M.E. 1996. In: Plant Tissue Culture Concept and Laboratory Exercises. Trigiano and Gray eds., CRC Press, Boca Raton, Florida
- Kartha, K.K. 1985. Cryopreservation of Plant Cells and Organs. CRC Press, Inc. Boca Raton, Florida, USA.
- Keller E.R.J., K.Schüler, H. Gäde, M. Fischer and K. Hammer. 1999. Present situation in the field collections of vegetatively propagated species in the Gaterleben Genebank. Pp. 21-24 in *Management of field and in vitro germplasm collections*, Proceedings of a consultation meeting - 15-20 January, 1996, CIAT, Cali, Colombia, F. Engelmann (ed.). International Plant Genetic Resources Institute, Rome.
- Kyte, L.A. and J. Kleyn. 2001. Plants from Test Tubes. An Introduction to Micropropagation, 3rd Edition. Timber Press, Portland, Oregon. USA
- Kumar, M.B. 1995. Genetic stability of micropropagated and *in vitro* cold-stored strawberries.

M.S. Thesis, Oregon State University.

Leifert, C. and W.M. Waites. 1994. Dealing with microbial contaminants in plant tissue and cell culture: hazard analysis and critical control points. Pp. 363-378 in P.J. Lumsden, J.R. Nicholar, and W.J. Davies (eds.). *Physiology, Growth and Development of Plants in Culture*. Kluwer Academic Publishers, Dordrecht, Netherlands.

Leskien D. and M. Flitner. 1997. Intellectual Property Rights and Plant Genetic Resources: Options for a *Sui Generis* System Issues in Genetic Resources No. 6. International Plant Genetic Resources Institute, Rome.

Libreros D. 2002. Bibliography on Access to Plant Genetic Resources and Intellectual Property Rights. IPGRI Regional Office for the Americas, Cali, Colombia.

Malaurie, B., Pungu, O., Dumont, R., Trouslot, M.F. 1993. The creation of an *in vitro* germplasm collection of yam (*Dioscorea* - spp.) for genetic resources preservation. *Euphytica* 65:113-122.

Marino, G., P. Rosati, and F. Sagrati. 1985. Storage of *in vitro* cultures of *Prunus* rootstocks. *Plant Cell, Tissue and Organ Culture* 5:73-78.

Mathur S.B. and O. Kongsdal. 2003. Common Laboratory Seed Health Testing Methods for Detecting Fungi. International Seed Testing Association, Bassersdorf, Switzerland.

Maxted N., B.V. Ford-Lloyd and J.G. Hawkes. 1997. Complementary conservation strategies. Pp. 15-39 in *Plant Genetic Resources Conservation*, N. Maxted, B.V. Ford-Lloyd and J.G. Hawkes (eds). Chapman & Hall, London.

Mix-Wagner G., H.M. Schumacher and R.J. Cross. 2003. Recovery of potato apices after several years of storage in liquid nitrogen. *CryoLetters* 24:33-42.

National Plant Germplasm Committee. 1986. Operations Manual for National Clonal Germplasm Repositories. U.S. Department of Agriculture, Beltsville, MD, USA.

Ng, S.Y.C. 1992. Micropropagation of white yam (*Dioscorea rotundata* Poir.). Pp. 135-159 in Y.P.S. Bajaj (ed.). *Biotechnology in Agriculture and Forestry*, Vol. 19, High-Tech and Micropropagation III, Springer Verlag, Berlin.

Ng, S.Y.C. 1994. Production and distribution of virus-free yam (*Dioscorea rotundata* Poir.). Pp. 324-328 in Proc. 5th Symp. ISTRC-AB.

Ng N.Q. and S.Y.C. Ng. 1999. Yam field genebank management at IITA. Pp. 16-18 in *Management of field and in vitro germplasm collections*, Proceedings of a consultation meeting - 15-20 January, 1996, CIAT, Cali, Colombia, F. Engelmann (ed.). International Plant Genetic Resources Institute, Rome.

Novak, F.J. 1990. *Allium* tissue culture. Pp. in H.D. Rabinowitch and J.L. Brewster (eds.) *Onions and Allied Crops. I. Botany, Physiology and Genetics*. CRC Press, Inc., Boca Raton, Florida, USA.

Obeidy, A.A. and M.A.L. Smith. 1990. Establishing axenic cultures from mature pecan embryo

explants on media with low water availability. *Plant Cell Reports* 9:463-465.

Old K.M., T.K. Vercoe, R.B. Floyd, M.J. Wingfield, J. Roux and S. Naser. 2003. FAO/IPGRI Technical Guidelines for the Safe Movement of *Acacia* spp. Germplasm. Food and Agriculture Organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Parfitt, D.E. and A.A. Almehti. 1986. *In vitro* propagation of peach: II. a medium for *in vitro* multiplication of 56 peach cultivars. *Fruit Varieties Journal* 40:46-47.

Pearson, M.N., Jackson, G.V.H., Zettler, F.W. and Frison, E.A. (eds.). 1991. FAO/IBPGR Technical Guidelines for the Safe Movement of Vanilla Germplasm. Food and Agriculture Organization of the United Nation, Rome/International Board for Plant Genetic Resources, Rome.

Peeters, J.P. and J.T. Williams. 1984. Towards a better use of genebanks with special reference to information. FAO/IBPGR Plant Genetic Resources Newsletter 60:22-31.

Pence, V.C. 1996. *In vitro* collection (IVC) method. Pp. 181-190 in M.N. Normah, M.K. Narimah and M.M. Clyde (eds.). *In vitro* Conservation of Plant Genetic Resources. Plant Biotechnology Laboratory, Faculty of Life Sciences, Universiti Kebangsaan Malaysia.

Pence V.C. and J.A. Sandoval. 2002. Controlling contamination during *in vitro* collecting. Pp. 30-40 in *In vitro* collecting techniques for germplasm conservation. Pence V.C., J. Sandoval, V. Villalobos and F. Engelmann (eds.), IPGRI Technical Bulletin N°7. International Plant Genetic Resources Institute, Rome.

Pence V.C., J. Sandoval, V. Villalobos and F. Engelmann (eds.). 2002. *In vitro* collecting techniques for germplasm conservation. IPGRI Technical Bulletin N°7. International Plant Genetic Resources Institute, Rome.

Rabinowitch, H.D. and Z. Orna. 1984. Collection, preservation, characterization and evaluation of *Allium* species growing wild in Israel: Selected Examples. Eucarpia, 3rd *Allium* Symposium, Wageningen, The Netherlands.

Reed, B.M. 1990. Multiplication of *Rubus* germplasm *in vitro*: A screen of 256 accessions. *Fruit Varieties Journal* 44:141-148.

Reed, B.M. 1992. Cold storage of strawberries *in vitro*: A comparison of three storage systems. *Fruit Varieties Journal* 46:98-102.

Reed, B.M. 1993. Improved survival of *in vitro*-stored *Rubus* germplasm. *Journal of the American Society of Horticultural Science* 118:890-895.

Reed, B.M. 2001. Implementing Cryogenic Storage of Clonally Propagated Plants. *CryoLetters*. 22:97-104.

Reed, B.M. 2002. Photoperiod Improves Long-Term Survival of In-Vitro Stored Strawberries. *HortScience*. 37: 811-814.

Reed, B.M. and A. Abdelnour-Esquivel. 1991. The use of zeatin to initiate *in vitro* cultures of *Vaccinium* species and cultivars. *HortScience* 26:1320-1322.

- Reed, B.M. and Y. Chang. 1997 Medium and Long Term Storage of *In vitro* Cultures of Temperate Fruit and Nut Crops. In: M.K. Razdan (ed.) Conservation of Plant Genetic Resources *In vitro*. Oxford and IBH Publishing Co. Pvt. Ltd., India.
- Reed, B.M. and P. Tanprasert. 1995. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. *Plant Tissue Culture and Biotechnology* 1:137-142.
- Reed, B.M., P.M. Buckley, and T.N. DeWilde. 1995. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In vitro Cellular and Developmental Biology* 31P:53-57.
- Reed, B.M., N. Okut, J. D'Achino, L. Narver, and J. DeNoma. 2003. Cold storage and cryopreservation of hops (*Humulus L.*) shoot cultures through application of standard protocols. *CryoLetters*. 24:389-396.
- Roca W.M., D. Debouck, R.H. Escobar, G. Mafla and M. Fregene. 2000. Cryopreservation and germplasm conservation at CIAT. Pp. 273-279 in *Cryopreservation of Tropical Plant Germplasm - Current Research Progress and Applications*. F. Engelmann and H. Takagi, 2000 (eds.), International Plant Genetic Resources Institute, Rome.
- Roca, W.M., Chavez, R. Martin, M.L., Arias, D.I., Mafla, G., Reyes, R. 1989. *In vitro* methods of germplasm conservation. *Genome* 31:813-817.
- Ruredzo, T.J. 1989. Progress Report on IBPGR-ILCA Tissue Culture Project. IBPGR Report Number 89/11 IBPGR, Rome.
- Ruredzo, T.J. 1991. A minimum facility method for *in vitro* collection of *Digitaria eriantha* spp. *Penzii* and *Cynodon dactylon*. *Tropical Grasslands* 25:59-65.
- Sackville Hamilton, N.R. and K.H. Chorlton. 1997. Regeneration of accessions in seed collections: a decision guide. *Handbooks for Genebanks No. 5*. International Plant Genetic Resources Institute, Rome.
- Sackville Hamilton, N.R., J.M.M. Engels, Th.J.L. van Hintum, B. Koo and M. Smale. 2002. Accession management: combining or splitting accessions as a tool to improve germplasm management efficiency. IPGRI Technical Bulletin N° 5. International Plant Genetic Resources Institute, Rome.
- Sakai, A., Kobayashi, S., and Oiyama, I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9:30-33.
- Salazar, L.E. 1996. Potato viruses and their control. International Potato center, Lima, Peru.
- Schäfer-Menuhr, A., H.-M. Schumacher, and G. Mix-Wagner. 1996. Long-term storage of old potato cultivars in liquid nitrogen. In: *Comprehensive Potato Biotechnology*, SM Paul Khurana *et al.*, eds., Chapman and Hall, UK.
- SGRP (2003). Booklet of CGIAR Centre Policy instruments, guidelines, and statements on genetic resources, biotechnology and intellectual property rights. Version II. System-wide

Genetic Resources Programme and CGIAR Genetic Resources Policy Committee.

Sheppard J.W. and V. Cockerell. 1996. ISTA PDC Handbook of Method validation for the detection of Seed-borne Pathogens. ISTA, Basserdorf, Switzerland.

Smith, R.A. 1980. Mechanisms of action of ribavirin. Pp. 99-118 in R.A. Smith and W. Kirkpatrick (eds.). Ribavirin. Academic Press, London.

Stover, R.H. and N.W. Simmonds. 1987. Bananas. Longman Group Ltd., UK.

Sutherland J.R., M. Diekmann and P. Berjak. 2002. Forest Tree Seed Health. IPGRI Technical Bulletin N° 6. International Plant Genetic Resources Institute, Rome.

Taylor, M. 2002. Taro. Pp. 65-67 in *In vitro* collecting techniques for germplasm conservation. Pence V.C., J. Sandoval, V. Villalobos and F. Engelmann (eds.). IPGRI Technical Bulletin N°7. International Plant Genetic Resources Institute, Rome.

Thorpe, T.A. (ed.). 1981. Plant Tissue Culture Methods and Applications in Agriculture, 1st ed. Academic Press, New York.

Towill, L.E. and E. Roos. 1989. Techniques for preservation of plant germplasm. Pp. 379-404 in L. Knutson and A.K. Stoner (eds). Biotic Diversity and Germplasm Preservation, Global Imperatives. Beltsville Symposia in Agricultural Research # 13. Kluwer, the Netherlands.

Towill L.E. and Y.P.S. Bajaj. 2002 (eds.). Cryopreservation of Plant Germplasm II: Biotechnology in Agriculture and Forestry, Vol. 50. Springer-Verlag, Berlin.

Van den Houwe, I, De Smet, K., Tezenas du Montcel, H., Swennen, R. 1995. Plant Cell, Tissue and Organ Culture 42:269-274.

Van Hintum Th.J.L., A.H.D. Brown, C. Spillane and T. Hodgkin. 2000. Core Collections of Plant Genetic Resources. IPGRI Technical Bulletin N° 3. International Plant Genetic Resources Institute, Rome.

Vasquez N., K. Salazar, F. Anthony, N. Chabrilange, F. Engelmann and S. Dussert. 2004. Variability in response of seeds to liquid nitrogen exposure within a coffee (*Coffea arabica* L.) core collection. In press in *Seed Science & Technology*.

Viss, P.R., E.M. Brooks, and J.A. Driver. 1991. A simplified method for the control of bacterial contamination in woody plant tissue culture. *In vitro* Cellular Developmental Biology 27P:42.

Viterbo, A., A. Altman and H.D. Rabinowitch. 1994. *In vitro* propagation and germplasm cold storage of fertile and male sterile *Allium trifoliatum* subsp. *hirsutum*. Genetic Resources and Crop Evolution 41:87-98.

Viterbo, A., H.D. Rabinowitch, and A. Altman. 1992. Plant regeneration from callus of *Allium trifoliatum* subsp. *hirsutum*, and assessment of genetic stability by isozyme polymorphism. Plant Breeding 108:265-273.

Walkey, D.G.A. 1990. Virus diseases. Pp. in H. D. Rabinowitch and J.L. Brewster (eds.) Onions and Allied Crops. II. Agronomy, Biotic Interactions, Pathology, and Crop Protection.

CRC Press, Inc., Boca Raton, Florida.

Wang Q., M. Mawassi, P. Li, R. Gafny, I. Sela & E. Tanne. 2003. Elimination of grapevine virus A (GVA) by cryopreservation of *in vitro*-grown shoot tips of *Vitis vinifera* L. *Plant Sci.* 165:321-327.

Westwood, M.N. 1989. Maintenance and Storage: Clonal Germplasm. *Plant Breeding Review* 7:111-128.

Withers, L.A. 1995. Collecting *in vitro* for genetic resources conservation. Pp. 511-515 in Guarino, L., Rao, V.R., and R. Reid, (eds). *Collecting Plant Genetic Diversity*. CAB International, Wallingford, UK.

Withers, L.A. and P.G. Alderson. (eds.) 1986. *Plant Tissue Culture and its Agricultural Applications*. Butterworths, London.

Yamada, T., A. Sakai, T. Matsumura, and S. Higuchi. 1991. Cryopreservation of apical meristems of white clover (*Trifolium repens* L.) by vitrification. *Plant Sci.* 78:81-87.

Yidana, J.A., L.A. Withers, and J.D. Ivins. 1987. Development of a simple method for collecting and propagating cocoa germplasm *in vitro*. *Acta Horticulturae* 212: 95-98.

Zandvoort, E.A., Hulshof, M.J.H. and Starisky, G. 1994. *Plant Cell, Tissue and Organ Culture* 36:309-316.

Zettler, F.W., Jackson, G.V.H. and Frison, E.A. (eds.). 1989. *FAO/IBPGR Technical Guidelines for the Safe Movement of Edible Aroid Germplasm*. Food and Agriculture organization of the United Nations, Rome/International Board for Plant Genetic Resources, Rome.

Zamora, A.B. and C.N. Paet. 1996. *In vitro* genebanking activities (Institute of Plant Breeding, college of Agriculture, University of the Philippines at Los Baños). Consultation on the management of field and *in vitro* genebanks. FAO/IPGRI.

IV APPENDICES

Appendix A. Example of a record keeping system for a genebank (NCGR).

These are the type of fields used in the database for the U.S. Germplasm System. Local information is electronically transferred to the national database (GRIN) so some fields are used to coordinate that transfer.

Structure for accession database:

Field	Field Name	Type	Width	Function of Field
1	ACTION	Character	1	Action to be done on GRIN
2	ACCPREFIX	Character	4	Accession prefix
3	ACCNO	Numeric	7	Accession number
4	CLOCAL	Character	4	Local accession number
5	LOCAL	Numeric	4	Local number
6	GENUS	Character	30	Genus name
7	SPECIES	Character	30	Species name
8	SUBSPECIES	Character	30	Subspecies name
9	VARIETY	Character	30	Botanical variety name
10	FORMA	Character	30	Botanical form name
11	TAXON	Character	100	Scientific name
12	TAXAUTHOR	Character	100	Authority
13	TAXNO	Numeric	8	Code number for GRIN
14	COMNAME	Character	30	Common name
15	PLANTIDACT	Character	1	Action on plant ID for GRIN
16	PLANTID	Character	40	Plant identity
17	IDTYPE	Character	10	Type of identification
18	IDRANK	Numeric	5	Rank identified (species, cultivar)
19	TOPIDFLAG	Character	1	All similar plants should have this
ID				
20	IDNOTE1	Character	60	Notes
21	IDNOTE2	Character	60	
22	IDNOTE3	Character	40	
23	OTHERID	Character	1	Other identifications exist
24	IDRECNO	Numeric	8	Identification record number
25	RESTRICT	Character	10	Biologically or legally restricted
26	SITE	Character	6	Location of accession records
27	WHYNULL	Character	10	Why this accession is no longer valid
28	CORE	Character	1	Part of the core collection Y or N
29	BACKUP	Character	1	Is there a second plant Y or N
30	BIOFLAG	Character	1	Is it from a foreign country Y or N
31	LIFEFORM	Character	10	Annual or perenial growth form
32	FORMREC	Character	2	Form received - plant, seed, <i>in vitro</i>

33	IMPRVSTAT	Character	10	Cultivar or wild
34	DATEREC	Character	10	Date received
35	DATECOL	Character	10	Date collected
36	AMTREC	Character	15	Amount received
37	DVLPACT	Character	1	Action by grin on developer
38	DVLPCODE	Character	6	Developer code
39	DVLPNAME	Character	65	Developer name
40	DVLPORG	Character	60	Developer organization
41	DVLPADD1	Character	60	Developer address
42	DVLPADD2	Character	60	
43	DVLPADD3	Character	60	
44	DVLPCITY	Character	20	Developer city
45	DVLPSTATE	Character	20	Developer state
46	DVLPZIP	Character	10	Postal code
47	DV LPCNTRY	Character	30	Developer country
48	DVLP HIST1	Character	60	History of development
49	DVLP HIST2	Character	60	
50	DVLP HIST3	Character	60	
51	DVLP HIST4	Character	60	
52	DVLPORIG	Character	1	Developer is the origin
53	OTHERDVLP	Character	1	Are there other developers? Y or N
54	DVLP SRCNO	Numeric	8	Developers country code numbers
55	DONORACT	Character	1	Action to be taken at GRIN on donor
56	DONORCODE	Character	6	Donor code
57	DONORNAME	Character	65	Donor name
58	DONORORG	Character	60	Donor organization
59	DONORCITY	Character	20	Donor city
60	DONORSTATE	Character	20	Donor state
61	DONORCNTRY	Character	30	Donor country
62	DONORHIST1	Character	60	Donor history information
63	DONORHIST3	Character	60	
65	DONORHIST4	Character	60	
66	DONORORIG	Character	1	Donor is the origin
67	OTHERDONOR	Character	1	Other donors of this material
68	DONOR SRCNO	Numeric	8	Donor country code
69	COLLACT	Character	1	
70	COLLCODE	Character	6	Collector's code
71	COLLNAME	Character	65	Collector's name
72	COLLCITY	Character	20	Collector's city
73	COLLSTATE	Character	20	Collector's state
74	COLLCNTRY	Character	30	Collector's country
75	COLLHIST1	Character	60	Collection history
76	COLLHIST2	Character	60	
77	COLLHIST3	Character	60	

78	COLLHIST4	Character	60	
79	COLLORIG	Character	1	Collector is the origin of this plant
80	OTHERCOLL	Character	1	Other collectors Y or N
81	COLLSRCNO	Numeric	8	Collection country code number
82	PEDACT	Character	1	Pedigree action needed on GRIN
83	PEDIGREE1	Character	60	Pedigree information
84	PEDIGREE2	Character	60	
85	PEDIGREE3	Character	60	
86	PEDIGREE4	Character	60	
87	DATEDEV	Character	10	Date developed or released
88	GEOACT	Character	1	Geography needed on GRIN
89	LATDEG	Character	2	Degrees latitude
90	LATMIN	Character	2	Minutes latitude
91	LATSEC	Character	2	Seconds latitude
92	LATHEM	Character	1	Hemisphere
93	LONGDEG	Character	3	Degrees longitude
94	LONGMIN	Character	2	Minutes longitude
95	LONGSEC	Character	2	Seconds longitude
96	LONGHEM	Character	1	Hemisphere
97	GEOTYPE	Character	3	Map or satellite location
98	ELEVATION	Character	5	Elevation of collection
99	HABITAT1	Character	60	Habitat information
100	HABITAT2	Character	60	
101	HABITAT3	Character	60	
102	HABITAT4	Character	60	
103	LOCALITY1	Character	60	Where collected, place names, etc.
104	LOCALITY2	Character	60	
105	LOCALITY3	Character	60	
106	LOCALITY4	Character	60	
107	COLLNOTES1	Character	60	Collection notes
108	COLLNOTES2	Character	60	
109	COLLNOTES3	Character	60	
110	COLLNOTES4	Character	60	
111	QUARACT	Character	1	Quarantine activity on GRIN
112	QUARTYPE	Character	10	Quarantine type
113	QUARSTATUS	Character	10	Quarantine status
114	DATEINQ	Character	10	Date in quarantine
115	DATEOUTQ	Character	10	Date out of quarantine
116	QUARSITE	Character	8	Quarantine site
117	QUARCMT	Character	60	Comments
118	QUARCMT2	Character	60	
119	CULTSYN	Character	60	Cultivar synonyms
120	TRANSLATE	Character	60	Translation from original language
121	WHYNAMED	Character	60	Reason for the cultivar name

122	NAMEDFOR	Character	60	Cultivar named for someone
123	LOCNAME	Character	60	Local name of plant
124	IPRACT	Character	1	International Property Rights Y or N
125	IPRTYPE	Character	10	Type of IPR
126	IPRID	Character	40	IPR identification
127	IPRNAME	Character	60	IPR name
128	IPRISSUE	Character	10	IPR reason
129	IPRASSIGN	Character	65	IPR assignment
131	IPRNOTE	Character	60	Notes
132	IPRNOTE2	Character	60	
133	IPRNOTE3	Character	60	
134	NOTESACT	Character	1	Activity needed on GRIN for notes
135	NOTES1	Character	65	Notes of interest
136	NOTES2	Character	65	
137	NOTES3	Character	65	
138	NOTES4	Character	65	
139	NOTES5	Character	65	
140	NARRATIVE	Memo	10	Additional information
141	CITACT	Character	1	Citation activity needed on GRIN
142	CITABRNAME	Character	20	Abbreviation of citation
143	CITTITLE	Character	60	Title of citation
144	CITTITLE2	Character	60	Second title cited
145	CITAUTHOR	Character	60	Author of citation
146	CITAUTHOR2	Character	60	Author of second title
147	CITYEAR	Character	4	Year of citation
148	CITREF	Character	60	Reference of citation
149	CITNOTES1	Character	60	Notes
150	CITNOTES2	Character	60	
151	CITNOTES3	Character	60	
152	CITNO	Numeric	8	Citation number
153	INFOSHEET	Numeric	4	Location of information sheet
154	ACID	Numeric	8	Accession identifier for GRIN
155	OLDPINUMB	Numeric	7	Old plant introduction number

Appendix B. Accession inventory database information for NCGR (Foxpro database).

Structure for local inventory database:

Field	Field Name	Type	Width	Function of Field
1	ACTION	Character	1	Action to be done on GRIN
2	ACCPREFIX	Character	4	Accession prefix
3	ACCNO	Numeric	7	Accession number
4	CLOCAL	Character	4	Local accession number
5	LOCAL	Numeric	10	Local accession number
6	STORECODE	Character	2	Form of plant (plant, seed, <i>in vitro</i>)
7	AVAILABLE	Character	4	Available for distribution Y or N
8	REASON	Character	4	Reason if not available
9	FLAG	Character	7	Special distribution conditions
10	DATEREC	Character	10	Date received
11	DATEQREL	Character	10	Date released from quarantine
12	G100WT	Numeric	4	Weight of 100 seeds
13	AMOUNT	Numeric	7	Number of seeds received
14	PLANTNAME	Character	65	Plant name
15	OTHERNUM	Character	65	Collector or breeder numbers
17	VIRSTATUS	Character	10	Virus status
18	ELISA	Character	65	ELISA virus test results
19	BIOASSAY	Character	65	Bioassay virus test results
20	VERIFYCODE	Character	10	Verified Y or N
21	VERIFYDATE	Character	10	Date verified
22	VERIFYNOTE	Character	65	Verification notes
23	PERCODE	Character	6	Donor code
24	COLDHARDY	Character	3	Cold hardy Y or N
25	GHLOCATE	Character	8	Greenhouse bench location
26	GHNO	Numeric	2	Greenhouse number
27	SHLOCATE	Character	8	Screenhouse bench location
28	SHNO	Numeric	2	Screenhouse number
29	SYPL	Character	4	Year planted
30	SHLABEL	Character	1	Needs a label, Y or N
31	TBLOCATE	Character	8	Tubehouse location
32	TBNO	Numeric	2	Tubehouse number
33	FIELD1	Character	5	Field location for 1st replicate
34	FYPL1	Character	4	Year 1st replicate planted
35	FIELD2	Character	5	Field location for 2nd replicate
36	FYPL2	Character	4	Year 2nd replicate planted
37	FIELDLABEL	Character	1	Needs a label, Y or N
38	TC	Character	4	Plant available <i>in vitro</i>
39	TCGRIN	Character	1	<i>In vitro</i> entered on GRIN Y or N
40	CROPTYPE	Character	20	Subgrouping within a genus for crop

type				
41	TRAITS	Character	40	Notes
42	INFOSHEET	Numeric	4	Location of information sheet
43	OLDPINUMB	Numeric	7	Old plant introduction number
44	OLDLOCAL	Numeric	7	Old local number
45	PREVTAXON	Character	65	Former taxon if changed
46	NOTE	Character	65	Notes on taxon changes

Appendix C. Basic CEC apple descriptors, passport data (IBPGR).

1. Accession Data

- 1.4 Other Numbers
 - 1.4.1 EC number
- 1.5 Scientific Name
 - 1.5.1 Genus 1.5.2 Species 1.5.3 Subspecies
- 1.6 Pedigree of Accession
- 1.12 Country where maintained
- 1.13 Site where maintained
- 1.15 Local name
- 1.16 Local clone/mutant/variant name
- 1.20 Genetic name
- 1.21 GRS clone/mutant/variant name

2. Collection Data

- 2.4 Country of collection or country where cultivar/variety was bred
- 2.17 Virus disease status
- 2.18 End use

Characterization and Preliminary Evaluation data

3. Site Data

4. Plant Data

- 4.1 Vegetative
 - 4.1.1 Propagation method 4.1.2 Chromosome number
- 4.2 Inflorescence and fruit
 - 4.2.1 Harvest maturity 4.2.2 Maximum storage life

Further Characterization and Evaluation

5. Site Data

6. Plant Data

- 6.1 Vegetative Rootstocks and/or interstocks
 - 6.1.6 Efficiency of mineral uptake
 - 6.1.6.2 Calcium
 - 6.1.7 Dwarfing
 - 6.1.12 Induction of precocious bearing in scions
- 6.2 Inflorescence and fruit scions
 - 6.2.19 Eating maturity

7. Stress susceptibility

8. Pest and disease susceptibility

9. Alloenzyme composition

10. Cytological characters and identified genes

11. Notes

Appendix D. Example of a plant distribution policy statement.

NCGR - Corvallis Distribution Policy Statement 8 December 2003

**Dr. Kim Hummer, Research Leader/Curator
Bruce R. Bartlett, Plant Distribution Manager**

The National Clonal Germplasm Repository (NCGR) at Corvallis complies with the USDA/ARS release policy to encourage continued free and unrestricted national and international exchange of germplasm for bona fide research.

The NCGR is a relatively small USDA/ARS facility responsible for safeguarding the preservation of 25 genera of temperate fruit and nuts. The germplasm is stored primarily as plants and seed. In most cases, two stock plants of each clone are maintained for distribution. Therefore, only a limited quantity of cuttings, scionwood, or plants is available each year for any single accession. Normally, two plants or cuttings, or 25 seeds, are provided for each accession requested. Larger quantities should be requested through commercial sources. Requests in excess of 10 accessions may be considered if justification is provided.

We ask that a statement concerning the intended use for the germplasm be provided by the requester. Timely, seasonal requests for bona fide research interests are distributed with highest priority. Requesters seeking plant material in exchange or because the collection at NCGR is the sole source are assigned a secondary priority. Other requests are honored as plant material is available. Amateur fruit growers: Please seek commercial or other scionwood sources BEFORE requesting material from the repository.

We would appreciate any research publications resulting from the use of the plant materials acknowledging NCGR-Corvallis as the supplier.

Germplasm with propagation restrictions, such as patents, plant variety protection, marketed under trademark, or propagation rights, is distributed by NCGR, an agent of the federal government, for research purposes only. Anyone wanting such germplasm for other use must contact the restriction holder directly.

Efforts are made by the NCGR staff to verify the identity of plant accessions, test for important diseases, and evaluate clonal qualities. Virus negative germplasm will be distributed whenever possible. NCGR will provide a written summary of identity verification and virus testing results for plant material for any requester. Historical information, including passport data, pedigree and development data, and previous evaluations, is also available. NCGR assumes no responsibility for identification or performance of distributed plant material.

Because limited plant material is available, orders frequently cannot be completely filled. Some

items may be marked as not available (NA) at the present time. A new request for these items should be submitted at a later date.

Foreign Requests

NCGR distributes germplasm to foreign requesters in compliance with federal quarantine regulations and restrictions of the United States and the recipient country. The requester MUST provide an import permit (IP), when required, with English translation. If possible, the IP should be open dated. This assists us in resending material that did not survive original shipment. Our USDA plant inspector can provide the phytosanitary certification only after we have received the required IP from the requesting country. Whenever possible, please send or FAX a copy of the IP with the original plant request.

Inquiries or requests for germplasm should be directed to: Curator, NCGR-Corvallis, 33447 Peoria Road, Corvallis, Oregon 97333, USA telephone (541) 738-4200, FAX (541) 738-4205, E-Mail: corkh@ars-grin.gov.

Domestic orders are sent First Class/Priority Mail. Foreign orders are sent International Air Mail or by courier through specific arrangement. Pre-paid shipping by the requester, whenever possible, is greatly appreciated.

When ordering, please group your order by genus, and provide us the following information:

Name, Address, Phone, FAX, E-Mail.

Statement of research interest.

Plant name and, where possible, local number.

Copy of Import Permit (with English translation), where applicable.

Do you have any special needs or conditions?

Timing? Packaging?

Will you accept Virus negative material only?

May we substitute duplicate clonal germplasm from a different source of a similar seedlot in the case of species material?

Do you have special postal or shipping requirements?

Appendix E. Plant distribution schedule from USDA/ARS Operations Manual (Hummer, 2003).

SCHEDULE OF PLANT AVAILABILITY: Plant material is generally available according to the schedule listed below. To expedite your order, **please request material six months to one year in advance.** Late requests can sometimes be accommodated, but propagules may be of inferior quality. **Spring bud break usually occurs in early to mid-February**, which limits dormant cuttings and scionwood distribution. Pollen and other propagules may be available by specific request. Information on plant evaluation after establishment by the recipient should be reported to the NCGR for entry into the Germplasm Resource Information Network (GRIN).

Genus	Seed	Scionwood	Budwood	Cutting	Misc.	<i>In vitro</i>
<i>Corylus</i>	Oct-Dec	January	----	----		Oct-Nov (layers) Few
<i>Fragaria</i>	All year	----	----	Jun-Sept (runners)	----	Yes
<i>Humulus</i>	All year	----	----	----	Jan-Feb (rhz.ctng)	Yes
<i>Mentha</i>	All year	----	----	----	All year (rhz.ctng)	Yes
<i>Pyrus</i>	All year	Dec-Jan	Aug-Sept	----	----	Yes
<i>Ribes</i>	All year	----	----	May-June (softwood) Dec-Jan (dormant)	----	Yes
<i>Rubus</i>	All year	----	----			
	Raspberry type			----	Dec-Jan (rt.ctng)	Yes
	Blackberry type				Sept-Nov (division)	Dec-Jan Yes
<i>Vaccinium</i>	All year	----	----	May-June (softwood) Dec-Jan (dormant)	----	Yes

Appendix F. Distribution procedures for plant germplasm (Hummer,2003).

Order Processing

- Request Received
 - By mail -GRIN
 - By phone -Information request
 - In person -e-mail
- Staff member writes request form
- Copy of letter, original import permit (IP), request form given to Distribution Technician (Dist. Tech.)
- Date received stamped by Dist. Tech.
 - Hard copy file prepared
 - requisition number assigned
- Data entered into:
 - Distwork.dbf
 - Dist96.dbf
 - GRIN order processing
- Confirmation (letter, fax, email) sent to requestor that order is pending
 - IP requested (when required)
 - Material Transfer Agreement paperwork sent (when required)
 - Shipping procedures determined in consultation with requestor
- Order shipment default
 - Domestic request=Priority Mail
 - Foreign request=International Airmail

Order Preparation

- Paperwork and computer file prepared
- In vitro* requests prepared by *In vitro* Technician and laboratory staff
- All other plant material prepared by Dist. Tech. in consultation with Plant Production Mgr. and Plant Pathologist
- Orders requiring a Phytosanitary Certificate (PC)
 - Dist. Tech. schedules inspection visit by APHIS
 - APHIS inspector (Ore. Dept. of Agric. inspector) contacted one week prior to inspection date and given information concerning the genus and country for each order
- Orders not requiring a PC (see Shipping)

Order Preparation

(interaction with APHIS plant inspector)

- In vitro* requests (whole or in part): One day prior to inspector's visit the *In vitro* Technician provides information concerning genera and specific amount of items per genus to Distribution
- All other requests: Dist. Tech. determines the specific amount of items per genus to be presented based on NCGR policy and consultation with Curator
- Dist. Tech. prepares Plant Inspection Summary Sheet from all requests to be inspected
- Material not likely to pass is removed in consultation with the Plant Pathologist
- APHIS inspector reviews plant material
 - If Import Permit regulations satisfied, a Phytosanitary Certificate (PC) is issued and Dist. Tech. ships plants
 - Plants not meeting requirements are removed from shipment and Dist. Tech. notifies requestor

Shipping

- All plant material (destination foreign or domestic) wrapped firmly to prevent potential damage
 - In vitro*: bubble wrap
 - Scions: moist towellette in plastic bag, scion ends waxed
 - Cuttings/Runners: moist towellette in plastic bag
- Domestic requests shipped U.S. Priority mail unless requestor has made other arrangements
- Foreign requests (no IP or PC required) shipped U.S. International Airmail unless requestor has made other arrangements (Customs sticker still required)
- Foreign requests requiring a PC shipped U.S. International Airmail unless requestor has made other arrangements
 - Customs sticker placed on outside of box
 - +Genus/Plantes pour recherche
 - +Value \$0.00
 - +Weight (to be determined)
 - Shipping pouch taped to outside of box (paperwork visible)
 - +Copy of IP (permit # visible)
 - +Copy of PC (certificate # visible)
 - +Copy of packing list
 - Duplicate copies of IP, PC and packing list to be inside of box

Paperwork Completion

- Database is updated by Dist. Tech.
- New pending list printed if request is not yet complete
- Arrange payment of PC's to ODA through purchase order
- Hard copies of packing list (completed and/or new pending) given to:
 - Distribution File
 - Computer Manager's File
 - In vitro* Tech. (when *In vitro*)

Appendix G. Sample plant tissue culture medium recipe sheets.

Example of tissue culture medium sheet from NCGR (Hummer, 2003).

MENTHA MEDIUM (MS based)

MULTIPLICATION

<u>Component</u>	<u>Conc./L</u>	<u>Amount Needed</u>
M & S Nitrates	10 ml	
M & S Sulfates	10 ml	
M & S Halides	10 ml	
M & S P,B,Mo	10 ml	
M & S Iron	10 ml	
M & S Vitamins	10 ml	
Sucrose	30 g	

Starpacks are 0 Hormone

Optional Peptone/Yeast for bacterial detection

Peptone	265 mg/l
Yeast	88 mg/l

Benzyladenine 0.5 mg/l 5 ml
(Stock sol. 10mg/100ml)

IBA 0.1 mg/l
1 ml (Stock sol. 10mg/100ml)

Bring to final volume and pH 5.7

Boxes and Tubes (Or agar only 6g)

Agar	3.0 g
and	
Gelrite	1.25 g

Star*packs (Or agar only 7g)

Agar	3.5 g
and	
Gelrite	1.45 g

Example of tissue culture medium sheet from CARDI (Bateson, personal communication).

CARDI TISSUE CULTURE LABORATORY

MEDIUM SHEET

Medium : Plantain Multiplication

Lab Code : BM-2

Quantity : 1 litre

Medium Constituents	To be added	DATE							
Stock soln. I	50 mls								
Stock soln. II	5 mls								
Stock soln. III	5 mls								
myo-inositol stock	12.5 mls								
Thiamine HCL stock	4 mls								
Pyridoxine HCL stock	5 mls								
Nicotinic acid stock	5 mls								
Glycine stock	20 mls								
BAP stock	50 mls								
Sucrose	30 grams								
Distilled water	Make up to 1 L								
Check pH	5.6 - 5.8								
Phytogel	2.8 grams								

Place tick in box as chemical added or activity completed.

Example of stock solution sheets from CARDI (Bateson, personal communication).

Stock solutions are made up monthly and stored at 2-8° C. The formulation of the macro and micro nutrients is as Murashige and Skoog (1962).

Stock soln. I contains	KNO ₃	1900 mg per 50mls
	NH ₄ NO ₃	1650 mg per 50mls
	CaCl ₂ .2H ₂ O	440 mg per 50mls
	MgSO ₄ .7H ₂ O	370 mg per 50mls
	KH ₂ PO ₄	170 mg per 50mls

Stock soln. II contains	MnSO ₄ .H ₂ O	22.3 mg per 5mls
	H ₃ BO ₃	6.2 mg per 5mls
	KI	0.83 mg per 5mls
	ZnSO ₄ .4H ₂ O	8.6 mg per 5mls
	Na ₂ MO ₄ .2H ₂ O	0.25 mg per 5mls
	CuSO ₄ .5H ₂ O	0.025 mg per 5mls
	CoCl ₂ .6H ₂ O	0.025 mg per 5mls

Stock soln. III contains	FeSO ₄ .7H ₂ O	27.85 mg per 5mls
	Na ₂ EDTA.2H ₂ O	37.25 mg per 5mls

myo-inositol stock soln.	=	8000 parts per million
Thiamine HCL stock soln.	=	100 parts per million
Pyridoxine stock soln.	=	100 parts per million
Nicotinic acid stock soln.	=	100 parts per million
Glycine stock soln.	=	100 parts per million
BAP stock soln.	=	100 parts per million



Appendix H. Protocols for plant tissue culture of *Fragaria* (Hummer, 2003).

a) Medium: Tissue culture plantlets of *Fragaria* are grown on medium containing MS salts and vitamins, sucrose 30 g/l, NaH₂PO₄ 170 mg/l, adenine sulfate 80 mg/l, BA 1 mg/l, IAA 1 mg/l, and GA₃ 0.01 mg/l at pH of 5.7 and solidified with 3 g/l agar and 1.25 g/l gelrite before autoclaving.
-Growth room conditions are 16 h photoperiod (25 mmol·s⁻¹·m⁻²) at 25°C.

b) Initiation/surface sterilization: *Fragaria* explants are taken from recently formed runners on plants in the greenhouse collection. Plantlets are surface sterilized by placing them in a 10% bleach solution (bleach is 5.25% sodium hypochlorite) with 0.1 ml/l Tween 20 and shaken on a rotary shaker for 10 minutes. Explants were then removed and rinsed twice with sterile water.

To detect internal contamination, explants are placed in 1/2 strength liquid MS medium with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.7. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group are all contaminated, consider antibiotic treatment (Tanprasert MS Thesis, 1996).

Multiplying plants suspected of contamination are streaked on petri plates containing a bacterial detecting medium (Viss, *et al.* 1991. *In vitro* 27P:42) containing sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, KH₂PO₄ 2 g/l, MgSO₄·7H₂O 0.15 g/l, and agar 8 g/l. The pH is adjusted to 6.9 before autoclaving. The base of each explant is streaked on the plate before planting in 16 mm tubes containing 5 ml multiplication medium. The plates are incubated for 48 hours at 24°C. Plantlets showing contamination on the plates are discarded.

c) Multiplication: Plantlets are multiplied on base medium as stated above.

d) Rooting: Healthy plants are rooted on regular base medium without hormones.

e) Additional information: Plantlets to be stored are placed on medium without hormones inside plastic tissue culture bags (medium with agar 3.5 g/l, Gelrite 1.45 g/l). After a cold hardening period, the bags are then placed in a storage room at 4°C in low light.

-Some plants in the greenhouse collection do not produce very many runners. A 500 ppm GA₃ spray combined with long day conditions has been helpful in inducing runnering in these plants.

Appendix I. Record keeping system for *in vitro* storage at NCGR (Reed, personal communication).

<u>Field Name</u>	<u>Field type</u>	<u>Width</u>	<u>Reason for field(Dbase IV)</u>
Local number	Numeric	8	Local identifying number
Former local number	Numeric	4	Old numbering system
Core	Logical 1		Is it part of the core collection
Plant name	Character	45	Cultivar or scientific name of plant
Other location	Character	12	Is it also stored in another location
Invitro	Logical 1		T or F , used for printing lists
Instorage	Logical 1		T or F, used for printing lists
Label	Logical 1		T or F, used for making labels
Explant	Date	8	Date explant taken
Multiplication medium	Character	12	Type of multiplication medium
Coldstore	Date	8	Date placed in cold storage
Store25C	Date	8	Date placed in warm storage
Reprop1	Date	8	Date removed for repropagation
Restore 1	Date	8	Date returned to storage (1st time)
Reprop2	Date	8	Date removed for repropagation
Restore 2	Date	8	Date returned to storage (2nd time)
Reprop3	Date	8	Date removed for repropagation
Restore 3	Date	8	Date returned to storage (3rd time)

Appendix J. Contamination and explant indexing for *in vitro* cultures.

Contamination detection for explants and multiplying plantlets at NCGR (Hummer,2003).

All explants are now initiated in an enriched liquid medium as follows:

a) Initiation/surface sterilization: explants are taken from recently formed runners or shoot tips of plants in the screenhouse collection. Shoots may also be forced from field grown branches, but should be treated for insects (Thrips, mites) with dormant oil or chemical sprays before forcing. Forced shoots should be placed in water with Floralife (florist mix) to inhibit bacterial growth. Plantlets are surface sterilized by placing them in a 10% bleach solution (bleach is 5.25% sodium hypochlorite) with 0.1 ml/l Tween 20 and shaken on a rotary shaker for 10 minutes. Explants are then removed and rinsed twice with sterile water.

b) To detect internal contamination, explants are placed in 1/2 strength liquid MS medium (salts and sucrose only) with 256 mg of peptone and 88 mg of yeast extract and a pH of 6.7. Contamination will look like cloudiness or flocculent growth in the medium. Use standard growing conditions in these steps. If no contamination shows after one week then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group are all contaminated, consider antibiotic treatment (Reed *et al.*, 1995 or P. Tanprasert, MS Thesis, 1996). Some plants do not tolerate submersion in liquid medium so they should be only partially submerged and shaken occasionally to rinse contaminants from the surface into the medium.

c) Multiplying plants suspected of contamination have their bases streaked on petri plates of 523 bacterial detection medium (Viss, *et al.* 1991. *In vitro* Cellular and Developmental Biology - Plant 27P:42) containing sucrose 10 g/l, casein hydrolysate 8 g/l, yeast extract 4 g/l, KH_2PO_4 2 g/l, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.15 g/l, and agar 8 g/l. The pH is adjusted to 6.9 before autoclaving. The base of each explant is streaked on the plate before planting in 16 mm tubes containing 5 ml multiplication medium. The plates are incubated for 2-10 days at 24°C. Plantlets showing contamination on the plates are discarded. Some latent bacteria may not be visible for several weeks. Plates should be checked with a dissecting microscope or a hand lens to verify the presence or absence of bacteria.

Detection of endophytic bacteria in banana tissue cultures at INIBAP (Van den houwe, personal communication).

Two media were found to be useful for indexing banana shoot tip cultures at INIBAP *Musa* Transit Center.

1. Bacto Nutrient agar supplemented with 1% glucose and 0.5% yeast extract at pH 7
2. 523 Medium : 10 g/l sucrose, 8 g/l casein hydrolysate, 4g/l KH_2PO_4 , 0.15 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 8 g/l agar at pH 6.

Appendix K. Antibiotic treatments for *in vitro* cultures (Reed et al., 1995).

At NCGR we have treated some bacterially contaminated *in vitro* plants with antibiotics. We only treat these plants because we are unable to get clean explants. The first choice is a clean explant. Ideally the plant chosen for treatment is newly collected, not one from a liquid culture of the bacterium or setting in a pool of bacteria. The fewer bacterial cells involved, the easier it is to get a clean plant.

The first steps toward a treatment are the antibiotic test on the bacterium and the phytotoxicity test of the plant. Once the antibiotic is chosen, the treatment concentration must be determined. This is often 4-10 X as much as the minimal bactericidal concentration (MBC). The upper concentration of antibiotic may be determined by its phytotoxicity. Combinations of antibiotics usually work better than singles, but you must be sure they are not antagonistic. Some are synergistic and are effective when two are combined but not individually. This can be determined through disc tests on agar plates.

It is also important in treating the plant that you use a small plant piece. The larger the explant, the more difficult it is to get the antibiotic to the bacteria. Usually a plant piece less than 1 cm long is best for treatment. In some cases apical segments survive treatment better than nodal segments.

Treatment design should be tailored to the plant and the phytotoxicity of the antibiotic. Some plants will withstand submersion in the antibiotic, others must be partially exposed or they die. The length of time in the antibiotic may range from 2-14 days. This will also depend on the plant response to treatment. Very short treatment times do not allow for movement of the antibiotic into the plant. Only bactericidal compounds should be used, bacteriostatic compounds will not provide lasting effects. Always test the antibiotic on or in the same medium you will use to treat the plant culture.

After treatment you must monitor each plant for bacteria for at least six months. It will take time to determine if the plants are really bacteria free. It may take a long time for a few bacteria to multiply to the point where they are noticeable to your detection method.

Appendix L. Inventory data taken for *in vitro*-stored cultures.

Data taken for cassava and yam at CARDI (Bateson, personal communication).

	MONITORING CODE					
	0	1	2	3	4	5
Height up tube		1/4	1/2	3/4	Full	
Leaf browning		10%	25%	50%	75%	Full
Root Develop.	None	small amount 2-3	moderate many roots	numerous roots		
Aerial roots	None	small amount	moderate amount	many		
Media browning	None	pale brown small amount around base of shoot	pale brown moderate amount around base of shoot	dark brown/black moderate amount around base of shoot	dark brown/black large amount	
Bacteria	None visible	faint halo in some	faint halo in all	distinct halo in some	distinct halo in all	heavily contaminated
Subculture	ASAP	1-2 months	2-3 months	to be assessed at a later date		

Appendix L. Inventory data taken for *in vitro*-stored cultures.

Data taken for *Corylus*, *Fragaria*, *Humulus*, *Mentha*, *Pyrus*, *Ribes*, *Rubus*, and *Vaccinium* at NCGR (Reed, personal communication).

<u>Field name</u>	<u>Field type</u>	<u>Width</u>	<u>Reason for field</u>
Genus	Character	10	Genus identification
Local number	Numeric	8	Identifying number
Storedate	Character	5	Date put in storage
Cells alive	Numeric	1	1-5 plants living
Total cells	Numeric	1	1-5 cells planted
Contaminated	Numeric	1	Number contaminated
Rating	Numeric	1	Condition rating 0 dead 5 excellent
Comment	Character	10	Other information

Plants rated 0, 1, or 2 are removed for subculture. Experimental materials may have additional evaluations included during inventory.

RATING SCALE

0	Dead, brown	3	etiolated, medium-green color
1	etiolated, pale, no green color	4	not etiolated, medium-green color
2	etiolated, pale green color	5	not etiolated, dark green

Appendix M: INIBAP germplasm health statement.

ITC Accession Number:

Accession Name:

Origin of Accession:

The material designated above was obtained from a shoot-tip cultured *in vitro*. Shoot-tip culturing is believed to eliminate the risk of the germplasm carrying fungal, bacterial, and nematode pathogens and insect pests of *Musa*. However, shoot-tip cultures could still carry virus pathogens.

SCREENING FOR VIRUS PATHOGENS

A representative sample of four plants derived from the same shoot-tip as the germplasm designated above has been grown under quarantine conditions for at least 6 months, regularly observed for disease symptoms and tested for virus pathogens as indicated below following methods recommended in the FAO/IPGRI Technical Guidelines for the Safe Movement of *Musa* Germplasm for the diagnosis of virus diseases.

Serology-ELISA	<input type="checkbox"/> BBTV- Banana bunchy top virus
	<input type="checkbox"/> CMV- Cucumber mosaic virus
	<input type="checkbox"/> BBMV- Banana bract mosaic virus
	<input type="checkbox"/> BSV- Banana streak virus
Electron microscopy	<input type="checkbox"/> Isometric virus particle - includes CMV
	<input type="checkbox"/> Bacilliform virus particle - includes BSV
	<input type="checkbox"/> Filamentous virus particle - includes BBMV

[P] = test positive, [N] = test negative, [] = test not undertaken.

DISTRIBUTION OF VIRUS PATHOGENS AND OTHER INFORMATION

(Example: BBTV and BBMV are not known to occur in country of origin)

The information provided in this germplasm statement is based on the results of tests undertaken at INIBAP's Virus Indexing Centers by competent virologists following protocols current at the time of the test and on present knowledge of virus disease distribution. However, neither INIBAP nor its Virus Indexing Center staff assume any legal responsibility in relation to this statement.

Signature

Date

This statement provides additional information for the official Phytosanitary Certificate issued by the plant quarantine authorities of Belgium.