IPGRI Handbooks for Genebanks No. 7

Technical guidelines for the management of field and *in vitro* germplasm collections

B.M. Reed, F. Engelmann, M.E. Dulloo and J.M.M. Engels

SGRP

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PREFACE

Plant species that are vegetatively propagated, that have long life cycles and/or produce nonorthodox 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. Further, 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 strategies and procedures for managing *in vitro* collections as routine and integral part of the overall conservation strategy of a crop genepool or collection.

The International Plant Genetic Resources Institute (IPGRI), on behalf of the System-wide Genetic Resources Programme (SGRP), with 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 at 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 input to this document.

These Guidelines are divided into 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 subsections. The first subsection, 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 subsection deals with *in vitro* collections. The laboratory and storage facilities required for the establishment and maintenance of *in vitro* collections are presented and the need for the establishment of detailed standard operational procedures is 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 are identified aiming at improving *in vitro* conservation of plant germplasm collections.

Boxes are included throughout the document to illustrate the various points addressed in the text through concrete examples of procedures developed by genebank curators, or to provide detailed information on specific subjects. Similarly, 13 appendixes 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.

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1. GENERAL CONSIDERATIONS FOR THE ESTABLISHMENT AND MANAGEMENT OF GERMPLASM COLLECTIONS

1.1 Establishment of the germplasm collection

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.

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 need 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; 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 1978). A single genotype might vary in phenotypic response from farm to farm because of 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 the 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, horti-culturalists, 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:

- 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 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 programmes,
- the potential range of genetic diversity available elsewhere,
- 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.

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 genepool, containing the related species that will not cross with the primary genepool under normal conditions, but may be utilized with extreme measures, e.g. embryo rescue and 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, postharvest 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.

1.2 Acquisition and entry of plants into the collection

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

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).

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. Seeds 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 sub-zero temperatures and on the development of handling systems before practical storage is achieved (Towill and Roos 1989).

Cultivars and rootstocks

Clones should represent known diversity, not all known cultivars. For domestic clonal cultivars, IPGRI has suggested that 250-500 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, IPGRI 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 labelling/numbering and record-keeping system (Appendixes 1 and 2)

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 backup should depend on the regularity of updating of the primary database. Many mainframe databases 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 that 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 reassigned 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 assign 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 (Appendixes 1-3). Options exist for registering new accessions: (i) register all plants as new accessions and verify identities, etc. later; and (ii) 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 3.3) 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 31).

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) (nonbinding). 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 that 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 notably found 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. Labelling, numbering, record-keeping systems and standard log-in procedures allow curators to easily track all accessions and link them with passport data.

1.3 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 1996a,b; 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 Box 9, page 31). 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 and 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 that 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 that may impair plant vigour, 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 in 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 (1996a,b) 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 antiserum for specific viruses. Curators should consider the health status and susceptibility of the plant when deciding how to conserve it.

1.4 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.

Box 1. Comparison of conservation options for coconuts (from Dulloo *et al.* 2005)

Type of material	*In situ* on farm/home gardens/natural habitats	Botanical gardens (living plants in gardens/greenhouses)
Mature plants	✓ Coconuts conserved widely on farm and in home gardens and natural stands exist on small isolated islands and atolls	✓ Occurs in botanic gardens but limited scope for conserving genetic diversity
Seeds and zygotic embryos	x Not feasible—seeds are recalcitrant, no natural soil seed banks	x Not feasible
Somatic embryos	x Not applicable	x Not applicable
Pollen	x Not applicable	x Not applicable
Apices	x Not applicable	x Not applicable
DNA	x Not applicable	x Not applicable

The costs, availability for pathogen eradication, distribution, collecting and safety duplication are also considered.

Importance of complementary methods

It is now well recognized that an appropriate conservation strategy for a particular plant genepool 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 genepool 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

'Conventional' genebanks (seed banks, field genebanks)	**Slow-growth conditions (short term)**	**Cryopreservation—liquid N (long term)**
✓ Field genebank is the most widely used conservation method so far. National and international coconut field genebanks exist	x Not applicable	x Not applicable
x Seeds are recalcitrant and too large; seed conservation not feasible	✓ Field collecting protocol established for zygotic embryos; *in vitro* culture functional	✓ Cryopreservation protocol has been established for zygotic embryos; suitable for long-term conservation
x Not applicable	x Mass propagation problematic. Not applicable	x Not applicable
✓ Possible, short-term conservation (2–6 months)	x Not applicable	✓ Coconut pollen can be cryopreserved and could be suitable for long-term conservation
x Not applicable	x Not applicable	✓ Cryopreservation protocol established; relatively low survival and regeneration of plants very difficult
✓ Storage as DNA libraries exists—value not known	x Not applicable	✓ Long-term storage possible (LN or –80°C freezer). Use of stored DNA questionable

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.* (2005) discuss the considerations and provide 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 that 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 mislabelling. 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

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, 5 to 10 for cassava, 10 to 12 for sweet potato, two to three for garlics, trees and shrubs, 6 to 10 for herbaceous plants, and 3 to 20 for bananas. Space and labour are the limiting factors. Plant characterization requires fewer plants, while 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 3 to 20, depending on the particular crop.

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 because of 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.

Safety duplication methods

Duplication using field plots is best done at noncontiguous 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

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 ministorage 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)

collections kept for active use. Seeds 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.

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 that commonly produce variants since they are more easily identified and rogued in the field than *in vitro*.

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.

Box 4. Advantages of germplasm conservation methods

Character	Field	Slow growth *In vitro*	Cryo
Medium term	+	+	–
Long term	–	–	+
Characterize	+	–	–
Evaluate	+	–	–
Virus elimination	–	+	–
Distribution	+	+	+/–
Base collection	+	+	–
Core collection	+	+/–	–
Safety duplication	+	+	+

(+= applicable; -= not applicable)

If cryopreservation is available, it can provide a secure backup at little continuing operational cost. Initial costs appear high, but cryopreservation procedures fit well with an *in vitro* facility and require few additional expenditures other than labour. 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 labour 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.

1.5 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 3)

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

The identity of plant accessions 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 a continuing process and 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 mislabelling 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

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

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.

Characterize accessions (Appendix 3)

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 e*t 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.

Box 6. Descriptor lists available from IPGRI for 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	*Pistacia* spp.	Yam
Fig	Pistachio (*Pistacia vera* L.)	

www.ipgri.cgiar.org/publications

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: (i) disease resistance; (ii) insect and mite resistance; (iii) soil pest resistance and tolerance to soil environments; (iv) plant hardiness and tolerance to other environmental stresses; (v) plant size, shape and productivity; and (vi) crop quality and nutritional factors.

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 3500 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 1939 to 1161. (Huaman and Stegemann 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 and 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) that 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 on the basis of the country's importance as a centre 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 operation 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 a documentation of procedures to provide continuity during changes in staff. The manual should document all the steps that 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 that 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 labelling 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

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 (Hummer 2003). Specific Safe Standard Operating Procedures included:

Field, screenhouses and greenhouses
Application of pesticides
Calibration of orchard sprayer
Respirator use
Mixing of fertilizers
Application of sodium monoborate
Transport of soil
Soil pasteurization
Power equipment operation
Laboratory safety check list
Autoclave operation
DMSO use
Medium for tissue culture
Microtechnique for stains and dyes in cells
Sterile transfer procedures
Virus testing procedures
Cryopreservation protocols
Hazardous waste permit

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).

Distribute germplasm (Appendixes 4–6)

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 that cannot 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 intellectual property rights (such as Plant Breeder's Rights) regulations.

Conform to the 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 Organization 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 an yet-to-be-finalized 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 Treaty coming into force, the Centres will be invited to enter into an agreement with the 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 backups in case of a catastrophic loss.

Provide information 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 whenever 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 the 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.

1. General considerations for the establishment and management of germplasm collections
2. Procedures for establishing field and *in vitro* collections
2.1 Establishment of the collection
2.2 Acquisition and entry of plants into the collection
3. References

2. PROCEDURES FOR ESTABLISHING AND MAINTAINING FIELD AND *IN VITRO* COLLECTIONS

2.1 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.

Establish and maintain vigorous, healthy plants

Select appropriate propagation methods

Propagation is necessary for establishing and utilizing clonal germplasm, but owing 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 that root sprouts will replace those that 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 favour 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 that 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 harbour 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 an 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. As many accessions enter genebanks without evaluation data, it is often difficult for curators to know this information in advance. If the problems are soilborne, 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 for 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 all these diverse genotypes may not 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 because of vandals, theft, wars, volcanoes, hurricanes, floods, earthquakes, animals and wildfires. Fencing may exclude vandals and large herbivores. Firebreaks 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 that 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 vigour, 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 weed-borne 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) programme will decrease the 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 the greenhouses/screenhouses to decrease pesticide use.

Elimination methods for virus and other diseases vary with crop type. Roguing 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 that require annual harvests must be properly cured, labelled 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 labelling, 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 minisett 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, each 3-m 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, which is necessary to ensure that tubers are in good conditions. Using the above described procedures, 2500 accessions of different yam germplasm have been successfully maintained at IITA since 1990. (Ng and Ng 1999)

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.

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 that are variable. These should be noted in the

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)

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 that primarily produce seed apomictically. Species that intercross easily lose superior genotypes when cross-pollination is not controlled and seeds are used. Studies on seed physiology and reproductive biology would identify grasses that 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 behaviour (Sackville Hamilton and Chorlton 1997). Additional aspects on the management of germplasm at the accession level are presented in Sackville Hamilton *et al.* (2002).

Evaluate genetic stability: Monitoring collections for genetic stability is not an easy task. Each crop has specific descriptors that 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 labelling of plants

Avoid mixing propagules: Mixing can easily occur in fields where roots, stolons, rhizomes and 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 labelling 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.

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 *et al.* (2003)
Allium	Diekmann (1996)
Cassava	Frison and Feliu (1991)
Citrus	Frison and Taher (1991)
Cocoa	Frison and Feliu (1989)
Coconut	Frison *et al.* (1993)
Edible aroids	Zettler *et al.* (1989)
Eucalyptus	Ciesla *et al.* (1996)
Grapevine	Frison and Ikin (1991)
Musa	Frison and Putter (1989)
	Diekmann and Putter (1996)
Pinus spp.	Diekmann *et al.* (2003)
Potato	Jeffries (1998)
Small fruits	Diekmann *et al.* (1994)
Stone fruits	Diekmann and Putter (1996)
Sugarcane	Frison and Putter (1993)
Sweet potato	Moyer *et al.* (1989)
Vanilla	Pearson *et al.* (1991)
Yam	Brunt *et al.* (1989)

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*, pag. 62).

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 that 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 that 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.

Research needs in the field genebank

As discussed before, field genebanks are very costly to maintain and are, in general, very susceptible to losing accessions for a variety of reasons (Maxted *et al*. 1997; Dulloo *et al.* 2001; Engelmann and Engels 2002). 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 that reflect the main constraints that field genebanks face.

Germplasm health

Pest and diseases are the major problems affecting field genebanks. Accessions introduced from different locations may

Box 10. An example of research carried out at field genebanks

Coffee collection in CATIE, Costa Rica

CATIE manages one of the largest field genebanks of *Coffea arabica* in the world. A total of 8590 coffee trees representing 1997 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 location of the field genebank at 602 m above sea level in a humid zone of Costa Rica does not provide optimal conditions for cultivation of either *C. arabica* or other coffee species. The cultivation method has been similar to the method of commercial plantations and is 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 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 2523 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 1533 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 them on to vigorous rootstocks of *Coffea 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 a strategy of rationalization based on passport and evaluation data will reduce by half the area of conservation and thus the cost of maintenance. (Dulloo *et al.* 2001)

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.

Adaptation to environment
Genebank materials include accessions from a wide variety of origins that 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.

Taxonomy
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).

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.

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 define core collections for field collections, thereby reducing collection size and costs. Such research can also help 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.

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 need 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.

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, selection of 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 roguing, labelling 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.

2.2 *In vitro* procedures

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 the 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, 1996; Kyte and Kleyn 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), hotplates 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 light-weight 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 isolated 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 µmol s^{-1} m^{-2} but most plant cultures require 50–200 µmol s^{-1} 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 daylength 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 are needed to regulate temperature, but the air should not flow directly

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 (J m^{-2} s^{-1})
- ergs per square centimetre per second (erg cm^{-2} s^{-1})

or in terms of quantum units:
- moles of photons per square metre per second (mol m^{-2} s^{-1})
- microeinsteins per square metre per second (mE m^{-2} s^{-1})

1 erg cm^{-2} s^{-1}=1 x 10^{-3} W m^{-2}
1 mmol m^{-2} s^{-1}=1 mE m^{-2} s^{-1}
=0.215 W m^{-2}=0.215 J m^{-2} s^{-1}

Quantum units are particularly suitable for measuring light where its energy may be utilized for photo chemical 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 → mE m^{-2} s^{-1} for 'Warm white'divide by 83
lux → mE m^{-2} s^{-1} for 'Cool white'divide by 80
lux → mE m^{-2} 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 that 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).

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 cannot 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.

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.

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, Dordrecht, The Netherlands.

George, E.F. 1993. Plant Propagation by Tissue Culture—Part 1. Exegetics Limited, UK.

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

Kyte, L.A. and J. Kleyn. 2001. Plants from Test Tubes. An Introduction to Micropropagation (3rd edn). Timber Press, Portland, Oregon, USA.

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

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 that shuts off the unit and sounds an alarm if the temperature varies by a set amount will prevent cultures from freezing or overheating; 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 cleanup. 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 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 labour requirements, cost and chance for contamination of cultures. The longer the 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.

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 that 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 disinfestation 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 for those with plant science and micropropagation backgrounds. The head laboratory technician or lead scientist should be prepared to spend 1–2 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 that 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.

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

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—Parts 1 and 2. Exegetics Limited, UK.

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

reference books is important for developing and improving protocols (see Box 13).

Develop protocols (Appendixes 7 and 8)
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 9)
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 labelling 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 that it can be easily traced**.**

Labelling: All possible sources of labelling errors should be identified and minimized. Ideally, labels should be computer printed. This will avoid labelling 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.

Introduce plant material into tissue culture

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

Explants (Appendixes 10 and 11)

Origin of material: All plant material selected for tissue culture should be collected from vigorous, healthy mother plants, tubers, corms, etc. Plant tissue culture vigour 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, harbour 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 and 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 1999). 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.

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.

Box 14. Examples of *in vitro* collecting

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

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

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

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

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

Miscellaneous species: Pence, V. (1996).

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

Choice of materials: It is important to use properly identified material with passport data and, when it is available, with 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 disinfestation (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 because of 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 10–15%. 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. Oversterilization results in browning, spotting or death, while understerilization 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 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 cannot 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 from 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 because of 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 be used 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 that meets the growth requirements for all accessions is required.

Contamination indexing (Appendix 10): Explants should be placed on a medium that favours 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 the 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 (gelan 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 2–4 days for at least 1 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, together 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 11): 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, http://plant-tc.coafes.umn.edu/listserv/ 1996).

Precautions should be taken to protect staff members from pesticide exposure, for example, by spraying during weekends 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

Box 15. Control of mite and thrips infestations

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

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

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).

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 buildup 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.

Culture storage

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

Slow-growth storage (Appendix 12)

Slow-growth storage lowers the risk of losing germplasm through handling errors, such as contamination and media errors; decreases mislabelling; decreases the risks of genetic instability; cuts down on labour; 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, email 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-h 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 nonadapted genotypes were lost during the first subculture, while in others, few or none were lost during 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 to 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 in 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 colour, defoliation, browning or tip necrosis may be indications of lack of viability.

Pretreatments are often useful for improving storage duration. Cold acclimatization (1 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 *et al.* 1985).

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 that 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 that 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 3 years. During that time period, one to three of five replicates were lost for each accession. On the basis of 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	*Manihot*	5 individual tubes
INIBAP	*Musa*	20 individual tubes
NCGR	*Corylus, Fragaria, Mentha, Pyrus, Ribes, Rubus, Vaccinium*	10 individual sections of plastic bags
CARDI	*Dioscorea, Manihot*	10 individual tubes

Subculture regime: Subculture of stored material can be handled in several ways. Two suggested methods are as follows: 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 12): 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 that 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 and then schedule inventories to match that time frame. For example, if 3 months is the shortest storage time for any genotype in that group, schedule the group's inventory for 3-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 12): 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 that might provide useful information include the number stored, alive, dead and contaminated; an evaluation of the plants' condition (good, fair, poor); colour; defoliation; elongation; rooting; multiplication; callus; and medium discolouration.

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 infomally 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 that 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 that 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 10 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: (i) visually examine tissue culture plants; (ii) observe tissue culture plants in the field or greenhouse through an entire growth cycle to observe for any changes in morphology and then either perform more frequent testing of those genotypes that are observed to be prone to somaclonal variation or randomly select a sample for testing; (iii) use isozyme analysis, being aware that field and tissue culture samples will have different results; (iv) test using DNA techniques such as RFLP, RAPD or AFLP, again realizing that 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.

Cryopreservation

Long-term storage of clonally propagated plants requires the use of ultracold 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. LN is usually supplied by specialized companies. Alternatively, LN supplies may be available from local hospitals,

industry or artificial insemination centres. Small LN manufacturing plants may be purchased, which produce from 100 to 900 l per month.

Safety: Special safety considerations apply when LN 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, safety glasses and closed-top shoes (so LN cannot enter and be trapped on the foot). LN safety considerations should be included in the 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 e-mail 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, labour 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 seeds are stored in the vapour phase, but most meristems are stored in the liquid phase.

Established techniques: Methods are available for use with cell suspensions, calluses, apical and lateral meristems, dormant buds, somatic embryos and zygotic embryos.

Box 17. General references for cryopreservation

Books

Bajaj, Y.P.S. (ed.). 1995. Cryopreservation of Plant Germplasm I. Biotechnology in Agriculture and Forestry, Vol. 32. Springer, Berlin, Germany.

Benson, E.E. 1999. Plant Conservation Biotechnology, pp. 139–154. Taylor & Francis, London.

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

Kartha, K.K. (ed.). 1985. Plant Cryopreservation. CRC Press, Boca Raton, Florida, USA.

Razdan, M.K. (ed.) 1997. Conservation of Plant Genetic Resources *In Vitro*. Oxford and IBH Publishing Co. Pvt. Ltd., India.

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

Journals

CryoLetters

Cryobiology

Plant Science

Plant Cell Reports

Occasionally in other plant science journals.

Controlled freezing (slow freezing, two-step freezing) of meristems requires pretreatment steps, cryoprotectants, and slow freezing (0.1–1°C min^{-1}) using a programmable freezer, generally to –40°C, followed by immersion in LN. Thawing is quick, with ice melting in 1–2 min (Reed and Chang 1997; Chang and Reed 2000).

Vitrification employs pretreatment and very viscous cryoprotectants. In this method, the length of exposure to cryoprotectants is critical as they may be very phytotoxic. After appropriate exposure to cryoprotectants, the samples are immersed in LN. Thawing is similar to that for slow freezing, and meristems should be removed from the cryoprotectant solution as soon as possible after thawing (Sakai *et al*. 1990).

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).

Box 18. Cryopreservation of plants

Part stored	Use
Apical meristems	Retain clonal stability
Dormant buds	Hardy woody plants
Somatic embryos	Fast clonal propagation
Zygotic embryos	Store recalcitrant seeds

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 that 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^{-1} to –30°C, held for 24 h and stored in the vapour 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 cannot 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 that 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 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 colletion database. Cryopreserved storage should be considered as 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

Box 19. Cryopreserved storage

Two approaches for cryopreserved storage

(i) 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.

(ii) Pear collection cryopreserved at the US 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 and 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.

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; and 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 that 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.

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.

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 1.5.

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 that 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 13)
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 cannot be transported to the western states because of the presence of Eastern Filbert Blight). Additional information on tests and treatments of the germplasm may be given in a GERMPLASM HEALTH STATEMENT, as shown in Appendix 13 (Diekmann and Putter 1996a,b).

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.

Research needs in the *in vitro* genebank

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 that 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 together 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 that 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.

1. General considerations for the establishment and management of germplasm collections
2. Procedures for establishing and *in vitro* collections
3. References

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APPENDIX 1

Example of a record-keeping system for a genebank (National Clonal Germplasm Repository)

These are the types of fields used in the database for the US Germplasm System. Local information is electronically transferred to the national database (GRIN), so some fields are used to coordinate the 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, *in vitro*
33	IMPRVSTAT	Character	10	Cultivar or wild

Field	Field Name	Type	Width	Function of Field
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	DVLPCNTRY	Character	30	Developer country
48	DVLPHIST1	Character	60	History of development
49	DVLPHIST2	Character	60	
50	DVLPHIST3	Character	60	
51	DVLPHIST4	Character	60	
52	DVLPORIG	Character	1	Developer is the origin
53	OTHERDVLP	Character	1	Are there other developers? Y or N
54	DVLPSRCNO	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	DONORHIST2	Character	60	
64	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	DONORSRCNO	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	

Field	Field Name	Type	Width	Function of Field
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

Field	Field Name	Type	Width	Function of Field
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
130	IPREXPIRE	Character	10	IPR expiry
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 2

Accession inventory database information for National Clonal Germplasm Repository (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, *in vitro*)
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
16	TAXON	Character	60	Taxon
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

Field	Field Name	Type	Width	Function of Field
38	TC	Character	4	Plant available *in vitro*
39	TCGRIN	Character	1	*In vitro* 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 3

Basic CEC apple descriptors, passport data (IPGRI)

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 4

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 5

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**	***In vitro***	
				Misc.		
Corylus	Oct–Dec	January	-----	-----	Oct–Nov (layers)	Few
Fragaria	All year	-----	-----	Jun–Sept (runners)	-----	Yes
Humulus	All year	-----	-----	-----	Jan–Feb (rhz.ctng)	Yes
Mentha	All year	-----	-----	-----	All year (rhz.ctng)	Yes
Pyrus	All year	Dec–Jan	Aug–Sept	-----	-----	Yes
Ribes	All year	-----	-----	May–June (softwood) Dec–Jan (dormant)	-----	Yes
Rubus	All year	-----	-----			
Raspberry type		-----	-----	Dec–Jan (rt.ctng)	-----	Yes
Blackberry type		-----	-----	Sept–Nov (division)	Dec–Jan	Yes
Vaccinium	All year	-----	-----	May–June (softwood) Dec–Jan (dormant)	-----	Yes

APPENDIX 6

Example of distribution procedures for plant germplasm: National Clonal Germplasm Repository (Hummer 2003)

Order Processing

1. Request Received
   - By mail
   - By phone
   - In person
   - GRIN
   - Information request
   - e-mail
2. Staff member writes request form
3. Copy of letter, original import permit (IP), request form given to Distribution Technician (Dist. Tech.)
4. Date received stamped by Dist. Tech.
   - Hard copy file prepared
   - Requisition number assigned
5. Data entered into:
   - Distwork.dbf
   - Dist96.dbf
   - GRIN order processing
6. Confirmation (letter, fax, e-mail) 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
7. Order shipment default
   - Domestic request=Priority Mail
   - Foreign request=International Airmail

Order Preparation

1. Paperwork and computer file prepared
2. *In vitro* requests prepared by *In vitro* Technician and laboratory staff
3. All other plant material prepared by Dist. Tech. in consultation with Plant Production Mgr. and Plant Pathologist
4. 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
5. Orders not requiring a PC (see Shipping)
6. Interaction with APHIS plant inspector
7. *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

8. All other requests: Dist. Tech. determines the specific amount of items per genus to be presented based on NCGR policy and consultation with Curator
9. Dist. Tech. prepares Plant Inspection Summary Sheet from all requests to be inspected
10. Material not likely to pass is removed in consultation with the Plant Pathologist
11. 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

1. 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
2. Domestic requests shipped US Priority mail unless requestor has made other arrangements
3. Foreign requests (no IP or PC required) shipped US International Airmail unless requestor has made other arrangements (Customs sticker still required)
4. Foreign requests requiring a PC shipped US 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

1. Database is updated by Dist. Tech.
2. New pending list printed if request is not yet complete
3. Arrange payment of PC's to ODA through purchase order
4. 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 7

Sample plant tissue culture medium recipe sheets

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

MENTHA MEDIUM (MS based)

MULTIPLICATION

Component	**Conc./L**	**Amount Needed**
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. 10 mg/100 ml)

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

Bring to final volume and pH 5.7

Boxes and Tubes (Or agar only 6 g)

Agar	3.0 g
and	
Gelrite	1.25 g

Star*packs (Or agar only 7 g)

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 ml								
Stock soln. II	5 ml								
Stock soln. III	5 ml								
myo-inositol stock	12.5 ml								
Thiamine HCL stock	4 ml								
Pyridoxine HCL stock	5 ml								
Nicotinic acid stock	5 ml								
Glycine stock	20 ml								
BAP stock	50 ml								
Sucrose	30 g								
Distilled water	Make up to 1 L								
Check pH	5.6–5.8								
Phytogel	2.8 g								

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 50 ml
	NH_4NO_3	1650 mg per 50 ml
	$CaCl_2 \cdot 2H_2O$	440 mg per 50 ml
	$MgSO_4 \cdot 7H_2O$	370 mg per 50 ml
	KH_2PO_4	170 mg per 50 ml
Stock soln. II contains	$MnSO_4 \cdot H_2O$	22.3 mg per 5 ml
	H_3BO_3	6.2 mg per 5 ml
	KI	0.83 mg per 5 ml
	$ZnSO_4 \cdot 4H_2O$	8.6 mg per 5 ml
	$Na_2MO_4 \cdot 2H_2O$	0.25 mg per 5 ml
	$CuSO_4 \cdot 5H_2O$	0.025 mg per 5 ml
	$CoCl_2 \cdot 6H_2O$	0.025 mg per 5 ml
Stock soln. III contains	$FeSO_4 \cdot 7H_2O$	27.85 mg per 5 ml
	Na_2 EDTA$\cdot 2H_2O$	37.25 mg per 5 ml
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 8

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_{-1}, NaH^2PO_4 170 mg l^{-1}, adenine sulfate 80 mg l^{-1}, BA 1 mg l^{-1}, IAA 1 mg l^{-1} and GA_3 0.01 mg l^{-1} at pH of 5.7 and solidified with 3 g l^{-1} agar and 1.25 g l^{-1} 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 screenhouse collection. Plantlets are surface sterilized by placing them in a 10% bleach solution (bleach is 5.25% sodium hypochlorite) with 0.1 ml l^{-1} Tween 20 and shaken on a rotary shaker for 10 min. Explants are 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 1 week, then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group is all contaminated, consider antibiotic treatment.

Multiplying plants suspected of contamination are streaked on petri plates containing a bacterial detecting medium (Viss *et al*. 1991) containing sucrose 10 g l^{-1}, casein hydrolysate 8 g l^{-1}, yeast extract 4 g l^{-1}, KH_2PO_4 2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.15 g l^{-1} and agar 8 g l^{-1}. 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 h at 24ºC. 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 screenhouse collection do not produce very many runners. A 500-ppm GA_3 spray combined with long-day conditions has been helpful in inducing runnering in these plants.

APPENDIX 9

Record-keeping system for *in vitro* storage at NCGR

Field Name	Field type	Width	Reason for field (Dbase IV)
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 10

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^{-1} Tween 20 and shaken on a rotary shaker for 10 min. 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 peptone and 88 mg yeast extract and pH 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 1 week, then go on to multiplication. If contamination shows, recollect new tips, sterilize and rinse as before. If a second group is all contaminated, consider antibiotic treatment (Reed *et al*. 1995). 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 containing sucrose 10 g l^{-1}, casein hydrolysate 8 g l^{-1}, yeast extract 4 g l^{-1}, KH_2PO_4 2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.15 g l^{-1} and agar 8 g l^{-1}. 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 Centre.

(i) Bacto Nutrient agar supplemented with 1% glucose and 0.5% yeast extract at pH 7.

(ii) 523 Medium: 10 g l^{-1} sucrose, 8 g l^{-1} casein hydrolysate, 4 g l^{-1} KH_2PO_4, 0.15 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 8 g l^{-1} agar at pH 6.

APPENDIX 11

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 sitting 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 times 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 antibiotics 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 to 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, because bacteriostatic compounds will not provide lasting effects. Always test the antibiotic on or in the same medium that you will use to treat the plant culture.

After treatment, you must monitor each plant for bacteria for at least 6 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 12

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 development	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		

Data taken for *Corylus*, *Fragaria*, *Humulus*, *Mentha*, *Pyrus*, *Ribes*, *Rubus*, and *Vaccinium* at NCGR.

Field name	Field type	Width	Reason for field
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 colour
1	Etiolated, pale, no green colour	4	Not etiolated, medium-green colour
2	Etiolated, pale green colour	5	Not etiolated, dark green

APPENDIX 13

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	[ ]	BBTV—Banana bunchy top virus
	[ ]	CMV—Cucumber mosaic virus
	[ ]	BBMV—Banana bract mosaic virus
	[ ]	BSV—Banana streak virus
Electron microscopy	[ ]	Isometric virus particle—includes CMV
	[ ]	Bacilliform virus particle—includes BSV
	[ ]	Filamentous virus particle—includes BMV

[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 Centres 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 Centre staff assume any legal responsibility in relation to this statement.

Signature Date

This statement provides additional information on the phytosanitary status of the plant germplasm described here in. It should not be considered as a substitute for the official phytosanitary certificate issued by the plant quarantine authorities of Belgium.

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