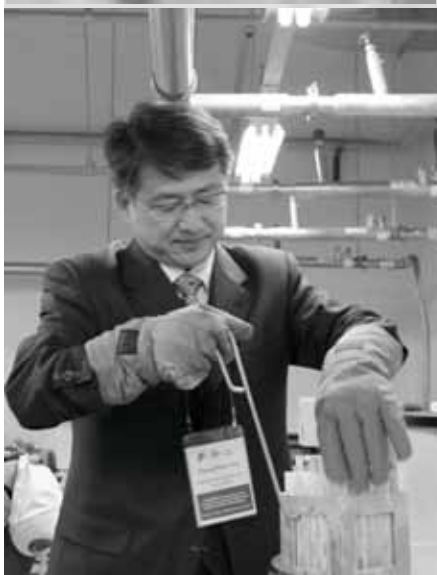
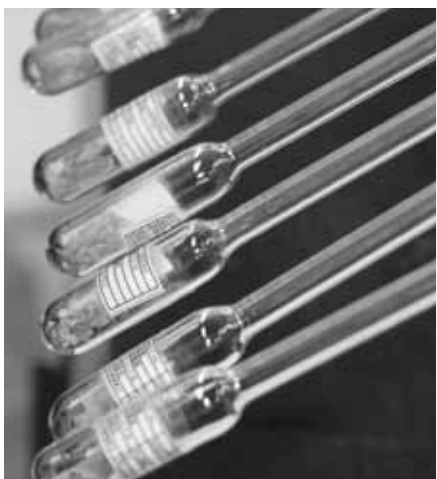


## A training module for the international course on the management and utilisation of field genebanks and *in vitro* collections

*Held at TARI, Fengshan, Taiwan on 16 -20 May 2011*







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**Bioversity International** is an independent international scientific organization that seeks to improve the well-being of present and future generations of people by enhancing conservation and the deployment of agricultural biodiversity on farms and in forests. It is one of 15 centres supported by the Consultative Group on International Agricultural Research (CGIAR), an association of public and private members who support efforts to mobilize cutting-edge science to reduce hunger and poverty, improve human nutrition and health, and protect the environment. Bioversity has its headquarters in Maccarese, near Rome, Italy, with offices in more than 20 other countries worldwide. The Institute operates through four programmes: Diversity for Livelihoods, Understanding and Managing Biodiversity, Global Partnerships, and Commodities for Livelihoods.

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**Module Prepared by:** Dr V Ramanatha Rao, Honorary Fellow, Bioversity International

**Photo Credits:** Leocadio Sebastian (Bioversity International) and Neil Palmer (CIAT)

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As the title of the training suggests, the course is focused on good management practices on the conservation and utilization of material in field and *in vitro* genebanks. The course aims to equip the participants with the latest knowledge on the best practices for managing field genebanks and *in vitro* collections.

The purpose of the module is to serve as a guide for lecturers and practicum instructors and other target users including technical staff in-charge of maintaining germplasm collections in field genebanks and in *in vitro* collections. This module is divided into lectures and discussions (PART 1), and practical hands-on sessions (PART 2).

As this module was prepared in advance of the training workshop, there can be additional information provided by the respective lecturers during the course. We encourage the lecturer and workshop participants to update the content of the module as maybe necessary.

We wish to thank Dr. Ramanatha Rao of Bioversity International for drafting this manual, Ms. Dorothy Chandrabalan for technical editing, and Ms. Katrina Borromeo for the editing, cover design and layout. We also appreciated the valuable inputs from Dr. Sheng-Chung Huang and colleagues from Bioversity International, TARI-COA and FFTC.

We hope that this module will guide the workshop participants as they apply the take-home lessons of the training workshop in their home countries.

Leocadio S Sebastian  
Regional Director  
Bioversity International  
Asia, Pacific and the Oceania (APO)



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# Introduction

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This training module was prepared for the International Training Workshop on the Management and Utilisation of Field Genebanks and *in-vitro* Collections. As the title suggests, the course is focused on technical procedures in field and *in vitro* genebank management. This module is in the same order of the course, which is divided into lectures and discussions (PART 1), and practical hands-on sessions (PART 2).

Field genebanks and *in vitro* collections are the options of *ex situ* conservation strategy for the conservation of germplasm of many plant species, that are vegetatively propagated or with recalcitrant seeds. At the same time, field genebanks can be difficult to manage, because they are expensive to maintain, require large space and carry high risks of loss. *In vitro* collections have the advantage of requiring less space for storage, the possibility of freeing plants from diseases, and convenience for exchanges; however they also are costly to produce and maintain. In addition, appropriate protocols for *in vitro* conservation of many plant species are yet to be finalized. Considering the challenges and costs involved, knowledge on effective management methods for these types of collections is vital for conserving the germplasm of a large number of important species. Thus, both the methods require considerable skills with the staff that manages such collections.

## Objectives

- To provide a sound scientific basis for establishing and managing FGBs and *in vitro* collections
- To impart to the participant latest knowledge and skills on the good practices in managing field genebanks and *in vitro* collections;
- To provide participants hands-on experiences and practical exercises on the managing field genebanks and *in vitro* collections;
- To update participants on the recent development in optimizing conservation and use of germplasm collections; and
- To provide a venue for sharing of experiences and networking among participants and host institutions for developing future collaborations on best in the future.



## PART I Discussion and Lectures





## LECTURE 1

# PGR conservation and use: principles and concepts and complementary conservation strategy

### Introduction

We can define biological diversity as the variation present in all species of plants and animals, their genetic material and the ecosystems in which they occur. This could be at three levels: genetic diversity (variation in genes and genotypes), species diversity (species richness) and ecosystem diversity (communities of species and their environment). During the past few years there has been increasing awareness on the holistic view of biodiversity, including agricultural biodiversity, conservation for sustainable utilisation and development. Sustainable conservation and use of PGR is a challenge facing us now, one of the many that we currently face. Plant genetic resources can include genotypes or populations of plants, representing cultivars, genetic stocks, wild species etc. Here an attempt is made to introduce the general concepts of PGR conservation and use along with principles of complementary conservation.

### Objectives

- Describe what are plant genetic resources and its functions
- Describe and discuss approaches to conservation
- Define and discuss what constitutes complementary conservation

### Description of the Lesson

This lecture deals with definition of plant genetic resources for food and agriculture and why they are important. It briefly describe the components of plant genetic resources (*ex situ* and *in situ*) and what, in broad terms, constitutes conservation of plant genetic resources (exploration, collecting and assembly; conservation; characterisation and evaluation; distribution; documentation; use). It explains different approaches to conservation and various extant methods under each approach in particular context. Emphasis is on conserving most genetic diversity in a given gene pool using complementary conservation strategy.

### Lessons to Learn

- Components of plant genetic resources for food and agriculture
- Approaches to conservation and what part of genetic diversity that each method under the two different approaches can help us to conserve
- No single approach/method can conserve all the genetic diversity that we would like to conserve

### Components of plant genetic resources

Crop germplasm collections are assemblies of genotypes or populations representative of cultivars, genetic stocks, and wild species etc., which are maintained in the form of plants, seeds, tissue cultures etc and populations in the wild or on-farm.

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Functionally, PGR constitute of landraces, advanced/improved cultivars and wild and weedy relatives of crop plants (either domesticated or non-domesticated). Landraces are distinct local races developed by farmers over generations, adapted to the many different agroecological conditions and interactions of natural and cultural environments to which crop species were gradually exposed and probably the most important of the genetic resources.

In general, the genetic diversity in cultivated plants is derived from wild ancestral species, modified by adaptations in response to cultivation. The modifications also take into consideration the physical, biological, cultural and socioeconomic factors of the environment. Due to highly specific nature of different environments, such domestication has resulted in many 'ecospecific' adaptations, which resulted in the formation of landraces, suited to local environments.

### **Centres of genetic diversity and genetic erosion**

The discoveries by Nikolai Vavilov during 1920-1940 were a major milestone in the field of PGR. Vavilov discovered what were first called the 'centres of origin of domesticated plants and animals'. Later these have been considered as 'centres of genetic diversity' and this is basically the current view, though there is some amount of confusion in the use of the terminology. This led to the expansion of gene pools of the plants, which are essential to human survival, which are frequently used by breeders for crop improvement and this has offered new opportunity for agricultural development.

### **PGR functions**

Given the seriousness of the problem of conservation and use of plant genetic diversity, there is an urgent need to try and assemble whatever genetic diversity is still available, especially of the material with a likely potential in the near future. The material thus assembled should be made widely available to users so that the material collected could be properly studied and used. To achieve this, the material must be characterized and evaluated. The characterisation and evaluation information should be documented properly and be made readily available to users along with the germplasm, which has been conserved. To complement these efforts, we should attempt to conserve and use the diversity *in situ* and on-farm. All this would require essentially five steps:

- Exploration, collecting and assembly
- Conservation
- Characterisation and evaluation
- Distribution
- Documentation
- Use

**Exploration and collecting:** Highest priority should be given to the collecting of material that is threatened with disappearance. For successful exploration and collecting, there is a need for well-coordinated effort with appropriate financial and manpower resources being available. However, there is strong need for the genetic resources institutes to create a situation in which exploration may expand and intensify in areas that have been either neglected or never explored.

**Conservation:** It is important to understand that conserving collected/assembled germplasm is for a long term and plans must be made accordingly. Conservation approaches and methods depend on the objectives of conservation and depend on time and space.

There are two approaches to conservation of PGR- *ex situ* and *in situ*. *Ex situ* conservation approach that conserved germplasm outside the site in which it evolved, generally comprises of the following methods: seed storage, field genebanks, *in vitro* storage, pollen storage, DNA storage and botanical gardens. Conservation of plant diversity using reserves/protected areas, on-farm and home gardens, is considered as *in situ* conservation approach. Each of this method will be briefly visited under the discussion for complementary conservation strategy.

**Characterisation and evaluation:** Collecting and conserving have significance in elucidating taxonomic status and evolutionary relationships between and within species. However, the main justification for genetic resource conservation is for utilisation in plant improvement. The key to successful use of variability from broad gene pools requires the knowledge of desirable traits available in the germplasm and this requires a systematic evaluation of germplasm. The role of characterisation and evaluation is basically to describe an accession with its various attributes - morphological, physiological, agronomical, biochemical, cytological and reaction to various stresses (biotic/abiotic). They help the curator to identify accessions, desirable genes or genotypes and, in general, they inform something about the variability/diversity of the available collection. The dividing line between characterisation and evaluation depends on genetics and genotype x environment (GxE) interactions. In general, effective evaluation is possible when there is close institutional and/or personal interaction between curators and breeders other crop improvement scientists, and where breeding objectives are reflected in evaluation programmes.

**Distribution, exchange and germplasm health:** It is important that all accessions in the genebank should be available to all those who wish to use them, either for crop improvement or other studies, under the usual conditions of germplasm supply. Often there may be some restrictions on the availability of germplasm, due to either the cost of seed production or because of national priorities.

**Documentation:** Progress in the field of plant genetic resources is related to the conservation of eroding genetic resources and utilisation of this material for crop improvement work. Success partly depends on the availability of information on the material being conserved. With increased international exchange of material, a certain amount of uniformity in data collecting, recording, storage and retrieval have become essential. Bioversity International, along with the Food and Agriculture Organization (FAO), has been playing a key role in bringing an understanding among the workers in many countries on these aspects. A computerized documentation system is now very common in many plant genetic resources centres. There is also increased attention being paid for documentation and management of data on *in situ* conservation.

**Utilisation of plant genetic resources:** One of the major objectives of conservation of PGR is to make genetic diversity available for immediate or future uses. New germplasm can (a) raise the genetic ceiling of improvement, (b) decrease vulnerability to biotic and abiotic stresses, and (c) add new developmental pathways and ecological adaptations to breeding material. There is the need to analyse genetic diversity and utilise it in plant improvement programmes in the future for enhanced sustainability and productivity

## Conservation approaches and methods and complementary conservation

It is well known that there are two approaches to conservation of PGR- *ex situ* and *in situ*. *Ex situ* conservation approach generally comprises of the following methods: seed storage, field genebanks, *in vitro* storage, pollen storage, DNA storage and botanical gardens. Conservation of plant diversity using reserves/protected areas, on-farm and home garden is considered as *in situ* conservation approach.

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## **Ex situ conservation**

**Seed storage:** Generally speaking, seed storage conservation method is an efficient and reproducible technique. The seeds are dried to optimal lower moisture content and stored at a low temperature. This method is almost universally applied to the orthodox seed species. Good storage conditions coupled with proper grow-outs are expected to increase longevity of shelf life of seeds. Seeds of most common species, can be maintained in this way for a number of years. Guidelines for proper handling and storage of seeds of many different crop species are available from Bioversity International and FAO. Work is also in progress on alternative methods of storage of seeds, such as the maintenance of seeds in imbibed storage, storage of seeds in liquid nitrogen at temperatures below -196°C and storage of ultra dry seeds. For some crop species standardised methods are already available.

**Living plants:** Many important varieties of field, horticultural and forestry species are either difficult or impossible to conserve as seeds (i.e. no seeds are formed or if formed, the seeds are recalcitrant) or reproduce vegetatively. Hence, they are conserved in field genebanks (FGB). FGBs require more space, especially for large plants such as tree species and they may be relatively expensive to maintain depending upon the location and the complexity of alternative techniques available. However, FGBs provide easy and ready access to conserved material for research as well as for use. For a number of plant species, the alternative methods have not been fully developed so that they can be effectively used

**Storage of tissues/cells:** Another method of *ex situ* conservation of germplasm is through the use of tissue or cell cultures. As seen earlier, there are a number of plant species, which cannot be conserved as seeds and presents different problems. It has also been noted that, generally, conservation of such material as plants in the field requires greater space, labour and funds and they also run the risk of being damaged by natural calamities and virus infections.

Hence the conservation of tuber, root, shrub and tree species becomes very difficult indeed. For some species, *in vitro* conservation is the only method available. Work on cryopreservation of tissue culture, so that these can be preserved for long periods, is also making rapid progress. Once these techniques are further refined, their large-scale adoption may be possible. Cryopreservation is an acknowledged method for long-term conservation of recalcitrant seeded plant species that include most of the tropical fruit tree (TFT) species. These examples showed that the work done so far is impressive. However, it must be realized that much more research still needs to be done to refine and standardise the existing cryopreservation protocols so that these could be effectively used for conservation for difficult to conserve plant species.

**Storage of pollen:** This is another important area that needs to be explored as a component of the conservation strategy for coconut genetic resources. Pollen storage was mainly developed

as a tool for controlled pollination of asynchronous flowering genotypes, especially in fruit tree species. In addition, pollen storage has also been considered as an emerging technology for genetic conservation.

**Definition of *in situ* and *ex situ* conservation strategies  
(United Nations Environment Programme - 1992)**

***Ex situ* conservation** - the conservation of components of biological diversity outside their natural habitats

***In situ* conservation** – the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties.



Even if it may not be considered to be a viable method for meaningful genetic conservation of genotypes, cryopreservation is likely to be more successful than other storage techniques routinely employed for pollen (e.g., under organic solvents, desiccation freeze drying, low temperature) in facilitating of hybridization when flowering is asynchronous or for use in next season. Thus, it can help in better utilisation of available genetic resources. Pollen can be easily collected and cryopreserved in large quantities in relatively small space. In addition, exchange of germplasm through pollen poses fewer quarantine problems compared with seed or other propagules. In recent years, cryopreservation techniques have been developed for pollen of an increasing number of species and cryobanks of pollen have been established for fruit tree species in several countries.

**DNA storage:** Storage of DNA is, in principle, simple to carry out and widely applicable and seems to be relatively easy and cheap. The progress in genetic engineering has resulted in breaking down the species and genus barriers for transferring genes. Transgenic plants have been produced with genes transferred from viruses, bacteria, fungi and even mice. Such efforts have lead to the establishment of DNA libraries, which store total genomic information of a plant in the form of DNA libraries. However, strategies and procedures have to be developed on how to use the material stored in the form of DNA. DNA is easy to extract from most plant tissues and can be stored for long periods in DNA banks. Basically this would consist of extracting and checking genomic DNA for quantity and quality using spectrophotometric techniques and gel electrophoresis. Samples are stored frozen at  $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$  and dried voucher specimens are stored as a backup. For successful use of this technology may require full integration with existing collections such as botanic gardens, herbaria and seed banks, and information retrieval systems that link such facilities, bioinformatic resources and other DNA banks. They also require efficient and well-regulated sample exchange procedures. However, strategies and procedures have to be developed on how to use the material stored in the form of DNA. The role and value of this method for PGR conservation is not completely clear yet.

**Botanical Gardens:** There are >1500 botanic gardens and arboreta in the world and the objectives of most of the gardens are to maintain essential ecological processes and life support systems and preserve a sample genetic diversity and ensuring sustainable utilisation of species and ecosystem. However, the botanical gardens, excepting a few well developed and funded ones, may play a limited role in the context of conservation and propagation and probably a greater role in public awareness and education. Number of accession and accession size in a botanical garden is limited most of these gardens cannot really reflect or conserve genetic diversity. There is a possibility that a few well-managed gardens can emphasise on conservation of certain groups of species as living collections (i.e. field genebanks).

### ***In situ Conservation***

This type of conservation is dynamic as opposed to the semi-static nature of *ex situ* conservation. One of the reasons given for choosing *in situ* conservation over *ex situ* is the need to maintain the evolutionary potential of species and populations. However, given the fact that human activities can cause habitat destruction and loss of biodiversity in some cases, and the maintenance of biodiversity in other cases, the need to complement it with *ex situ* conservation effort is now well recognised. In general, research and monitoring is needed at three levels for successful *in situ* conservation: the assay of genetic variation represented within a target species in a particular area (ideally by studies of intraspecific morphological and molecular variation and the diversity as recognized by local users, including farmers); regular inventory of species numbers; and observation of general ecological condition and habitat alteration, including farming systems.

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**Biosphere Reserves/Protected Areas:** In general, the biodiversity at the species and ecosystem level can only be conserved through *in situ* conservation. Various types of protected or semi-protected areas that are identified to be rich in diversity of ecosystems and/or species are used in this method. Conservation of wild species crop relatives in genetic reserves involves the location, designation, management and monitoring of genetic diversity in a particular, natural location. However, it must be remembered that genetic reserves are often not very accessible for use. Additionally, the monitoring and management may not be optimal due to difficult conditions under which these need to be performed. For the same reason, characterisation and evaluation may be limited. The reserves are also vulnerable to natural and human made disasters.

**On-Farm Conservation:** *In situ* conservation of agrobiodiversity or on-farm conservation involves the maintenance of traditional crop cultivars (landraces) or farming systems by farmers within traditional agricultural systems. Many farmers still use landraces, which are developed by farmers and strongly adapted to the local environment. This method of conservation has been gaining importance in recent years, though farmers have been using, mostly unconsciously, it for centuries. In the case of agrobiodiversity, the effects of growers-practices are of paramount importance. Systematic documentation of farmers' knowledge of diversity and uses is needed. Sustainable *in situ* conservation efforts require community participation, control of land rights in local communities, education, extension and development of environmental awareness. Of equal importance is the principle that any *in situ* conservation programme must also benefit the local communities. Management by local communities can often be developed to effectively link conservation and use. It is important to consider indigenous knowledge, people's participation and co-operation between local people, researcher and conservationists and nongovernmental organizations (NGOs). The methods of management and benefits to local communities in maintaining and using this diversity must be considered while implementing an *in situ* (or on-farm) conservation programme. Much progress has been made in understanding the scientific basis of on-farm conservation of agrobiodiversity in recent years.

**Home Gardens:** Home garden conservation is very similar to on-farm conservation however, the scale is much smaller. In most rural situations, home gardens tend to contain a wide spectrum of species, such as vegetables, fruits, medicinal plants and spices, than on-farm plots. As it is akin to on-farm conservation, the dynamic nature of this conservation technique has the same advantages. Home garden, as a single unit, has very little value in terms of conservation, but a community of them in a given area may contribute significantly to the conservation and direct use of genetic diversity. Most of such diversity could be somewhat unique/rare as the people tend to grow unique materials in their gardens and also underutilised species. However, the system is vulnerable to changes in management practices.

Home gardens are also known to be testing grounds for farmer-home gardener, as well as, a location for testing out some of the wild and semi wild species. Thus, in rural areas, the home gardens may continue to play a role in genetic diversity conservation as well as development.

## Complementary Conservation Strategy

As we have seen just now, there are two main approaches to conservation of PGR: *ex situ* and *in situ*. It is important to emphasize that these two approaches are complementary in nature. Conserving a genepool should employ a combination of methods, from nature reserves to genebanks as no single method can conserve all the diversity. The appropriate balance between different methods employed depends on factors such as the biological characteristics of the genepool, infrastructure and human resources, number of accessions in a given collection and its geographic site and the intended use of the conserved germplasm. For any given genepool the extent of a particular method used may defer from that used in another genepool and there is now increased emphasis on developing and implementing complementary conservation strategies for various important genepools.

To develop the complementary conservation strategy it should not only consider the technical aspects of specific methods. It also needs to consider the practical and political aspects. This is particularly important for *in situ* conservation.

Selection of the appropriate method or methods should be based on a range of criteria, including the biological nature of the species in question, practicality and feasibility of the particular method chosen (which depends on the availability of the necessary infrastructure) as well as the cost-effectiveness and security afforded by its application. For any given genepool the extent of a particular method used may defer from that used in another genepool. A combination of methods, from both *in situ* and *ex situ* approaches, should be applied so that one method complements the other. Such system can provide a strategy that optimally conserves maximum diversity within the target genepool.

For example, if one considers the conservation of Citrus genepool, at the simplest level, one has citrus species that have orthodox seeds, intermediate seeds or recalcitrant seeds. All other things being equal, it could be said that the Citrus species that produce orthodox seeds are best conserved in seed genebank. However, this can be complicated by other considerations, for example, objective of conservation, polyembryony etc.

Species that produce recalcitrant seeds, depending on the level and stability of the technology available, may be conserved as tissue under slow growth conditions or cryopreservation or in field genebanks. If the decision is to use former, we need to know the gestation period for *in vitro* conserved material to become useful. In this way, it may be possible to determine which part of the gene pool can be conserved using which method, thus establishing CCS for *Citrus*. As one can see, there is a need for complete information to make the appropriate choices. However, attempts to develop models at genus (such as *Citrus*) or groups of species level may be futile, as the number and type of parameters to be considered for any one gene pool varies greatly and this can make development of a model system virtually impractical.

**Table 1. Factors to consider when establishing plant collections.**

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

(+= applicable; -= not applicable)

There are several factors to consider when establishing plant collections (See table 1). The best storage method or combination of methods can vary with the crop type or perhaps with the genotype in question. It is important to maintain field collections for evaluation, characterisation, 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 regued in the field than *in vitro*.

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## Cost-effective conservation

There is no need to re-emphasize the need to efficiently conserve and sustainably use plant genetic resources by all countries, as stressed by CBD and GPA. The responsibility of conserving plant genetic resources of a country, either in *in situ* and/or *ex situ* lies with the country. Like any other activities, conservation of PGR requires an investment and economic questions thus become important. The value of genetic resources is considered important because of the belief that genetic resources are extremely valuable, so much so that we cannot afford the predicted rate of extinction during the next century (Brown 1990). The genetic resources have uncertain potential value. However, limited budgets necessitate ranking and cooperation of all concerned to pool the diminishing resource and to share the expertise. Keeping in mind the limitations of each approach and method, it is prudent on the part of genetic resources community to work rigorously to develop different methods and cost effective protocols, which can provide options for a complementary conservation strategy for these globally important plant genetic resources. In the area of *ex situ* conservation, increased efforts on cryopreservation of regenerable tissues of these crops to enhance our capacity to conserve the genetic resources of difficult-to- conserve species on a long-term basis. There is the scope as well for further progress to be made in DNA banking, though current situation precludes the use of such stored DNA. Development of On-farm conservation strategies as well as *in situ* conservation in natural habitats, as most of the tree species do occur naturally in the wild, can further enhance the chances of developing a complementary conservation strategy for a very large group of genetic resources. This could include various methods of *ex situ* conservation coupled with *in situ* conservation.

## Further Reading

- Brown, A. H. D., D.R. Marshal, O.H. Frankel and J.T. Williams (eds). 1989. The Use of Plant Genetic Resources. Cambridge University Press, Cambridge, UK.
- Centro Internacional de Agricultura Tropical (CIAT), Universidad Nacional de Colombia-Sede Palmira and Bioersity International, Red de Instituciones Vinculadas a la Capacitacion en Economia y Politicas Agricolas en America Latina y el Caribe (REDCAPA) and Centre technique de cooperation agricole et rurale (CTA). 2007. Multi-institutional distance learning course on the *ex situ* conservation of plant genetic resources. Cali, Colombia.
- Frankel, O.H. and Bennett, E. (eds). 1970. Genetic Resources in Plants - Their Exploration and Conservation. Blackwell, Oxford and Edinburgh.
- Frankel, O.H. and J.G. Hawkes (eds). 1975. Crop Genetic Resources for Today and Tomorrow. Cambridge. 492 p.
- Frankel, O.H. and M.E. Soulé. 1981. Conservation and Evolution. Cambridge University Press, Cambridge.
- Guarino, L., V. Ramanatha Rao and R, Reid. (eds). 1995. Collecting Plant Genetic Diversity. Technical Guidelines. CAB International on behalf of IPGRI in association with FAO/ IUCNIMEP, Wallingford, UK. 748p.
- Drew, R. and S. Ashmore. 2003. Complementary Conservation Strategy for Tropical Fruit Species. Consultancy Report under ADB funded project on 'Conservation and Use of Native Tropical Fruit Species Biodiversity in Asia'. IPGRI South Asia Office, New Delhi, India. 68 p.
- Engelmann, F. and J.M.M. Engels. 2002. Technologies and strategies for *ex situ* conservation. Pp. 89-104 in Managing Plant Genetic Diversity. (J.M.M. Engels, V. Ramanatha Rao, A.H.D. Brown and M.T. Jackson, eds.). CAB International and IPGRI, Wallingford and Rome.

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- Ramanatha Rao, V. and Bhag Mal, 2002 Tropical fruit species in Asia: diversity and conservation strategies. Pp. 179-190 in Proceedings of the International Symposium on Tropical and Subtropical Fruits (Rod Drew ed.). Acta Horticulturae 575. International Society for Horticulture Science. 405 p.
- Saad, M.S. and V. Ramanatha Rao, Eds. 2001. Establishment and Management of Field Genebank, a Training Manual. IPGRI-APO, Serdang. 122P
- Zhao YanHua; Wu YongJie; Chang, Y. J.; Reed, B. M.; Reed, B. M. 2008. Cryopreservation of fruit and ornamental trees. Pp. 387-420 *in* Plant cryopreservation: a practical guide (B.M. Reed, Ed). Springer-Verlag GmbH, Heidelberg, Germany,



## LECTURE 2

### Germplasm conservation and related phytosanitary measures

#### Introduction

It is not just enough to conserve genetic diversity, it must be maintained in healthy condition and should be exchanged through healthy propagules. This is the main responsibility of the genebank curator. Maintaining disease free material in seed and cryobanks may be easier but in the case of field genebanks it requires much effort and many processes put in place. 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.

#### Objectives

- Describe constraints to maintain healthy genebank
- Describe and discuss appropriate measures to maintaining healthy genebanks
- Define and discuss phytosanitary measures needed for exchange of healthy propagules

#### Description of the lesson

- Maintenance of material free of pathogens of quarantine interest
- Facilitating the availability of germplasm without plant health risks to users

#### Lessons to learn

- Acquire healthy germplasm into collections
- Maintain disease free germplasm in collections
- Distribute healthy germplasm/propagules to those who request

#### Conservation of healthy germplasm

The objectives of field genebanks are to maintain healthy, vigorous plants and to minimize risk of loss or genetic change. 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. There is no doubt that activities to strengthen the conservation and use of plant genetic resources worldwide, with special emphasis on the needs of developing countries are of utmost importance. However, care must be taken not to introduce new pathogens inadvertently. With the high genetic diversity of plants, a diversity of pathogens is often associated. The diversity of plant genotypes is much needed for the selection of resistant varieties, which may be the only promising control strategy for some plant diseases.

Clearly, the movement of plants or plant parts between countries or continents entails the risk of introducing exotic plant pests or pathogens. Less-developed countries often lack adequate plant quarantine and diagnostic facilities, and are especially vulnerable to the damaging effects of newly introduced diseases.

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It is extremely important that the risk is recognised, and that a minimum risk transfer form of germplasm is chosen, such as *in vitro* plantlets instead of seeds or other form of propagules. An effective phytosanitary system acts as a filter and not a barrier to germplasm exchange. It keeps pathogens out and allows germplasm to pass. As some countries have stronger systems than others the plant breeders and the germplasm community should also give due attention to pathogens. In addition to the risk of spreading pathogens to new areas (there are numerous examples where this has happened with germplasm), there is also the risk of the collections, or part thereof, being destroyed by disease epidemics. This risk is particularly high in field genebanks.

The choice of phytosanitary measures (exclusion, import permit stating certain conditions, certification according to the requirements of the importing country, standard quarantine certificate, post-entry isolation and observation) depends on the risk. The instrument of pest-risk analysis helps to make the correct choice, provided the required data are available.

Bioversity International (formerly the International Plant Genetic Resources Institute (IPGRI)) has published jointly with the Food and Agricultural Organization (FAO) Technical Guidelines for the Safe Movement of Germplasm. Since the publication of these guidelines, further research was conducted. Germplasm health needs to be considered not only at the point of exchange, but at any stage of germplasm management (collecting, multiplication, evaluation and characterization, storage and distribution). Cooperation among breeders/ germplasm curators and regulatory organizations is essential. Consultation should occur regularly, particularly at early planning stages for collecting, establishing field genebanks, etc. Germplasm should be exchanged only for immediate use or for safety duplication.

### **Pre-acquisition/establishment**

The efforts to maintain and distribute healthy germplasm start even before a collection is established with collection of, as much as possible, propagules from healthy plants and phytosanitary protocols for material introduced from other genebanks. Some are described here under.

**Site selection:** In the case of field genebank, 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.

**Choice of conservation method:** 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.



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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.

### **Considerations when sourcing material**

Always consider carefully what type of material you want and really need to source. Check if you need to bring the plant material well in advance of registering the material in well in advance to satisfy post-entry quarantine requirements. Check whether the taxa are listed as known hosts for certain pests and diseases or are prohibited under the plant health regulations of your country and whether the species known to be invasive or regarded as pernicious weeds. It is worth remembering that, historically, exchanges of plant materials have been a major factor in the introduction of invasive species around the world!

Where are you sourcing material from? Import of some materials prohibited under plant health regulations are only prohibited from certain countries where particular pests or diseases are known to occur. Check if there suitable within the country sources that could be used instead. Location of sources should be considered not only in the context of pest and disease risks but also from the perspective of shipping costs and sustainability, in much the same way as 'food miles'.

One may not be able to obtain a phytosanitary certificate for wild-source material. Such material has generally been exposed to a wider range of pests and diseases than has material of cultivated origin, and it may not have been produced under controlled conditions. Applying for a plant import licence for importing natural-source expedition material can take several months; and, before such a licence is granted, you may need to improve your on-site facilities to ensure effective containment. Whole plants can harbour a wide range of pests, diseases and viruses on all parts of the plant. Often, symptoms only emerge several months into the isolation process, once material is in active growth. Trees and large woody plants may be infested by wood-boring beetles and other cryptic pests, which often have long life cycles and may not be evident for several months, or even years, after arrival.

If possible, conduct nursery visits to your suppliers in person, to check the quality of material and ask questions. Plant health standards for exports vary in different source countries. Consider how long the shipment will spend in transit. Length of time in transit equates to the length of time for pests and diseases to hatch or develop. As an institutional practice, develop good procedures for inspecting all consignments on delivery and check carefully for pest and disease symptoms, as well as matching goods to invoices. If, for any reason, you are not satisfied with the shipment, reject the material. As far as site demands will allow, aim to conduct inspections in an isolated or contained area away from other collections. This allows for material to be isolated and pests to be contained immediately if a problem is found.

**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 Bioversity

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Much of what follows relates to standard agricultural/horticultural best practice and will already be commonplace in most institutions. However, it is worth emphasising some of the principles as they can make a dramatic difference to the general health of collections and the rates of transmission of pests, diseases and viruses.

- Sterilise tools, such as secateurs, pruning knives and saws, between cuts, and especially between different plants.
- Use clean new pots, canes, cane caps etc. for new batches of material and sterilise materials between uses.
- Be aware of humans as vectors.
- Wash hands or wear gloves between handling new batches of material and other collections
- Be aware of disease transmission on soil particles and take measures to contain any soil and growing medium received with new batches of material. Sterilise and dispose of infested soil appropriately.
- If the growing area permits, space plants adequately to ensure they are not touching.\
- Avoid over-watering and generating large amounts of run-off.
- Place sticky traps among batches of new material to trap and monitor any emerging pests.
- Control weeds in and around glasshouses and isolation houses, as weed species may provide alternative hosts for many pests and diseases.

### **Isolation and quarantine facilities**

- If it is not possible to install separate isolation or quarantine-standard facilities, there are several aspects to consider. Even in a small-scale operation, some precautions can be adopted
- Isolation facilities should be distinctly separate and sited away from other growing areas, e.g. in a separate glasshouse or polytunnel; but, if this is not possible, a separate area within a glasshouse or even a single bench can be beneficial.
- Access should be limited. Take steps to minimise the risk of cross-contamination.
- A quarantine glasshouse may be possible to divide it into separate sections with different
- environmental regimes
- Depending on the organism to be contained use appropriate grade of vent-screen netting to prevent its escape.
- Minimise and contain water run-off.
- Before draining to a soak-away, water can also be sterilised by heat or ultraviolet treatment.
- Avoid plants being in direct contact with the ground.
- Incorporate disposal facilities into the facility to minimise the risk of spreading infected materials.
- Use dedicated equipment in isolation facilities and in each compartment of isolation houses.

**Quarantine Periods:** The life cycles of pests include stages that are not easily observed and are sometimes difficult to target with treatments. As a consequence, pests that are present on plants may not be detected by initial inspection, and treatments before they were exported may not be 100% effective. It is for these reasons that a period in quarantine is required.

To implement an effective quarantine system, it is necessary to become familiar with the life cycles of the pests that you might encounter, particularly notifiable pests. The life cycle of a pest is dependent on the host plant and the temperature. In many cases, increasing the temperature of the quarantine and isolation area will reduce the duration of the pest life cycle. The quarantine period should be calculated as the duration of the period free from the pest.

This is not simply the period from egg to adult emergence (length of life cycle), but how long a potentially fecund female could live and produce offspring. The basic calculation for a minimum period free from a pest is:

maximum recorded developmental time+ period of female longevity

There are also many situations where the pest life-cycle is particularly complex, and it is necessary to monitor for more than one life stage, since in certain situations the pest may follow an alternative life cycle or symptoms may not be evident. It is good practice to keep dormant plants in quarantine until they have been in active growth for some time, allowing possible cryptic pests to present themselves.

### **Post-collection establishment concerns**

**Collection management:** 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.

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

**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. Several tools and methods for the identification of viruses have been described in the literature.

**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.

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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.

### **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. 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. 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.

**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.

### **Plant disease diagnostics**

The traditional method of identifying plant pathogens is through visual examination. This is often possible only after major damage has already been done to the crop, so treatments will be of limited or no use. To save plants from irreparable damage by pathogens, farmers have to be able to identify an infection even before it becomes visible. Is this possible? What happens when pathogens attack a plant? An attack by disease-causing organisms generates a complex immune response in a plant, resulting in the production of disease-specific proteins involved in plant defense and in limiting the spread of infection. Pathogens also produce proteins and toxins to facilitate their infection, before disease symptoms appear. These molecules play vital role in the development of plant diagnostic kits.

Advances in molecular biology, plant pathology, and biotechnology have made the development of such kits possible. These kits are designed to detect plant diseases early, either by identifying the presence of the pathogen in the plant (by testing for the presence of pathogen DNA) or the molecules (proteins) produced by either the pathogen or the plant during infection. These techniques require minimal processing time and are more accurate in identifying pathogens. While some require laboratory equipment and training, other procedures can be performed on site by a person with no special training. So far, diagnostic kits have been designed to detect diseases in crops such as rice, potatoes, papaya, tomatoes, and banana. Similar kits are also increasingly important for identifying genetically modified organisms (GMOs) in shipments of conventional crops.

### **DNA-based diagnostic kits**

DNA diagnostic kits are based on the ability of single stranded nucleic acids to bind to other single stranded nucleic acids that are complementary in sequence (referred to as homologous).

The tool used in DNA diagnostic kits is the Polymerase Chain Reaction (PCR). There are 3 steps involved in PCR. The DNA is first unwound, and its strands separated by high temperatures. As the temperature is lowered, short, single-stranded DNA sequences called primers are free to bind to the DNA strands at regions of homology, allowing the (Taq) polymerase enzyme to make a new copy of the molecule. This cycle of denaturation-annealing-elongation is repeated 30-40 times, yielding millions of identical copies of the segment. The primers in PCR diagnostic kits are very specific for the genes of a pathogen, and amplification will occur only in diseased plants. The primers in PCR diagnostic kits are very specific for the genes of a pathogen, and DNA amplification will occur only in diseased plants.

Several PCR-based methods have successfully been adapted for plant pathogen detection. Real-time PCR (RT PCR) follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified, using fluorescent dyes, as it accumulates in the reaction mixture after each cycle. It offers several advantages over normal PCR, including: reduced risk of sample contamination, provision of data in real time and simultaneous testing for multiple pathogens. Real-time PCR protocols are among the most rapid species-specific detection techniques currently available.

DNA microarrays are also of great use for simultaneous pathogen detection. This is important, as plants are often infected with several pathogens, some of which may act together to cause a disease complex. Microarrays consist of pathogen-specific DNA sequences immobilized onto a solid surface. Sample DNA is amplified by PCR, labeled with fluorescent dyes, and then hybridised to the array.

PCR-based diagnostics is very sensitive compared to other techniques; detection of a small amount of DNA is possible. PCR can also help farmers detect the presence of pathogens that have long latent periods between infection and symptom development. Moreover, it can quantify pathogen biomass in host tissue and environmental samples, and at the same time detect fungicide resistance. PCR-based detection, however, is expensive compared to protein-based diagnostic methods, and also requires costly equipments.

### **Protein-based diagnostic kits**

The first step in a defense response reaction is the recognition of an invader by a host's immune system. This recognition is due to the ability of specific host proteins, called antibodies, to recognize and bind proteins that are unique to a pathogen (antigens) and to trigger an immune reaction. Protein-based diagnostic kits for plant diseases contain an antibody (the primary antibody) that can either recognize a protein from either the pathogen or the diseased plant. Because the antibody-antigen complex cannot be seen by the naked eye, diagnostic kits also contain a secondary antibody, which is joined to an enzyme. This enzyme will catalyze a chemical reaction that will result in a color change only when the primary antibody is bound to the antigen. Therefore, if a color change occurs in the kit's reaction mixture, then the plant pathogen is present. The enzyme-linked immunosorbent assay (ELISA) method makes use of this detection system, and forms the basis of some protein-based diagnostic kits. ELISA kits are very easy to use because test takes only a few minutes to perform, and does not require sophisticated laboratory equipment or training.

There are already numerous ELISA test kits available on the market. Some of them detect diseases of root crops (e.g. cassava, beet, potato), ornamentals (e.g. lilies, orchids), fruits (e.g. banana, apple, grapes), grains (e.g. wheat, rice), and vegetables. ELISA techniques can detect ratoon stunting disease of sugarcane, tomato mosaic virus, papaya ringspot virus, banana bract mosaic virus, banana bunchy top virus, watermelon mosaic virus, and rice tungro virus.

One of the first ELISA kits developed to diagnose plant disease was by the International Potato Center (CIP). It can detect the presence of all races, biovars, and serotypes of *Ralstonia solanacearum*, the pathogen that causes bacterial wilt or brown rot in potato.

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They also developed a kit that samples for the presence of any of the following sweet potato viruses: SPFMV (sweet potato feathery mottle virus), SPCSV (sweet potato chlorotic stunt crinivirus), SPMSV (Sweet potato mild speckling virus), SPMMV (Sweet potato mild mottle virus), SwPLV (Sweet potato latent virus), SPCFV (Sweet potato chlorotic fleck virus), SPCaLV (Sweet potato caulimovirus), and C-6 (new flexuous rod virus).

### **Distribution of disease free germplasm**

All efforts must be made to supply recipients disease free propagules of germplasm conserved, especially when one is sending materials to distant regions in a country or outside the country.

*In vitro* germplasm conservation presents different advantages one which is easy and convenient for international distribution. But International exchanges need more for safe international exchange. You need to know the plant material on genetic level, and over all on the phytosanitary level. On the phytosanitary level, various viruses have been described on many crops. Indexing techniques allow highlighting a certain number of viruses in each crop species. Indexation for virus detection have been systematically standardized during the during the, establishment of a *in vitro* germplasm collection (can be used for field genebank as well). If pathogens are found, use eradication techniques. The use of *in vitro* techniques allows to, be free from fungus, bacteria, and other pests. Only viruses could be present on the plant and have to be eradicated. Different techniques exist that can be used (as noted earlier these include: meristem culture, thermotherapy and/ or chemotherapy. Electrotherapy (used on potato), or apex micrografting (used on Lemon tree or vine) need to further researched.

Exchange and distribution of plant material could be done by two ways (depending on severity of quarantine problems that go with the crop species):

- With non aseptic plant material (tubers, aerial tubers, seeds, nodal cuttings from the vine)
- With plant material in aseptic conditions (micro-nodal cuttings, microtubers, aerial microtubers, apices, zygotic or somatic embryos, callus and cells suspension)

### **Further Reading**

Long, H., F. Inches and K. Treseder. 2006. Guidelines on Legislation, Import Practices and Plant Quarantine for Botanic Gardens and Kindred Institutions. (Judith Cheney, ed.). PlantNetwork: UK and Ireland

Reed, B.M., F. Engelmann, M.E. Dulloo and J.M.M. Engels. 2004. Technical guidelines for the management of field and *in vitro* germplasm collections. IPGRI Handbooks for Genebanks No. 7. IPGRI: Rome.

Saad, M.S. and V. Ramanatha Rao, Eds. 2001. Establishment and Management of Field Genebank, a Training Manual. IPGRI-APO, Serdang. 122P.

<http://www.isaaa.org/resources/publications/pocketk/22/default.asp> - Pocket K No. 22: Plant Disease Diagnostics.

## LECTURE 3

# Acquisition and entry of fruit germplasm into the collection- collecting and maintenance

### Introduction

*In vitro* facilities and FGB is a method that available in most genebank centres that conserve asexually propagated and perennial plant species. Whereas, those that are kept in the form *in vitro* culture must be regenerated and grown in the field before they could be used, plants/trees in filed genebank are available for immediate use. However, the *in vitro* collections require much less space and may even cheaper in the long run. *In vitro* genebanks have additional needs of special regeneration methods and may take bit longer to become useful genetic resources; may be longer than those conserved as see. These unique features of each genebank type govern the acquisition of material into a genebank.

### Objectives

- Discuss briefly types of material that is generally conserved in field and *in vitro* genebanks
- Understand sampling strategies for different kinds of materials and other management practices needed
- Discuss criteria for material to be acquired for conservation in filed and *in vitro* genebanks
- Apply the principles with suitable modifications to fruit species

### Description of lessons covered

This discussion covers types of material to that can be conserved, appropriate sampling strategies, the procedures needed to genebank 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. It also touches upon the criteria for prioritizing for acquisition.

### Lessons to learn

- Types of material for acquisition to genebanks (FGB and *in vitro*)
- Sampling strategies and procedures for management of material in the genebanks
- Criteria for prioritizing acquisitions

## Collecting

**Why collect:** The main reasons for collecting tropical fruit tree species are as follows:

- To safeguard the diversity threaten by genetic erosion or even extinction
- To meet the needs of breeding programmes on particular traits or materials
- To fill the gaps in existing diversity

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**Where to collect:** According to the purpose of collecting missions, the locations should be identified for the collecting. Generally, the following principles should be considered when determining the localities for collecting missions:

- The region rich in diversity of fruit species
- The region where there exist special traits or types requested by breeders or other researchers.
- Remote areas where no representatives in existing collections

To identify the regions for collecting, it is necessary to review existing data, including literature, herbarium collections and other information. Once the collecting locations are identified, the route for collecting trip should be designed and transportation should be arranged accordingly.

**When to collect:** The collecting time is critical for a successful mission. Although the collecting can be performed during the whole year, the most suitable period for observation and collecting will be fruit maturity period, during which the best traits and plants or fruits can be easily identified. Usually a mission will take several weeks to complete according to the purpose of the collecting. Therefore, the time for carrying out collecting should be carefully considered during the planning of the mission.

**Types of collecting:** There are many types of collecting according to the purposes of the missions. Follows are some types of missions suitable for collecting tropical fruit tree species:

- **Multi-species:** a region will be targeted and an attempt is made to sample as much as possible of the diversity of as many as species as possible. Multi-species collecting mission will be performed by a multi-discipline team composed of experts working on different relevant species.
- **Species-specific:** the species-specific collecting mission is targeted on a broad genepool of specific crop, including wild species. But sometimes it may also just focus on particular needs and sample special genes or genotypes. The collecting team may be composed of experts working only on the targeted crop.

**Sampling strategy:** Development of sampling strategies is important for collecting missions. How collectors sample germplasm in the field will largely rely on such strategies. Defining a strategy is often difficult because species differ in crucial way, many plant populations have different complex genetic structures and samples may be used in different ways. A sampling strategy will provide information on understanding breeding system, regeneration method, seed storage behaviour, and genetic structure of the target species.

**Breeding system:** Although many fruit trees are outcrossing species, they are mostly clonally propagated crops. Thus the sampling strategy depends on whether one is collecting clonally propagated material from orchards and home gardens or from natural populations. In the clonally propagated material, there is almost no gene flow between populations. There are more variations among populations than within populations or types. The extent and level of genetic divergence among populations are crucial for determining the sampling. Both variation within and among populations should be sampled during germplasm collecting. There are many parameters used to describing genetic variation within and between populations. The most popular parameter used is allele numbers or richness because the users of genetic resources can adjust the frequencies of specific desired alleles at their own will. The samples should include average number of alleles for a large number of marker loci of the natural populations.



**Regeneration method:** Most of fruits are clonally propagated through grafting buds and other tissues. Therefore, there is no doubt that shoot cutting with a number of nodes is a major part of plant for sampling. However, few species such as jackfruit are seed propagating species. Therefore, seeds should be collected for these species.

**Seed behaviour:** Most of fruit species produce recalcitrant seeds, but a few species in *Citrus* and *Garcinia* produce orthodox seeds. This information will help collectors to determine which methods are used to process and store the collected seeds.

**Sampling diversity:** Theoretically, the number of alleles in a population will be increased in proportion to the logarithm of sample size. However, the size of sample should be determined by resources available and the cost per sample. It was suggested that the sample should cover 95% of the alleles that occurred in the target population at frequencies greater than 0.05. Increasing the certainty level higher than 95% or decreasing the allele frequency to lower than 0.05 will dramatically increase sample size.

**Number of sampling sites:** For a target region, 50 sites should be considered per species. Of course, the number can be adjusted according to the size and ecogeographic conditions of the region. For example, if a collecting mission is planned for Southern Yunnan, China, the first step is to divide the region into a number of sub-regions (10-12) according to ecological condition. Then the sampling activities will be carried out in each sub-region.

**Number of individual plants per population:** In each site, the seeds or cuttings of 10-15 individual plants per population should be sampled if the fruit trees are propagated by seeds, e.g. jackfruit. If the trees are clonally propagated, a bulk sample of 2-4 vegetative propagules from 10-15 randomly chosen individual clones.

If however there is always the possibility of loss of samples during the transportation, and hence the number should be increased, and usually double the number is recommended. If the number of individual plants cannot reach 10-15, more sites should be considered in a region. If seeds are collected from a clonally propagated cultivar as at a given site, it can either be kept as single-tree samples and given the same number as any vegetative sample or bulked in a cultivar sample assigned a distinct number.

**Choice of individuals:** Generally, random sampling is used in selecting individuals at a site. However, biased sampling of rare phenotypic individuals is used to capture some important genes such as disease resistance. Additionally, keeping phytosanitary conditions in mind, it is recommended that the sampling is done from apparently healthy trees. This would help to reduce the risk of bringing diseased propagules into genebank.

**Number and type of propagules per plant:** For fruit species, vegetative cutting, fruits, seeds or pollens can be collected according to species and purpose of the collecting missions. Vegetative cuttings are most popularly used for collecting fruit tree species as they produce recalcitrant seeds, or even no seeds. The number of cuttings to collect will be depended on the needs for conservation and use. If the collected materials need to be distributed to other researchers or genebanks, more cuttings should be collected. Otherwise, 5-10 cuttings/propagules per plant should be enough (but see above). As seeds or pollens require less space in the transportation or storage in genebank, adequate amount should be collected.

**Equipment:** Collecting of germplasm involves is working on trees in the field. Sometimes collectors also need to stay overnight outside in the open. The equipment should meet the needs of transportation, camping and medicinal medical care. Therefore, sufficient equipment will be the basis of a successful collecting mission (for details see Guarino et al. 1995).

**Training:** Members of collecting team should be trained to understand the objectives of the mission, sampling techniques, use of relevant equipment, familiar with route and possible environments, etc.

### Methods of regeneration of species

Citrus: Vegetative, seed  
Jackfruit: Seed  
Litchi: Vegetative  
Mango: Seeds and Vegetative  
Mangosteen: Vegetative  
Rambutan: Vegetative

**Conduct collecting missions:** The survey and collecting mission should be carried out in time according to the plan. As the collecting mission involves working outside in fields and orchards, it will be greatly affected by local climatic changes. Some extra time should be allocated in order to get enough time for the planned activities.

**In the field:** The collecting team should work closely with local farmers or guides, who know where the diversity is distributed. Different types of target fruit species are grown in field or home gardens in

communities. At the same time, interviews with farmers should be conducted about the uses of local varieties, particularly the value, traits, e.g. fruit quality, other uses, disease resistance. The germplasm should be sampled according to the strategies for different species. The time timing is most important for collecting tropical fruit tree seeds. The collecting, transportation and processing should be completed within 30 days in order to keep the viability of the seeds. And transportation to HQ should be organized in appropriate packages.

**Documenting collecting:** It is important to properly record the relevant information during collecting and after returning to base. All samples should be correctly labelled and should have information on:

- Collecting identifier (or collecting organization)
- Name(s) of collector(s)
- Collecting number (or collector's number)
- Collecting date
- Type of material

### Seed storage behaviour

Citrus: Recalcitrant, intermediate, orthodox  
Jackfruit: Recalcitrant  
Litchi: Recalcitrant  
Mango: Recalcitrant  
Mangosteen: Recalcitrant and orthodox  
Rambutan: Recalcitrant

### Filling collecting form

When a site is selected and samples are taken, the relevant data should be recorded on a collecting form. Bioversity have developed a collecting form covering information on description of environments of a site, identity of the sample, basic traits of the sample, etc. There is indigenous knowledge associated with conservation and use of germplasm. Indigenous knowledge is not only useful to local farmers, but also scientists in further develop and use the germplasm. Local farmers should be asked about the method of propagation of the trees, major features of different varieties and the differentiation between varieties in the village. Also the genetic information including the sources of mother trees, adaptability, and resistance of the varieties should be gathered from farmers. The information on local uses of fruit trees will be important for researchers to choose materials for breeding programmes.

## Criteria for acquisition of germplasm

As noted earlier, genebanks cannot go on taking in germplasm in unlimited numbers and quantities due to space, financial and other considerations. Some level of discretion needs to be used and each genebank, depending on its objectives, can set up criteria for prioritization of material that is registered in its storage facilities. Each genebank needs to have a germplasm acquisition policy. Any such policy needs to build into the present and future germplasm needs for each crop.

- **The species' state of conservation** - based on sufficiency of its representation in collections so that conservation activities do not duplicate already existing ones.
- **Urgency for conservation** - depends on threat to the species or population within a crop species. For wild related species, the *IUCN Red List of Threatened Species* may be consulted.
- **Contributions in terms of genetic variability** - selected species and populations may provide additional diversity.
- **Potential usefulness of the species** - species that contribute to the satisfaction of basic needs (e.g., food, medicines, and housing).
- **Relative cost of conservation** - depends on the capacity of the conservation unit.
- **Cultural importance to the community** - contribution to the aesthetic, symbolic, or cultural value of a species for a community (i.e., the role that it fulfils in cultural or religious activities may determine whether it should be conserved.)

## Maintenance

### Types of material introduced into field and *in vitro* genebanks

**Plants with recalcitrant seeds:** Many plant species, especially of tropical origin, produce recalcitrant seeds. Although the number is not as big as those that produce orthodox seeds Many of them are economically important such as mango (*Mangifera indica*), oil palm (*Elaeis guineensis*), rubber (*Hevea brasiliensis*), avocado (*Persea americana*), durian (*Durio zibethinus*) and many other tropical fruit species produce recalcitrant seeds that cannot be successfully stored for long periods (see Cromarty et al. 1990 and Hong et al. 1996).

**Asexually propagated plant species:** Some plant species, such as banana (*Musa* spp.), pineapple (*Ananas comosus*), taro (*Colocasia esculenta*) etc., reproduce mainly through vegetative means such as tubers, root, suckers, crown, etc. They rarely produce seeds; thus field and/or *in vitro* genebank is a better way to maintain their genetic resources.

**Genotype conservation:** For many species of plants that are cross-pollinated and can be vegetatively propagated the genotypes can be kept intact by growing them in the field in the form of clones or developing *in vitro*/cryopreservation methods for them. Growing these plant materials as clones in filed genebank enable us to maintain genotypes and breeders can directly evaluate and select. Superior genotype can be isolated and could be used as varieties or as parents for further breeding. Seeds from these plants usually give rise to segregating progenies. The segregation is even greater for polyploid plant species such as hexaploid sweetpotato (*Ipomoea batatas*), tetraploid black pepper (*Piper nigrum*) and potato (*Solanum tuberosum*).

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Storing the seeds of these species might be more relevant for gene conservation but not for genotype conservation. Furthermore, a much larger number of seeds must be stored in order to capture all possible genes from the particular genotypes. However, one may not be able to obtain the same genotypes by growing their seeds. It is normally a tedious and long drawn process to isolate desirable genotypes from segregating progenies. For example, in sweetpotato, a breeder normally grows thousands of seeds to increase his/her chance to isolate desirable genotypes.

**Plants with very long life cycle:** Many plant species have a very long life cycle. They take many years to mature. Seeds grown from such plants may take more than 10 years to mature and start flowering, which is disadvantage if that germplasm needed for immediate use. Maintaining such plants with long gestation period in field genebank is advantageous, as the plants stay there for many years. Once they reach maturity (flowering and fruiting) then they are in a ready-to-use form. They can be continuously evaluated and crossing can be done at any time once flowers are available. Unlike those grown from seeds there is no waiting time for it to reach maturity.

**Cultivars and rootstocks:** Clones should represent known diversity, not all known cultivars. For domestic clonal cultivars, BIOVERSITY 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, Bioversity 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. Decision should also be based on observed variation with a species and its subspecies (intraspecific diversity). A collection's deficiencies should be noted according to established criteria and collecting prioritized accordingly.

**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. 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.

**Appropriate sampling strategies:** Information about population and reproductive biology, phenology, ethnobotany, taxonomy and accessibility must be considered when designing a collecting strategy. Each crop type varies 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 *In vitro* germplasm collection management. Refer to Guarino et al. 1995 for details.

## Labelling/numbering and record-keeping system

Record keeping should include plant catalogues, images (photographs, drawings), characterisation 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.

### Types of Seed

**Orthodox seeds** are desiccation tolerant and may be stored in the dry state for predictable periods under defined conditions. Unless debilitated by zero-tolerant storage fungi, orthodox seeds should maintain high vigour and viability at least from harvest until the next growing season.

**Recalcitrant seeds** are those that undergo little, or no, maturation drying and remain desiccation sensitive both during development and after they are shed.

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, characterisation, 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.

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 may be specified by both the donating and the receiving country. If there are no restrictions, it is wise to have an in-house policy 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. BIOVERSITY has produced jointly with FAO technical guidelines for the safe movement of germplasm for a number of crop species.

## Characterisation, evaluation and utilisation

Plant germplasm require characterisation and agronomic evaluation to be effectively used by breeders. Thus, all germplasm collections must be grown in the field for this purpose. In the case of short-term crops they undergo a few generations of field planting for characterisation and agronomic evaluation.

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Perennial tree species such as fruit trees take longer time to mature. Such germplasm materials are normally grown in FGB for characterisation and evaluation. Since they are going to be in the field for a longer period of time they might as well be grown and be maintained for the purpose of conservation as well. Furthermore, materials grown in FGB can be directly used for selection.

### **Access and benefit-sharing arrangements**

Distribution is an essential part of genebank management so that the conserved germplasm is effectively used by breeders and other users. Discuss what such arrangements could be. International collecting must be conducted in accordance with the requirements of the Convention on Biological Diversity (CBD) (legally binding), 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 (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 utilisation of these resources on a complementary and mutually reinforcing basis.

### **Further Reading**

- Cromarty, A.S. R.H. Ellis and E.H. Roberts. 1990. Handbooks for Genebank No. 1. The design of seed storage facility for genetic conservation. IBPGR, Rome.
- Guarino, L. V. Ramanatha Rao and R. Reid, eds. 1995. Collecting Plant Genetic Diversity - Technical Guidelines. CAB International, Wallingford.
- Hong, T.D., S. Linington and R.H. Ellis. 1996. Seed Storage Behaviour: a Compendium. Handbooks for Genebanks: No 4. International Plant Genetic Resources Institute, Rome.
- Reed, B.M., F. Engelmann, M.E. Dulloo and J.M.M. Engels. 2004. Technical guidelines for the management of field and *in vitro* germplasm collections. IPGRI Handbooks for Genebanks No. 7. IPGRI: Rome.
- Saad, M.S. and V. Ramanatha Rao, Eds. 2001. Establishment and Management of Field Genebank, a Training Manual. IPGRI-APO, Serdang. 122P.

## LECTURE 4

### Diversity and genetic considerations in FGB and *in vitro* genebanks

#### Introduction

To effectively collect and conserve plant genetic resources, there is a need to have a sufficient understanding of some of the genetic principles of plant genetic resources (PGR) exploration and conservation especially those related to the structure and distribution of the genetic diversity of species to be conserved as well as the genetic diversity of the materials that are being conserved. Therefore, in this lecture, attempt is made to introduce some of the genetic background related to PGR collecting and conservation. Because conservation itself normally deals with a collection of individuals, its genetics is also shifting, from the usual genetics of individual to the genetics of a large number of individuals or a population, from dealing with a small variation to a large variation, and from a time scale of few years to a time scale of thousands or probably millions of years to happen.

#### Objectives

- Discuss briefly genetic diversity and its distribution
- Discuss briefly different breeding systems, ecological adaptations, Effect of isolation and selection, Genetic variability and PGR exploration and conservation and other factors that affect the genetic structure of accessions in genebanks
- Summarize genetic considerations when establishing genebanks, especially field genebanks that require optimization of accession size and field plot techniques to maintain and manage genetic diversity

#### Description of lessons covered

The lecture will discuss genetic consequences of various biological features like breeding systems and issues that need to be carefully considered before the establishment of a genebank for cost efficient conservation of a broad range of genetic diversity.

#### Lessons to learn

- Genetic diversity is central to conservation of plant genetic resources
- Genetic effects of a range of biological factors and their significance in conservation of plant genetic resources
- Issues that need to be considered while establishing and managing genebanks, in particular field genebanks

#### Genetic variability (diversity) and its distribution

It is well recognized that genetic diversity in plant species is the sum total of the genes/alleles contained in its genepool. Each gene consists of hundreds of nucleotides, each capable of base substitutions and with additional permutations possible through sequence arrangements, additions and deletions. Theoretically the potential number of allelic states at a single locus is virtually infinite. Therefore, in a natural population, there exists genetic diversity.

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In genetics term, natural populations are said to be polymorphic for a few or many loci or natural population is said to show polymorphism. It has been reported that in any given population, between 20 to 50 percent of the gene loci exist in two or more allelic forms. Understanding how these various alleles are distributed is fundamental for an effective and efficient conservation programme.

**Ecological adaptation:** Close relationship between some characters in a population and its habitat in which they are expressed has been reported many times in the literature. Collections made from separate geographical areas can differ significantly. For instance, some characters are common in accessions collected from certain regions but not in the other regions. When the rice germplasm collection of IRRRI was extensively screened for drought resistance, the largest proportion of resistant types was found in upland rice collected from Africa, South America, in hill rice from Laos, and among early ripening rice of Bangladesh. In other words, plants can evolve for some characters to fit themselves under conditions prevalent in particular geographical areas. The pattern of variation can be a type which steadily follow a clear and distinct clinal pattern (for instance variations observed as we move from cold and wet to hot and dry), or in a patchwork or mosaic pattern in the case of mountain habitat diversity. Marked local differentiations are likely to develop in self-pollinated species because of the limitation imposed on gene exchange/gene flow due to its mating systems, while they may be more dispersed in outbreeding species.

Evolution is the main mechanism in ecological adaptation. Evolution is a process that converts variation within a population into variation between populations, both in space (race formation and speciation) and in time (the evolution of phyla). The driving force of selection is mostly natural selection for or against certain genetic variants occurring within a population. The relevance of this theory of evolution to genetic variability is that the genotypic constitution of a population is in fact a consequence of the innate capacity of the organisms to vary (variation), and of populations to respond to environmental pressures by differentiation into a range of distinctive gene pools of considerable diversity through a process called adaptation (natural selection). Adaptation works on variability. If there were no variability, populations would have become extinct when environment change. Most of these adaptive traits are quantitative characters, e.g. stature, reaction to biotic and abiotic stresses. Adaptive process also involves its interaction with environment and this process leads to ecotype formation. Therefore, when planning for a PGR collection, it is important to collect samples from each of the various environments that exist in the target area with view to sample all possible alleles which are rare in certain environment might be common in other environments. In other words, alleles that we may miss collecting in one area, we may be able to find it easily in other areas.

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.

**Effect of breeding systems:** In many species, a major change under domestication is increased self-pollination. Yet, wide variation in rate of outcrossing indicates that the evolutionary processes might favour a mixed mating system. The primary consequences of inbreeding are increased homozygosity and a greater chance of selection among homozygotes to change the gene frequencies rapidly. New arising mutants are likely to be lost rapidly if they are unfavourable recessives.

Inbreeding species would have high interpopulation variability and less intrapopulation variation compared to outbreeders. Inbreeders also have greater colonizing ability due to higher dispersal rates, higher reproduction under low density and under low pollinator environment. Hence generally speaking, populations of inbreeding species have a simple genetic structure, consisting of a number of inbred lines, genetically homozygous and homogeneous, several individuals representing each line in the population, some variations between lines and very little heterozygosity.



This makes conservation of genetic resources self-pollinated species much simpler, compared to outbreeding species in which the population in general has higher within population variability. Maintaining this variation within a cross-pollinating species is complex both in terms of germplasm collecting and maintenance in genebanks (particularly in field genebanks) and later regeneration and sampling for utilisation.

On the other hand, genetic structure populations of outbreeding species with high rates of gene flow through pollen or seed dispersal, would approach some sort of equilibrium, thus precluding the species from developing differentiated populations on a microgeographic scale. So, as in the case of species with high migration rate, there is no point in sampling many populations from small or closed geographic areas.

Breeding system also determines the number of plants and the number of seeds to be sampled from a population. For both inbreeders and out breeders, it has been shown that the number of plants collected per population rather than that of seeds per plant is the primary determinant of the success of sampling. In a predominantly selfing population, the decrease in plant number causes a sharp reduction in the probability of capturing representative of all the alleles especially rare alleles. However, in an outbreeding population, such a decrease does not make much difference in the probability of sampling all representative alleles, unless the number of plants and seeds are very small. The probability is a little increased if the number of seeds per plant is increased in outbreeding population, and thus the practice of collecting more seeds per plant when dealing with outcrossing population is advisable.

**Effect of isolation and selection:** We know that populations tend to have similar gene frequencies if they are kept close because of smooth gene flow due to the migration of pollen or seed or other planting materials. The effective isolation distance to prevent such gene flow varies with plant species and location and still is a subject of argument by many researchers. The effects of isolation and selection can be:

- All alleles present but the frequencies change. Populations remain polymorphic for the same alleles but have them at different frequencies. In another words, no allele is absent in one population but present in another. Samples can be collected from a few populations collected on a regional basis.
- Some alleles would have become fixed and some alleles are lost entirely. At some point, due to sheer distance or topography (e.g. presence of mountain), total isolation between two populations may happen in which, through a process of selection, allele fixation occurs creating distinct populations. In other words, it is no longer possible to obtain the same alleles from a population because the allele is simply no longer present in that population. Therefore in collection for conservation, the species must be represented by as many samples as there are different populations, if we are to preserve all the variability in the species.

In most cultivated crops, sufficient uniformity exists in the cultural practices. This in fact acts against divergence. But cultural practices are not the only selection force that is operating. Ethnic preference is another, as well as differences in environment.

### **Genetic variability and PGR exploration and conservation**

Conservation of large number of accessions in a genebank is expensive and requires well trained personnel. There is always a limit to the number of samples or accessions that can be conserved, evaluated and utilized. Decisions with regard to number and size of samples to be conserved should be based on sound scientific principles. Understanding some of the parameters, which are used to describe allelic diversity, richness and evenness, is useful in making such decisions. This specially true with the information on allelic richness because, in most of the conservation work, we are normally interested in capturing at least one representative of all the alleles that are present in the population rather than maintaining a certain level of frequency of each allele.

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Usually, the three questions that are most frequently asked by those who plan to collect plant genetic resources for the purpose of genetic conservation are:

- Optimum number of sites to sample
- Optimum distribution of sampling sites within the area
- Optimum number of plants to sample per site

To determine the most effective distribution of the sampling sites, one needs to know the population structure of the species to be collected in the target area. Obviously we should locate most of the collection sites in areas where there is maximum diversity as indicated by data from genetic diversity studies. For instance, a sample size  $n = 50$  having an average of 2.40 alleles per locus, in terms of allelic richness, is more diverse than the one with 2.20 alleles per locus. Therefore, if choice has to be made, collecting samples from the former should be given more priority. Similarly, in terms of allelic evenness, a sample with three equally frequent alleles is more diverse than the one with a single predominant allele and two rare ones and thus the former should be given higher priority in collection and conservation. However, prior information on genetic diversity is mostly not available. The objective collection for field genebank or in vitro bank is to obtain maximum diversity with minimum sample size and number. Both random and non-random sampling may be used while collecting samples and decision would depend on whether material being collected is asexually reproducing species or sexually reproducing perennial species (which usually are the subjects of field and in vitro genebanks. Non-random sampling selects only those with clear morphological characters. For more on sampling see Guarino et al (1995).

**Habitat Diversity:** As stated earlier on, genetic differentiation within species is strongly correlated with environmental heterogeneity. Therefore, species that grow in a wide range of ecological conditions are expected to diverge genetically according to habitat conditions in which they live. So sampling sites should also be located in the whole range within the target areas. In other words, there is a need to increase the number of populations (or sites) to be sampled, probably at the expense of the number of plants collected per samples or site.

**Migration:** When the rate of migration in a species is said to be high, it means that the species is experiencing a high level of gene flow, largely through the dispersal of pollen and/or seeds. When this happens, populations are more likely to have the same genes and thus intrapopulation diversity is expected to be lower. Hence, the sampling strategy would be to collect samples from a few of sites or to widen the distance between sites. Under such situation, it is also advisable to increase the number of plants per sample. Migration is also related to breeding system.

**Effect of population size:** Several factors such as competition, pests and diseases, abiotic stresses, isolation, geological change, climate, natural disasters, and humans can cause some plant species to become rare or even extinct. In fact, if one examines carefully, all these factors enhance isolation of any plant population leading to a decrease in its size and genetic erosion. While propagules are collected from a small number of plants (sample size is small), leading to a reduction in numbers. Such a reduction in numbers has been called 'bottleneck'. A bottleneck leads to a drastic reduction in genetic diversity relative to the original population and this is more related to founder effect in species that are transported to a totally different geographical location. For the purposes of genetic diversity conservation, more important is the reduction diversity due to genetic drift. Genetic drift or allelic drift is the change in the frequency of a gene variant (allele) in a population due to random sampling. The alleles in the offspring are a sample of those in the parents, and chance has a role in determining whether a given individual survives and reproduces. A population's allele frequency is the fraction of the copies of one gene that share a particular form. Genetic drift may cause gene variants to disappear completely, and thereby reduce genetic variation. The effect of genetic drift is larger in small populations, and smaller in large populations.

Since in plant genetic resources conservation we deal with small populations, managing genetic drift through adequate sampling at the time of collecting and then sampling for germplasm regeneration during genebank management is very important. Effective conservation is inclusive of a reduction in number of regenerations during the shelf life of an accession.

## **Measuring genetic diversity**

### **Morphological markers**

Ordinary average, or arithmetic mean, which along with the range (a measure of dispersion or spread), tells us something about the distribution of values in population for a particular trait. However, this is not a measure of variation. Standard deviation informs us to the extent of dispersion or deviation from the mean in a population. Variance is derived from standard deviation and it is more useful because it is additive and it can be partitioned. In all the above statistics we are only dealing with one trait at a time (univariate analysis), without taking into account any associations between different traits for which measurements are recorded. Most often, in the study of genetic variation we will be dealing with several traits that are either interdependent or somehow influence the expression of other traits. This leads to study of genetic diversity using several characteristics at the same time and such analyses are grouped under multivariate analysis.

Multivariate analysis deals with the simultaneous variation of two or more dependent variables. Generally the studies on multivariate analysis start with a correlation matrix, which is a symmetrical table of correlation coefficients for each variable with all the others. Given the standard deviations of each variable, one can obtain covariances. One would be able to some sort of structure of the population under study for the matrix of correlations. There are many methods that can be used for grouping the variables according to the magnitude and relationships among their correlations. These methods are known as cluster analysis. Multivariate techniques, such as discriminant function, canonical variate analysis, principle component analysis, or factor analysis can specify characters (variables) that best represent or summarise certain patterns of variation.

### **Biochemical Markers**

Analyses of allozymes (also known as isozymes, and are allelic variants of enzymes) provide an estimate of gene and genotypic frequencies within populations. Such data can be used to measure population subdivision, genetic diversity, gene flow, genetic structure of species, and comparisons among species. Allozymes have been used in studying outcrossing rates, population structure and population divergence, such as in the case of crop wild relatives. Despite being a good indicator of variation within the genome, because it is based on only a single set of genes, those genes may not be the most representative of variation within a species. Also, although these markers allowed large numbers of samples to be analyzed, comparisons of samples from different species, loci, and laboratories are problematic.

### **Molecular Markers**

Of the methods cited above, the most definitive way of measuring biodiversity, in particular intra-specific diversity, is at the genetic level where examination of molecular markers and their genetic distance from one another determines the degree of relatedness between organisms. This is of major significance to agriculture as it is varietal diversity which provides the range of adaptive traits from which farmers then select varieties appropriate for specific environments, nutrients, etc.

Molecular markers are divided into:

- Non-Polymerase Chain Reaction (PCR) based techniques: These include Restriction length Polymorphisms (RFLPs) and minisatellites analysis (also known as Variable Number of Tandem Repeats (VNTR) analysis).
- PCR-based techniques: These include DNA sequencing and Sequence-Tagged Sites (STS).

The latter method has undergone technological development and has spawned a variety of methods; the most well-known are microsatellite analysis (also known as Simple Sequence Repeats (SSR)), Random Amplified Polymorphic DNA (RAPD) analysis and Amplified Fragment Length Polymorphism (AFLP).

**Table 2. Comparative assessment of different molecular genetic screening techniques.**

Characteristics	Isozyme	RFLPs	RAPDs	Sequence-tagged SSRs	AFLPs	PCR sequencing
Development costs	Low	Medium	Low	High	Low	High
Level of polymorphism	Medium	Medium	Medium	High	Medium	Medium
Automation possible	No	No	Yes/No	Yes/No	Yes/No	Yes
Cost of automation	Low	Medium	Medium	High	High	High
Repeatability	Low	High	Low	High	Medium	High
Level of training required	Low	Low	Low	Low/Medium	Medium	High
Cost (\$ per assay)	High (2.00)	High (2.00)	Low (1.00)	Low (1.50)	Medium (1.50)	High (2.00)
Radioactivity used	No	Yes/No	No	Yes/No	Yes/No	Yes/No
Samples/day (without automation)	30-40	20	50	50	50	20

### What diversity is measured and the methods employed

- **Diversity in single genes**
  - Biochemical analysis
  - Mendelian analysis
- **Polygenic diversity**
  - Multivariate analysis of morphological variation in traits whose expression is determined by more than one gene
- **Latent diversity of genome**
  - Genealogical analysis
  - Analysis of cytoplasm donors
  - Molecular (DNA) analysis and probes

## What diversity is measured and the methods employed

### ▪ Pedigree complexity

- Genealogical characteristics

### ▪ Performance-based complexity

- Analysis of genotypic variance and genotype by environment interactions
- Analysis of yield variance at farm, district, national, or regional level

### ▪ *Ex situ* diversity

- Analysis of numbers of accessions within and among species
- Morphological analysis of accessions

### ▪ Spatial diversity

- Number of cultivars by percentage of area
- Percentage distribution of area by cultivar

### ▪ Temporal diversity

- Average age of cultivars
- Rate of cultivar replacement

## Genetic Considerations for establishing and managing germplasm collections

So far we have seen various factors that have a bearing on genetic diversity in conserved germplasm, and that genetic diversity is central to our conservation efforts. We have also noted certain genetic principles of plant genetic resources exploration and conservation especially those related to the structure and distribution of the genetic diversity of species to be conserved as well as the genetic diversity of the materials that are being conserved. Considerations for ensuring genetic integrity and maintenance of genetic diversity include issues like:

- Breeding systems
- Isolation requirements,
- Adequate number of trees,
- Genetic drift etc.

Nevertheless, we know very little on reproductive biology and breeding systems of many plant species. In addition, in the case of FGB for plant species, for practical reasons, different accessions may have to be planted together. Most often, in the conservation of perennial crops the main consideration is on elite material (maintained as clones) and less on genetic diversity found in different populations. Hence some of the stringent genetic considerations are often ignored. Nevertheless, a few of these issues for any eventual genetic diversity conservation of many plant species in genebanks are briefly discussed below.

**Range of diversity:** To establish a good field genebank, accessions displaying a range of diversity need to be planted. For this reason and from the point of population genetics, the principle of random sampling of genotypes from a given source population should be followed. In practice, both elite materials and genetic stocks are also kept in FGB. Therefore, as far as possible, even if the random sampling is not followed, it is essential to sample a range of diversity to be represented in the FGB. In addition, seedlings or clones of elite material may be included as selective sample.

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Thus the FGB can include populations/genotypes representing a range of diversity, elite materials, genetic stocks and some unique materials.

**Plot size and shape:** The size of the plot depends mainly on the breeding system and diversity in the sample and on the number of trees/plants planted. It is be most appropriate if the material planted in a field genebank can be representative of the source population. For raising seedlings and transplanting, the best methods should be used to ensure maximum survival and vigour. Larger plot sizes with more number of plants are better for outcrossing species. However, most genebanks are not really for very long-term conservation (compared to for e.g., cryopreservation), with 3-5 generations being realistic time frame. Hence smaller plots are acceptable. If characterisation and evaluation need to be combined with conservation, larger plot sizes as dictated by statistical principles should be used to avoid biases due to competition and xenia.

It is well established that square plots are better than row planting for reducing pollen contamination). The minimum number required per entry is the number of seedling/plants that represent the genetic diversity of the population while the maximum number is determined by the resources available for maintaining and managing the collection. The number of plants is also determined by the size of the original sample and the frequency of alleles to be conserved. There are a few genetic principles to be kept in mind while taking material out of the genebank for either testing in another location or for exchange as this has implications on the size as well as management of the FGB plot. From the point of genetic principles underlying regeneration, promoting random mating is recommended so as to increase the effective population size to mitigate the effects of genetic drift. However, in perennial species, cuttings or scions are used for distribution.

Some of genetic issues have been considered in this section. These considerations must be taken into account when making decisions. It is also clear that one cannot make a “practical” decision without influencing the “genetic” impact of the practice on the populations maintained in FGB. It is important that the curators or FGB managers are aware of the pros and cons of the decisions made.

True genetic conservation may not be possible but knowing the boundaries and being able to channel plant conservation on the basis of the knowledge and application of genetic parameters.

### **Summary of steps to be undertaken particularly for field genebank**

In the discussion presented here, it has been assumed that the collecting has been effectively carried out, keeping the sampling of genetic diversity in mind, and that all quarantine requirements have been completed. No attempt has been made to review extensively the information available on genetic diversity or quarantine problems and only brief description of conservation methods other than field genebank has been provided, as appropriate to the occasion. Since other methods of conservation are not fully viable, the emphasis is on establishing and managing field genebanks. So to establish, maintain and manage a field genebank for many species, the following critical checklist of steps is suggested. This is by no means an exhaustive list of steps to be taken but only the important considerations that determine the effectiveness and sustainability of the field genebank. These also assume that the issues considered in the earlier discussion were taken into account, decisions have been made and the consequences noted.

1. Agreement on precise functions of the collection
2. Selection of site, based on the criteria established (more on site selection in another lecture)
  - Agreement on obligations and responsibilities of host institutes and other stakeholders involved

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3. Establishment of infrastructure and facilities
    - Legal aspects and exchange protocols (ownership, conditions of release, IPR issues, benefit sharing, use of MTA and other mechanisms) as agreed by all partners
  4. Establishment of a FGB
    - Assure comprehensiveness of collection by including as much genetic diversity as appropriate from the sub-region
    - Consider carefully the sampling techniques (random vs. non-random, and the need for deviation)
    - Assure that there is no duplication of accessions as this directly increases cost of FGB
    - Determine the need for having replications, the number etc., based on the objectives of FGB
    - Determine through discussions and by actual visitations the accessions and the number of accessions to be included in FGB, and number of plants per plot
    - Establish nursery of vigorous and healthy seedlings
    - Lay out square blocks of equal size
    - Plan space for present and future accessions (as much as possible) to be randomized in the FGB
    - Follow all protocols for safe movement of germplasm
    - Ensure that grafting/rootstock production etc., facilities be put in place for exchange of material
    - Accept more material into FGB as they become available by going through all the steps discussed
  5. Maintenance of a FGB
    - Take all the necessary agronomic and plant protection measures to maintain a healthy stand of trees
    - Take all the measures feasible to protect FGB from adverse environmental conditions, physical stresses etc.
    - Make sure that a safety duplication is established and all the needs of health care are fulfilled
    - Document all accessions as well as activities carried out in FGB by establishing and running an appropriate information management system
    - Provide linkages to other methods of conservation, if any, such as in vitro conservation of zygotic embryos, pollen preservation etc.
  6. Ensuring access to material in FGB
    - Ensure physical availability of the material
    - Keep the plants in healthy condition
    - Characterise/evaluate the material in FGB according to agreed principles
    - Provide for production of propagules through established procedures
    - Make available the information on the material conserved in the FGB to all users
    - Exchange material using appropriate propagules that can be made disease free

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## Some specific considerations for *in vitro* conservation

### Genetic stability and integrity

While conserving plant genetic resources, it is important to maintain the genetic stability and integrity of target species and take care that cultural system should not cause genetic changes. For example, calli are not accepted as they cause genetic instability.

### Genetic changes

Genetic changes may occur in germplasm stored under *in vitro* conditions due to unusual selective environments of culture conditions and response of plant tissues *in vitro* is not predictable. Literature is replete with many observations on genetic stability caused mainly due to instability of undifferentiated tissues or single cells.

Somaclonal variation is another issue that needs to be guarded against. It is a general term for all variability generated in plants derived from culture and usually happens during dedifferentiation of cells at regeneration and is the cause of most concern in *in vitro* conservation. Presently, for most species culture methods are available that take care of this problem. Plants regenerated from meristems, shoot tips, axillary or apical buds are normally genetically stable. So genetic stability of *in vitro* cultures should be monitored with different genetic markers, the best would be DNA markers. Somaclonal variation can be minimized through culture of organized meristems initiated from terminal or axillary buds, protoplasts, leaf pieces or callus should not be used for conservation.

Management problems related to slow growth storage can be overcome through minimizing the workload of subculturing. Slow growth with frequent subculturing may cause variation in ecological adaptation and may lead to accumulation of mutations over time. This problem may not be true in the case of cryopreservation.

Impact of eliminating diseases and maintaining material in identical environmental conditions with little or no natural selections are not well understood. Similarly, the genetic consequences of acclimatization and elimination by acclimatization difficulties during the process of cryopreservation may lead to further genetic drift and hence one must guard against it.

Genetically, use of organized meristems such as terminal or axillary buds or growing points can help reducing genetic changes in conserved material. One needs to be aware of the problems of adaptation under different ecological conditions and acclimatization problems. In addition, it is important to be aware of genetic consequences of *in vitro* and cryopreservation and make efforts to reduce such impacts so that genetic diversity can be effectively conserved.

For successful conservation of clonally propagated material one must realize the problems in sampling genetic diversity as only small range of genetic diversity will generally be available in target gene pool and hence need to maintain larger number of accession. This basic principle is generally ignored under the assumption that clonally propagated materials tend to be homogeneous and homozygous.

In the conservation of material *in vitro* that are naturally seed producers but propagated through asexual means, one should focus on zygotic embryo conservation which is appropriate for genetic conservation. However, choice of material is dependent mostly on available technology. What is important is to be aware of genetic consequences of our conservation decisions.



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## FURTHER READING

- Brown, A.H.D., M.T. Clegg, A.L. Kahler and B.S. Weir. 1990. *Plant Population Genetics, Breeding, and Genetic Resources*. Sinauer Associates Inc., Sunderland, Massachusetts
- Frankel, O.H. and E. Bennet, eds. 1970. *Genetic Resources in Plants- their Exploration and Conservation*. IBP handbook No 11. Blackwell Scientific Publications, Oxford and Edinburgh.
- Ford-Lloyd, B. and M. Jackson. 1986. *Plant Genetic Resources - an Introduction to their Conservation and Use*. Edward Arnold, Baltimore, Maryland.
- Frankel, O.H. and J.G. Hawkes, eds. 1975. *Crop Genetic Resources for Today and Tomorrow*. International Biological Programme 2. Cambridge University Press, Cambridge.
- Guarino, L. V. Ramanatha Rao and R. Reid, eds. 1995. *Collecting Plant Genetic Diversity - Technical Guidelines*. CAB International, Wallingford.
- Japan International Cooperation Agency. 1989. *Exploration and Collection of Plant Genetic Resources. Part 1. Seed Propagated Crops*, JICA technical assistance activities for genetic resources projects.
- Mohd Shukor Nordin and Mohd Said Saad. Genetic considerations in filed genebank conservation. Pp. 66-72 *in* Establishment and Management of Field Genebank, Training Manual (Mohd Said Saad and V. Ramanatha Rao, Eds.) IPGRI-APO, Serdang.
- Ramanatha Rao, V. And B.M.C. Reddy. 2010. Tropical Fruit Genetic Resources Conservation. Pp. 83-98 *in* Horticulture to Hort-Business (K.L. Chadha, A.K.Singh and V.B. Patel, eds). Westville Publishing House, New Delhi.



## LECTURE 5

### Establishment, maintenance and use of field genebank

#### Introduction

Optimising genebank efficiency involves choosing between many different conflicting demands for limited resources i.e. managing different genebank activities efficiently. For example, allocation of resources to running or improving management of a genebank must be assessed against allocating those resources to networking with other genebanks. The potential benefits of investment in collecting new diversity must be judged against investing in better conservation or utilisation of existing collections. Such broader issues are beyond the scope of this publication, which focuses on the issue of optimising the genetic information contained in a given set of accessions i.e. a collection.

#### Objectives

- Understand the options and considerations in developing effective collection and genebank management strategies;
- Discuss the important elements of management of the genebank and the collections;
- Discuss options for efficient and cost effective management of field and *in vitro* collections
- Discuss issues related to accession identification and characterisation, collection rationalization, core collections etc.
- Understand the need and components of a facilities operation manual and general distribution policies

#### Description of lessons covered

This section deals with the general topics of collection and genebank management. The later is as applied to field and genebanks (*in vitro* will be dealt separately). Most of the collection management discussed applies to *in vitro* genebanks as well. Information is included on accession identification and characterisation, collection rationalization and the designation of core collections. Additionally, the development of a facilities operation manual and general distribution policies are discussed. 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.

#### Lessons to learn

- General principles of field genebank management
- Development of field genebank operation manual

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

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The identification of an accession provided by the collector or donor be rechecked, if necessary, by crop experts and/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. 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**

Each accession in the collection should be characterized using standard descriptors. Bioversity has produced a series of descriptor lists, which provide an international format and a universally understood 'language' for plant genetic resources data. Bioversity and FAO have also published a List of Multi-Crop Passport Descriptors, which is a reference tool to provide international standards to facilitate germplasm passport information exchange across crops. Bioversity has also developed Descriptors for Genetic Markers Technologies which help in standardizing and documenting information about genetic markers. If published descriptors are not available for particular crop/species, it would be worthwhile first to develop a list at country or genebank level and use it for characterisation to conform to standards agreed by the researchers who work on the particular plant species.

Characterisation is mainly for diagnostic purposes, and helps identify and eliminate duplicate accessions, renders the collection more useful to plant breeders and may improve plant maintenance. Nevertheless, in some genebank staff need to be very careful about eliminating duplicates (it is easier said than done), characterisation descriptors along with passport data some level of rationalization can be attempted. In some cases, chromosome counts are helpful or required, especially while dealing with less known taxa and polyploidy species. Cooperative research with nearby universities may provide evaluation data.

### **Make characterisation data accessible**

Characterisation data should be available to plant breeders, agronomists and horticulturalists for selecting breeding material and improving crop types and to other researchers for further studying the genetic resources. Ease of access to characterisation data makes 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.

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. For example, the accessions in a genebank could be sorted out using passport information and morphological data using computerized database containing data on key morphological characters. This helps in sorting out similar accessions coming from the same geographical area. These potentially duplicate accessions could then be further compared for all visible characters under the same environment, preferably in more than one location and for more than one season (depending on the number of quantitative traits involved (to reduce the genotype X environment interactions).

Morphologically identical accessions should also be compared by electrophoretic analysis of their total proteins and some key enzymes extracted from appropriate plant parts. This can be further refined by the use of molecular markers. Any efforts to eliminate duplicates and/or rationalize collection should be based on sound scientific principles and not on arbitrary decisions.

New acquisitions should be compared right away 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 and hence at least one or two growouts in one location may be necessary. 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.

### **Identify core collection**

Core collections are a subset of the entire collection representing the range of genetic diversity. The accessions chosen should 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. 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. Most often than not the need for a core collection depends on 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. It is important to note that the development of core collection is not mandatory for a genebank with fewer accessions which is known to be not representative of the diversity. It is not a tool to reduce the number of accessions in a genebank but a way to make increased use of accessions in very large collections which becomes difficult to sample from. For field genebank, most often it is not needed as the number of accessions would generally be low and also it would only be a list of accessions that figure in the core; it is not a separate collection to be planted and managed.

### **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. This manual should also record the processes described earlier, such as identification of material etc.

**Develop protocols:** Staff members can detail the work that they do, including step-by-step procedures for complicated tasks. It is very important to create a documentation system even before the first accession arrives on site. 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 inclusive of data from collecting expeditions, field management, characterisation, etc. This should be easily accessible to the genebank staff and interested researchers. 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.

### 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?](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](http://www.ipni.org/ipni/query_ipni.html)

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

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

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

### Develop labelling and numbering system:

Computer-generated labels from authenticated lists of species and cultivars helps avoid spelling and numbering errors. Each accession should be identified separately, even though it may be a duplicate cultivar from a different source. Separate types of labels may be required for field and *in vitro* accessions, but the same accession number should be used. Any material used for labelling should be long lasting.

**Train staff:** Well-trained staff are especially important to maintain the health of the plants, detect diseases and perform standard characterisations. The type and level of training can 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.

### Distribute germplasm

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.

### Important genetic evaluations include:

Disease resistance

Insect & mite resistance

Soil pest resistance & tolerance to soil environments

Plant hardiness & tolerance to other environmental stresses

Plant size, shape & productivity

Crop quality & nutritional factors

**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 require cancellation of the order.

Waiting to check regulations until the shipment is ready to send can 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, although the conditions for access and benefit sharing are yet to be finalized. Use a material transfer agreement (MTA) while distributing material. If your genebanks deals with plant species that are not in the Annex 1 of the Treaty, then follow guidelines for them as developed by your country or institute.

**Solicit information on plant evaluation and characterisation from users:** The users of the germplasm normally 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 along with propagules. Associated information about an accession adds to its value to a plant breeder or scientist. Accurate identity, pedigree, evaluation, and characterisation 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.

### Field procedures

Below are various genebank management procedures that apply to both field genebank and *in vitro* genebank. This may not be exhaustive, but if you do additional activities your genebank, add those to the operational manual to keep track of what happens in the genebank on day to day basis. Any pertinent observations and adaptation/modifications to the processes carried out can be added to the manual. This record keeping is important so that the genebanks function continuously in case of movement of critical staff and avoids the need to work from memory of one or more persons which can be easily lost, leaving the rest of the staff in confusion. 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.

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## 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. A well-managed nursery should be established. 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 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 can be healthier and safer if planted under appropriate soil and climatic 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 for a facility is influenced by crop type and use. For example, outcrossing 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 information on pests, is helpful. The site should be easily accessible and should be free of any legal problems such as ownership etc. It would be useful to project land use change and developmental activities in the vicinity to prevent any damage to the land in the near future.

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 establishing 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.

**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 pests. Such protection, when necessary, is provided best under screenhouse (SH) culture. The need for an SH depends on whether growing in the field would expose the plants to lethal or debilitating pests or temperatures. This question must be answered for each different crop collection. For crop species susceptible to viruses that are transmitted by insect vectors or pollinators, the screenhouse offers protection when used properly. 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 soil-borne, move susceptible plants to fumigated plots. If disease affects foliage, treat susceptible accessions with pesticides more frequently. After harvest, disinfect and treat propagules to prevent storage losses.

**Select appropriate planting procedures:** Field preparation and spacing 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 and/or actual barriers to prevent clones from mixing. Accessions with different morphologies may be planted in adjacent plots for ease of identification when creeping or spreading occurs. Invasive clones may require planting in cans, pots or boxes or even concrete structures to reduce mixing or competition with less vigorous accessions. Accessions may need to be planted in groups according to vigour, height, branching habit or lodging tendencies and similar harvesting period. Replicates of perennial crops may be planted together to allow ease of 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 depend on the crop and the intended uses of the collection (conservation, evaluation, distribution). Follow standard cultivation practices for particular crop species. However, 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 characterisation 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. A well-designed integrated pest management (IPM) programme helps decrease the chemical pesticide use and protect workers and the environment.

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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. Rouging diseased plants from fields can decrease the spread of certain pathogens. Insect-borne viruses can be contained only in screened enclosures. Virus elimination procedures are discussed in an earlier section. Soil fumigation may be required for some crops.

**Harvest and store propagules properly:** Crops 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.

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.

### **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. 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, characterisation data should be used to determine which the correct propagule is. 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 usually have some members that are variable. These should be noted in the database and carefully monitored. Herbarium specimens or photographs of accessions may also be useful for verifying the identity of questionable specimens.

Accessions from which seeds are collected for propagation (grasses and legumes) can lose genetic information over time if too few individuals of an accession are maintained and self pollination occurs in a percentage of cases. This problem is less in accessions that primarily apomictic.

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.

**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. Most collections 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 helps 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 reporting effort helps minimize errors.

**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. Accessions must be managed to provide propagules as well as to maintain healthy collections. 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 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. Fragile propagules may require express delivery services.

### **Constraints and research needs**

As discussed before, field genebanks are very costly to maintain and are, in general, very susceptible to losing accessions for a variety of reasons. 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 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.

Pest and diseases are the major problems affecting field genebanks. Accessions introduced from different locations may be infected with diverse arrays of pathogens. Infected accessions can become major sources of pathogens that can be transmitted to other accessions.

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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.

### **Further Reading**

Reed, B.M., F. Engelmann, M.E. Dooloo and J.M.M. Engels. 2004. Technical guidelines for the management of field and *in vitro* germplasm collections. IPGRI Handbooks for Genebanks No. 7. IPGRI: Rome.

Saad, M.S. and V. Ramanatha Rao, Eds. 2001. Establishment and Management of Field Genebank, a Training Manual. IPGRI-APO, Serdang. 122P

## LECTURE 6

# Detection, diagnosis and elimination of plant viruses for vegetatively propagated germplasm

### Introduction

One of the important aspects of good management of genebanks is the health status of plant genetic resources conserved and health certification procedures adopted for material acquired and distributed. In maintaining and distribution of pest free germplasm, plant quarantine procedures play an important and critical role. Plant health staff should recognise the intrinsic value of plant genetic resources and that germplasm exchange promotes crop productivity through new crop varieties.

### Objectives

- Discuss germplasm health issues and consider that the health status and susceptibility of the plants when deciding how to conserve them
- Understand procedures that are important in establishing and maintaining a healthy collection
- Discuss and describe the importance and ways and means for providing pathogen-free plants to those that request for germplasm
- Understand that the major difficulty is often the lack of techniques or antiserum for specific viruses

### Description of lessons covered

#### Research needs include:

- Causes of genetic instability
- Cryopreservation & other alternative conservation techniques
- Causes of seed recalcitrance
- Low-input maintenance strategies
- Improved storage facilities for annual crops
- Improved crop descriptors & descriptors for non-Annex1 crops
- Extension of storage time of planting material
- Improved disease indexing

This lecture covers germplasm health issues and procedures that are important in establishing and maintaining a healthy collection and providing pathogen-free plants to those that request for the same. 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.

#### Lessons to learn

- Understand the importance and role played in establishing and maintaining pest-free, as far as possible, germplasm collections.
- Health issues and procedures that

need to be considered while establishing and managing genebanks, in particular field genebanks.

## Research needs

**Adaptation to environment:** 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 taxonomic identity of accessions of many unknown wild relatives poses problems and taxonomic research along with ploidy information is needed for their proper description and management.

**Reproductive biology:** 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:** Research on genetic diversity can help define core collections for field collections, thereby reducing collection size and costs; can help better characterize the field collection and identify gaps in collections; can also help determining the causes for genetic instability, including mutations, drifts and shifts in field collections.

**Genebank management:** Research is needed to improve management and maintenance of field collection: methods of propagation, cultural practices, protocols need to be established for maintaining an accession according to its breeding behaviour, recovery of fertility in herbaceous plants, improved storage facilities for annual crops and the extension of storage time of planting material before planting out.

**Economics:** Research on alternative methods for conservation (e.g. seed, *in vitro*, cryopreservation or *in situ* conservation), studies seed physiology to define conditions to allow conserving seeds rather than whole plants and thus reduce necessity of maintaining field genebanks.

## General

### Important practices

- Considerations when sourcing material
  - Risk assessment of intended imports
  - Type of material being acquired
  - Source of material being acquired
  - Final destination of material
- Isolation and quarantine
  - Basic hygiene and best practice
  - Isolation and quarantine facilities
  - Isolation and quarantine procedures
  - Identification of problems
  - Treatment methods
  - Disposal methods
  - Education and staff training

## Germplasm health issues

While establishing and managing *in vitro* and field genebank, issues related the health of germplasm conserved need to be considered. In the case of the former, issues involved are mainly ante-conservation (i.e. prior to storage), while in the case of field genebank, health issues need to be considered from before introduction into genebank to distribution of pest free germplasm.

It would always be best to establish field genebank in generally in areas free from important pests if possible for two main reasons. One is the risk of the entire collection, or part thereof, being destroyed by pests or diseases. The other is the risk of spreading pests and pathogens to new areas, which may easily happen with exchange of infected germplasm. However, it may not always be possible to establish field genebank in pest free areas and hence germplasm health issues need to be dealt with from the start of the genebank. Germplasm health aspects need to be considered not only at the point of exchange, but at any stage of germplasm management. During collecting, care must be taken that germplasm is collected only from healthy trees. In the regeneration and multiplication process, plant protection measures including pesticide application may be required. If an evaluation of traits like resistance to pathogens is done under conditions of high disease pressure, e.g. with artificial inoculation, a careful evaluation of the material with regard to its use in regeneration or exchange is essential. Here we will discuss requirements to maintain germplasm health in a collection.

### 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.

Small facilities without resources to hire a pathologist, entomologist etc, options, such as establishing linkages with university laboratories need to be considered. 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 published jointly between FAO and BIOVERSITY.

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. 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.

### Detection, diagnosis and elimination

**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.

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**Diagnosis and identification:** The lack of rapid, accurate, and reliable means by which plant pathogens can be adequately detected, identified, and quantified is one of the main limitations in plant disease management. Traditionally, the most predominant techniques used to identify plant pathogens relied upon culture-based morphological approaches or graft transmission. The major limitations of these methods, however, are the reliance on the ability of the organism to be cultured, the time consuming and laborious nature, and the requirement for extensive taxonomical knowledge, all together often complicating timely disease management decisions.

In the last ten years there has been an explosion in plant virus outbreaks, because of either direct or indirect activities of humans. The ease of transporting plant materials and virus vectors, and collecting relatives of crop plants from the wild, has spread viruses widely throughout the world. The most important step in managing a virus disease is correct identification. Tests for diagnosis should be fast, accurate and inexpensive. Current diagnosis techniques are broadly divided into serological procedures, nucleic acid procedures and combinations of both.

The techniques available have evolved significantly in the last few years to achieve rapid and reliable detection of pathogens, extraction of the target from the sample being important for optimising detection. For viruses, sample preparation has been simplified by imprinting or squashing plant material or insect vectors onto membranes. To improve the sensitivity of techniques for bacterial detection, a prior enrichment step in liquid or solid medium is advised. Serological and molecular techniques are currently the most appropriate when high numbers of samples need to be analysed. Specific monoclonal and/or recombinant antibodies are available for many plant pathogens and have contributed to the specificity of serological detection.

Molecular detection can be optimised through the automatic purification of nucleic acids from pathogens by columns or robotics.

New variants of PCR, such as simple or multiplex nested PCR in a single closed tube, co-operative-PCR and real-time monitoring of amplicons or quantitative PCR, allow high sensitivity in the detection of one or several pathogens in a single assay. The latest development in the analysis of nucleic acids is micro-array technology, but it requires generic DNA/RNA extraction and pre-amplification methods to increase detection sensitivity. The advances in research that will result from the sequencing of many plant pathogen genomes, especially now in the era of proteomics, represent a new source of information for the future development of sensitive and specific detection techniques for these microorganisms. Molecular techniques can circumvent many of these drawbacks, especially if they make use of the polymerase chain reaction (PCR).

In general, these methods are much faster, more specific, more sensitive, and more accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. Perhaps even more important, these techniques allow detection of nonculturable microorganisms. However, whereas these methods are routinely used in the diagnosis of human diseases, they are not yet widely used for routine plant pathogen detection. One of the reasons is that, although generally most of these assays are reliable, they target only a single pathogen, making comprehensive screening of complex samples relatively unprofitable. Therefore, the major challenge currently is the development of multiplex pathogen detection assays that, in addition, allow quantification and are suitable for implementation in practice.

Let us look at the procedures in some order:

### **Serological procedures**

**Enzyme-linked immunosorbent assay (ELISA):** ELISA is not a new technique, although it is widely used throughout the world because of its accuracy, simplicity and low cost. The technique utilizes the ability of antibodies raised in animals to recognize proteins, usually the coat protein, of the virus of interest. Antibodies are fixed to the surface of a well within a microtitre plate, and a sap extract from the plant is added to the well. If the virus of interest is present in the plant, it will bind to the antibodies fixed on the surface. Any unbound extract is washed-off before a secondary antibody that recognizes the first antibody is added.



The secondary antibody allows for indirect detection of the virus because it has a reporter molecule attached to it, usually an enzyme that acts on a substrate that changes colour, which is detected visually by a calibrated microtitre plate spectrophotometer. With careful calibration, ELISA can be quantitative as well as qualitative. This method can be used for testing multiple plants for a single virus using one well per plant sample, or alternatively a single plant can be simultaneously tested for many viruses on a single plate with different antibodies coated to each well in duplicate or triplicate for reproducibility. The major constraint of the method is the requirement for polyclonal or monoclonal antibody sera specific for each virus of interest that does not cross-react with plant proteins, but cross absorption with plant sap avoids this problem substantially.

**Tissue blot immunoassay (TIBA):** Tissue blotting, like ELISA, utilizes antibodies raised against viruses. Sap from the plant tissue is expressed onto blotting paper; nitrocellulose or nylon membranes and the virus is detected by labelled probes, often chemiluminescent. The procedure is less labour-intensive than ELISA, rapid, sensitive, simple (no virus extraction is required), inexpensive (minimal equipment is needed), suitable for surveys of 1000 to 2000 samples per day, and the samples can be taken in the field and processed some time later.

Kits are available for a number of viruses, notably from the International Centre for Agriculture Research in the Dry Areas, which offers kits for 19 viruses of legumes. Quartz crystal microbalance (QCM) immunosensors In this novel technique for plant virus detection, a quartz crystal disk is coated with virus-specific antibodies.

**Quartz crystal microbalance (QCM) immunosensors:** In this novel technique for plant virus detection, a quartz crystal disk is coated with virus-specific antibodies. Voltage is applied across the disk, making the disk warp slightly via a piezoelectric effect. Adsorption of virus particles to the crystal surface changes its resonance oscillation frequency in a concentration-dependent manner. It is therefore qualitative and quantitative. The developers of the technique claim that it is as sensitive but more rapid than ELISA, and economical. In the first described use of QCM for plant viruses, as little as 1 ng of particles of Cymbidium mosaic virus and Odontoglossum ringspot virus were detected in crude sap extracts. .

## **Nucleic acids procedures**

**Reverse transcription–polymerase chain reaction (RT–PCR) and PCR:** RT–PCR and PCR are popular techniques for detection and identification of RNA and DNA plant viruses respectively. The procedures are extremely sensitive, fairly inexpensive and require minimal skill to perform. In the case of RNA viruses, a cDNA strand complementary to the virus is made with reverse transcriptase (RT). Oligonucleotide primers, flanking part of the genome of the virus, are extended by a thermostable DNA polymerase in a series of denaturation and extension steps that exponentially increase the target DNA. For DNA viruses, the RT step is unnecessary.

PCR-based methods can be adapted to high-throughput applications<sup>5</sup>. In addition to detection of the virus, an additional advantage of the method is that the amplicon can be sequenced to provide further data about strain types. Possible drawbacks of the method are the need for a thermocycler, which can be expensive, and sequence information for design of primers. With databases containing ever-growing numbers of virus sequences, access to sequence information for many viruses is possible. Careful primer design is crucial, whether to detect only a single strain, or all the members of a genus.

The sensitivity of the method is its major advantage. An RT–PCR assay of Cucumber mosaic virus in lupin grain was able to reliably detect one infected seed in one thousand healthy seeds. High sensitivity can easily lead to false positive results from contamination; so adequate controls are essential. Knowledge of the nucleotide sequence is required in order to design oligonucleotide primers.

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Reverse transcriptase synthesizes a cDNA strand from which a fragment flanked by the primers is amplified by a thermostable DNA polymerase under a cyclical temperature regime. The amplicon is visualized on an agarose gel. There are a number of variations on the basic technique, designed to increase sensitivity, alter specificity or allow automation of detection. The most common are listed below.

- Multiplex RT–PCR: Multiple species or strains are detected in a single reaction by combining oligonucleotide primers specific for different viruses.
- Fluorescence RT–PCR using Taqman<sup>®</sup> technology: Two primers flank the sequence of interest and a third fluorescently labelled primer anneals between them.
- Competitive fluorescence PCR (CF–PCR): This is a variation on the above technique. It is used to simultaneously differentiate between virus strains and multiple virus infections.
- Immunocapture PCR (IC–PCR): This combines capture of virus particles by antibodies with amplification by PCR.
- Nested PCR: In this method, two PCRs are carried out with the first reaction increasing the amount of template for the second.
- Restriction fragment length polymorphism (RFLP): RFLP is used in combination with PCR to identify differences between viruses based on the presence or absence of restriction enzyme-recognition sites.
- Labelled probes: Nucleic acid hybridization of DNA or RNA probes has the advantage of being able to detect the nucleic acid of the virus in both forms, single-stranded and double-stranded.
- Arrays: Arrays, both microarrays and macroarrays, have been used for some years as a tool for visualizing relative changes in global expression levels of mRNAs, as well as single nucleotide polymorphism typing and host–pathogen interactions. Microarrays are high-density arrays with spot sizes smaller than 150 microns. Macroarrays are generally membrane-based with spot sizes of greater than 300 microns.
- Probes for microarrays: Because the employment of array technology for plant virus detection is recent, commercial plant virus arrays are not available and therefore must be made individually. Of primary importance in making an array is probe design. Probes determine the sensitivity of the array and the amount of information that they provide.

As has been seen in the last ten years, plant viruses are becoming more widespread and there are real threats of new virus epidemics. It is therefore essential that the movement of viruses around the world be documented and quarantine restrictions put in place where necessary.

**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. 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. Concerned staff should keep abreast with research on diagnostic methods so that cheaper and more effective methods could be adapted over the years.

## 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. 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. 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.

### Further Reading

George, E.F. 1993. Plant propagation by Tissue Culture – Part I. Exegenetics Limited, London, UK.

George, E.F. 1993. Plant propagation by Tissue Culture – Part II. Exegenetics Limited, London, UK.

I-APO, Serdang. 122P

Lievens, B. and B.P.H.J. Thomma. 2005. Recent Developments in Pathogen Detection Arrays: Implications for Fungal Plant Pathogens and Use in Practice. *Phytopathology*. y 95:1374-1380.

Reed, B.M., F. Engelmann, M.E. Dulloo and J.M.M. Engels. 2004. Technical guidelines for the management of field and *in vitro* germplasm collections. Handbooks for Genebanks No. 7. IPGRI: Rome.

Saad, M.S. and V. Ramanatha Rao, Eds. 2001. Establishment and Management of Field Genebank, a Training Manual. IPGR

Webster, C.G., S. J. Wylie and M.G.K. Jones. 2004. Diagnosis of plant viral pathogens. *Current Science*. 86(12): 1604-1607.



## LECTURE 7

### Management of diseases in field genebank

#### Introduction

Several countries have established field and *in vitro* genebanks. Many of these are managed by norms set by the respective institutions in which they are located and operate with varied degrees of efficiency. It would be important for the participants to understand that it is not just enough to maintain maximum genetic diversity of crops of their interest by they must be maintained free of pests and diseases and all efforts must be made to distribute pest free propagules.

#### Objectives

- Understand the pest problems of genebanks and how to deal with them
- Understand that the pest can not only result in losses of material conserved (in some cases may result in total loss) but also affect the use of germplasm conserve

#### Description of lessons covered

Participants will be briefed on pest and disease problems that affect genebanks and ways and means to deal with them. It will also touch on the importance of producing disease free material both for acquisition as well distribution.

#### Lessons to learn

- Better understanding of genebank management and health state of the collection.
- Methods for control of pests in genebanks and production of pest free material.

#### Field management

- Keep the plots well weeded to avoid competition.
- Eliminate plants growing off-row. Rogue plants that are genuine mixtures.
- Irrigate the field after establishment of the plants and when subsequently needed.
- Do not allow the leaves to wilt at any stage.
- Apply fertilizer at the recommended rate for the crop in that area.
- Inspect the field regularly and preferably daily to ensure that the plants and plots are in good condition and take action quickly if any problems are noted.

#### Pest and disease control

Pests, diseases and weeds must therefore be controlled by other means during germplasm regeneration. Lines, which are highly susceptible to a prevalent pest or pathogen, could be wiped out, although they may be sources of resistance to other pests or pathogens, or have other useful characters.

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Curators are often reluctant to use pesticides, which might be phytotoxic to germplasm. To avoid the use of pesticides, hand weeding and regeneration of germplasm at locations unfavourable for epidemics are recommended.

The various resistant and susceptible lines may carry pathogen races of different virulence. The question of races is important because crop resistance may break down upon introduction of new races. Unfortunately, in the centres of genetic diversity of crops their pathogens are equally diverse and particularly virulent races may be found there. Field inspection of germplasm is different from that of commercial seed production. If a seed-increase field of several hectares is planted with one variety and properly sampled and carefully inspected, one can obtain reliable information on the disease situation in the field with germplasm regeneration. However, thousands of lines, which differ greatly in their susceptibility to pests and pathogens, are grown in a small field.

Sample areas for inspection are therefore inadequate. Each one must be inspected separately, which is a time-consuming task. As with all field inspection, it is difficult to detect diseases occurring at a low incidence, and yet it is important from a quarantine point of view.

Vegetatively propagated material, either as plants, corms, cuttings, bulb or buds, is assessed as the highest risk category involving the potential for spread of all stages of all types of pests such as insects, mites, fungi, bacteria, nematodes and viruses. In allocating pest risk assessments to planting material from a number of sources, recognized that materials grown under some kind of supervised cultivation have lesser risk than those collected in the wild. This is primarily because the former would have little, if any, pest occurrence.

Crop protection is an important aspect in field genebank management. At all stages of crop growth they are susceptible to pests. Prevention is an important step in controlling infestations and some phytosanitary measures should be practised. There are several methods to control pests, chemical control being still the most popular. The present trend of pest control is integrated in approach, i.e. utilisation of more than one method of control such as combination of chemical and biological. Wild boar, monkeys, birds, bats and insects are common pests that can damage the plants and feed on flowers and fruits and therefore interfere with data collection.

- Coordinate periodic field inspections with pathologists and virologists during the growing season.
- Spray with appropriate chemicals when necessary. Spray with fungicide to control mildew during the rainy season or when using irrigation and with insecticide at the first sign of insect damage.
- Rogue out any infested material to eliminate all parasites before harvesting any plant material and burn the residues.

### **Pests – checklist**

The increasing international trade in agricultural products is inevitable. Thus to prohibit possible pest entry most developed countries have imposed strict quarantine restrictions. Developing countries have also been imposing plant quarantine regulations but, in most cases, not as stringently as in the developed countries. Most countries have national and regional lists of pests. For such pests, close vigilance is maintained by plant quarantine services of many countries to guard the country against entry of these exotic pests, while those found in certain areas in the region are kept in seclusion.

Many countries are largely dependent on agriculture. If pests could slip through ports into our crop and, then the outcome could be disastrous. For instance, if by sheer accident, the dreaded South American Leaf Blight that is present in the tropical America, is introduced into Malaysia, the multiplier effect of one such accident will spill over the border into Thailand, Indonesia or even the Philippines and other countries where rubber is a thriving industry.

## Staff training

The disease situation in genebank, especially field genebank, can be dynamic. Hence staff needs to be well trained in modern methods of pest control with minimum usage of pesticides. They must well-versed with integrated pest management aspects so that the genebank is maintained as much as possible pest free and be able to deal with occasional outbursts of pests. This will need continuous updating of their skills and regular refresher course.

In the case of *in vitro* collections, once clean material is entered into genebank, controlling pests in collections may be of less importance than cross-contamination. However, when propagules are produced for the purpose of exchange of material all care must be taken, including pest management.

## Conclusion

A germplasm collection usually consists of material from different origins. Frequently, information on the distribution of pests and pathogens in the country is lacking or incomplete. This is more so with plant materials from the forests, where very little or no information is available with regards to their pest and disease status. It is thus difficult to determine the appropriate test methods. Therefore, Plant Introduction Stations are often faulted for the introduction and spread of new plant pathogens.

Detection techniques, which are acceptable to plant quarantine, must be reliable, efficient, simple and technologically feasible. It would be of great help if the plant quarantine officer has adequate knowledge on the current disease status of the materials imported.

Regardless of the affiliation, importers of germplasm and propagated plant material must strictly follow the Plant Quarantine Regulations in their respective country and in addition they must observe the Importation Regulation of Plant Materials of the Department of Agriculture. Ideally all consignments should require post entry quarantine, including appropriate treatments upon arrival. Subsequent inspections and detections for pest should be carried out. Certain material can be detained in the post entry quarantine screen house for a period of time for symptom development and indexing for virus disease. Materials are normally released after they have been certified as pest free.

A combination of the activities of plant pathologists and plant breeders is required to ensure that clean material is exchanged on a worldwide basis, and the cooperation already underway has reduced the disease compliment of some crops. The technique of tissue culture, being an effective and efficient method of transferring and sorting genetic material still has a degree of risk and should be combined with other psychopathological techniques. Presently there is no inventory on pests and diseases on plants grown in wild. A concerted effort is needed for listing of imported pests and pathogen generally present in these species.

## Further reading

Reed, B.M., F. Engelmann, M.E. Dulloo and J.M.M. Engels. 2004. Technical guidelines for the management of field and *in vitro* germplasm collections. IPGRI Handbooks for Genebanks No. 7. IPGRI: Rome.

Saad, M.S. and V. Ramanatha Rao, Eds. 2001. Establishment and Management of Field Genebank, a Training Manual. IPGR





## LECTURE 8

# Management of *in vitro* collection and cryopreservation

### Introduction

It has been recognized that for most of the difficult-to- conserve species, such as the ones with recalcitrant seeds, asexually propagated ones, seed conservation is not possible. *Ex situ* conservation options for this type of plant species are field genebank or *in vitro* genebank. So far we have discussed about the establishment of management of field genebank. Now we turn to how best we can utilize the *in vitro* option for conserving the genetic diversity in difficult-to- conserve plant species.

### Objectives

- Understand the method of *in vitro* conservation
- Understand the management process of *in vitro* conservation and cryopreservation

### Description of lessons covered

This section described the physical requirements for a plant tissue culture laboratory that is an essential part of *in vitro* genebank 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. The longer the plants can be stored without transfer or subculture, the lower the cost and the more secure the accessions. This section discussed the development of tissue culture systems, research techniques, procedures and record keeping. Cryopreservation may be used as base collection storage for clonal crops. Immediate research needs are also discussed.

### Lessons to learn

- Better understanding of *in vitro* genebank management
- Generation of new ideas that can enhance efficiency of *in vitro* genebank development and management

### *In vitro* procedures

**Laboratory facilities:** *In vitro* culture and storage facilities are quite variable. This section highlights the important points to consider and provides examples from *in vitro* genebanks.

A basic laboratory requires a clean, tightly constructed room in a building with adequate lighting, electricity, heat/cooling system, water and ventilation. 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.

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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. Some are mentioned below briefly. For more details consult Reed et al (2004).

- Sterile transfer facilities are of utmost important and this can be done through **designing** to minimize foot traffic and outside airflow into the room where transfers are done and laminar flow cabinet is necessary for any sterile work.
- Equipment should include laminar flow benches, pH meters, balances, sterilization equipment, 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.
- For cryopreservation, a reliable source of liquid nitrogen (LN), tanks, dewar, vials and specific chemicals will be needed in addition to tissue culture facilities.
- 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.
- Culture growth rooms with temperature control, lighting and shelving, as well as culture storage rooms are needed.
- Light requirements range from 10 to 1000 lumens m<sup>2</sup> but most plant cultures require 50–200 lumens. Some research may be necessary to determine the appropriate levels for the genera in question.
- Ventilations systems or air conditioners are needed to regulate temperature, but the air should not flow directly onto the cultures. Common growth room temperatures range from 22 to 28°C, but will depend upon the requirements of the genera.
- 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.

### **Culture storage facilities**

The storage of *in vitro* cultures, whether in warm or cool conditions, requires certain precautions.

- Cold-storage facilities require reliable and fail-safe equipment and alarm systems. Whether the storage is at 15°C or 0°C, a malfunction can overheat or freeze valuable cultures.
- Cultures that are stored at growth room temperature require the same monitoring as those in the cold room.
- 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.

### **Staffing**

The education and experience level of the tissue culture staff will affect the quality and efficiency of the laboratory operations.

- All lab staff should be trained in various aspects of lab procedures, although some level of specialization may be necessary, to avoid breakdown of work if one or two staff go away.

- Hold regular refresher courses to update staff on new techniques that may become available over the years.
- The number of personnel required will depend upon the collection's size and diversity, the amount of research conducted and the results desired.

## 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. Many problems that arise in tissue culture laboratories can be eliminated through careful maintenance and cleanliness of the facilities and equipment.

**Develop a procedures manual:** As noted in the case of field genebank, all procedures and recipes should be written out in detail and placed in a procedures manual as a reference guide for workers.

**Establish a tissue culture system:** The wide diversity of genetic resources available in genebanks requires 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.

**Research techniques:** To begin to develop media recipes and protocols, it is necessary to compile literature on the *in vitro* techniques pertinent to the genera to be collected and grown in culture. A reference library of pertinent literature and reference books is important for developing and improving protocols.

**Develop protocols:** Protocols and media for initiating, multiplying, rooting and storing plants from each species or group of accessions must be developed. Standardised stock solution recipes are essential for repeatable media. Standard protocols for storage and repropagation should be written down and dated.

**Design a record-keeping system:** 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.
- Keep track of each accession's explanting date, source, initiation medium, multiplication etc. using the *in vitro* database identifying plants by the same labelling system as the field genebank to traced back to the mother plant when necessary.
- Identify all possible sources of labelling errors and minimize them.

## Explants

- Collect plant material for tissue culture from vigorous, healthy mother plants, tubers, corms, etc. *In vitro* collecting may be useful in some cases.
- Discover through experience the number of explants that must be collected to obtain the number of plantlets needed for establishment, multiplication and storage.
- Number of explants needed may vary with season, climate, propagation rate, crop, genetic stability, demand or distribution rate, facility, stress and storage situation.

- Identify It is important to use properly identified material with passport data and, when it is available, with plant health and other characterisation data.
- The explants need to be surface disinfested and chlorine bleach with a surfactant added is the most common and safest disinfectant for laboratory workers.
- 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.
- 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*.
- 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.
- Explants should be placed on a medium that favours microorganism growth. 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.
- 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.
- 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.

### **Multiplication**

- 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.
- Genebanks with diverse genotypes usually require additional research to determine techniques suitable for a range of genotypes
- Data collected on explants should include initiation date, medium, growth conditions, hormones and parent plant health. Morphological characterisation 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.

### **Culture storage**

Some of the options for storage of cultures and procedures for determining storage conditions are listed below. For more information

## Slow-growth storage

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.

- Carry out an extensive literature review on slow-growth techniques.
- Note that the available storage options vary from facility to facility.
- Each genus has specific requirements but often, standard techniques for related plant types can be successfully used with some modification
- Determine an appropriate storage temperature for a genus or species by linking 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
- The most economical solution may be to create minimal conditions in which all the genera will survive at a desired level.
- Pretreatments are often useful for improving storage duration.
- There are no specific rules on the number of replicates that should be held in storage. Determine the number of replicates to store by finding out the number that are lost during a given time period.
- Suggested methods for sub culturing are:
  - One or a few are removed and subcultured or
  - Half of the replicates are subcultured at one time and the remainder are held until it is known that the first group is healthy and growing.
- Maintain an inventory of stored plants and monitor to assess their viability and need for reculturing.
- As storage length varies greatly within genera and species, determine the shortest period between which reculture within the genus and then schedule inventories accordingly.
- Standardise what data are taken and how the inventories for each genus or species are conducted to gain valuable information on the collection that can help 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.
- Note that duplicating the collection in at least two places for safety is very important.
- 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.
- Monitor genetic stability. Note that somaclonal variation, while a problem with plants regenerated from single cells, callus or adventitious buds, is not common in plants micropropagated from axillary buds. Monitor more closely those plant species that display variability under field conditions should also be closely monitored *in vitro*.
- Carryout standard assessment of genetic stability at regular intervals. Note- Since the chance of variability is low for most genera, assessment may be of low priority when establishing an *in vitro* collection.

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- Develop a simple genetic variability monitoring system using techniques available to the facility.

## **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**

- A well-equipped tissue culture laboratory including a reliable liquid nitrogen (LN) source, storage dewars and vented handling containers.
- For safety considerations, 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.

### **Techniques**

Many techniques are still at the research phase; however, others are currently used to store genetic resources.

- The use of cryopreservation in a laboratory requires some research and screening of plant materials before a storage regime can be implemented.
- Carryout a thorough review of the literature
- Test initially to determine which cryopreservation methods are best suited to the genus to be frozen.
- Recovery of cryopreserved plants can be improved by modifying plant culture, pretreatments, cryoprotectant type, cryoprotectant exposure time, freezing or thawing rates and recovery medium.
- Most seeds are stored in the vapour phase, but most meristems are stored in the liquid phase.
- Methods are available for use with cell suspensions, calluses, apical and lateral meristems, dormant buds, somatic embryos and zygotic embryos.
- Controlled freezing (slow freezing, two-step freezing) of meristems requires pretreatment steps, cryoprotectants, and slow freezing (0.1–1°C min<sup>-1</sup>) using a programmable freezer, generally to –40°C, followed by immersion in LN.
- Thawing is quick, with ice melting in 1–2 min
- Vitrification employs pretreatment and very viscous cryoprotectants
- 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

- Vitrification methods for meristems usually require preculture of sucrose rich medium, cryoprotectant treatment and dehydration by vitrification solution. Then meristems are plunged into LN. The cryopreserved meristems are thawed in hot water at about 40 oC quickly (1 min) to avoid ice formation into cells, then remove the vitrification solution immediately because it's harmful for cells. After thawing, the meristems are diluted with 1.2 M sucrose solution, and placed on solidified medium.
- For encapsulation-dehydration method, meristems are encapsulated in alginate beads and pretreated in sucrose solution, then dried using silica gel and plunged into LN. Cryotubes with encapsulated meristems are thawed in hot water at about 40 oC quickly (1-2 min) and placed on solidified medium.

### ***In vitro* germplasm collection management**

- 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.
- During cryopreservation storage, keeping the temperature below -130 oC is necessary to avoid ice formation into cells. As the temperature of grass transition is -115 oC, cells of cryopreserved samples must not be above -115 °C.

### **Storage procedures**

- Once the samples are frozen, they cannot be warmed and refrozen. Transfer from one LN container to another must be done very quickly (in seconds, not minutes).
- 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).
- Check and fill dewars on a regular basis.
- The number of replicates stored depends on the survival rates achieved, crop type, speed of propagation, stability in culture, ease of propagation and material available for storage.
- Consider cryopreserved storage as a base collection for a field collection and not the only form for conserving an accession.
- Set up a database 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 using field observation for morphological traits and If facilities are available use molecular techniques. However, note that 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.

### **Distribution**

- Distribution pressures vary with the type of collection. Collections providing virus-free planting stock may have very different requirements from those providing minimal propagules to plant breeders.

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- Timing of multiplication for virus-free stock may include storage of the propagules until the time of distribution.
  - Store greater numbers of accessions under heavy demand than other accessions, so that they can be distributed without a long repropagation time.
  - Form of distribution varies with the plant genotype involved.
  - Growth stage will vary with the crop and the purpose of the genebank.

#### **Procedures for recipients of dispatched cultures**

- Provide recipients information on medium, handling instructions, culture conditions and historical information
- Determine that the requesters' ability to culture or acclimate the *in vitro* plants and provide assistance to recipients in some cases or a different form of plant may be sent that is more easily used.
- Include written protocols and an evaluation/information form with shipments to aid improvements to the shipping procedures.

#### **Phytosanitary and quarantine considerations:**

- Phytosanitary certification and import permits are usually required for international shipments.
- Additional information on tests and treatments of the germplasm may be given in a GERMPASM HEALTH STATEMENT

#### **Packaging and shipping:**

- Consider the amount of time required for a shipment.
- Choice of shipper and correct packing procedures improve the plants' condition on arrival.
- Consider temperature requirements to avoid freezing or overheating in transit.
- Use appropriate packaging material.
- Use appropriate labels for the exterior of the package.
- Keep records of plants requested, plants distributed, recipient information and shipping costs.

#### **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.
- Investigations on the effects of plant growth regulators and growth retardants for optimizing slow-growth storage to minimize their use
- Determining the effects of light quality and quantity, temperature and light interactions, and propagule size and growth stage.



- 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.
- 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
- Studies on the applicability of cryopreservation methods to a wide range of species and genotypes
- Studies on cryotherapy - 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

### Further Reading

- Hammer, K. and Y. Teklu. 2008. Plant Genetic Resources: Selected Issues from Genetic Erosion to Genetic Engineering. *Journal of Agriculture and Rural Development in the Tropics and Subtropics*. 109(1): 15–50.
- Khoury, C. B. Laliberté and L. Guarino. 2010. Trends in ex situ conservation of plant genetic resources: a review of global crop and regional conservation strategies. *Genetic Resources and Crop Evolution* 57(4): 625-639,
- Matsumoto, T., 2002. Cryopreservation of in vitro-grown apical shoot tips of wasabi (*Wasabia japonica*) by different procedures. *Biotechnology in Agriculture and Forestry* 50. Cryopreservation of plant germplasm II, Springer-Verlag, pp.180-195.
- Reed, B.M., F. Engelmann, M.E. Dulloo and J.M.M. Engels. 2004. Technical guidelines for the management of field and in vitro germplasm collections. IPGRI Handbooks for Genebanks No. 7. IPGRI: Rome.
- Sakai, A., D. Hirai and T. Niino. 2008. Development of PVS-Based Vitrification and Encapsulation-Vitrification Protocols. *Plant Cryopreservation: A Practical Guide*. Springer. pp.33-58.



## LECTURE 9

# Legal issues in field genebank and in vitro genebanks /acquisition policy / quarantine regulations / access and benefit-sharing arrangement

### Introduction

Plant genetic resources were once considered as common heritage and were freely exchanged. However, now nations have sovereign right over the plant genetic resources that occur with their borders and hence policies and rules have evolved controlling their assembly, use and distribution. In the case of field genebank, which requires substantial amount of land resources, legal issues with regard to land and other issues have to be considered as well.

### Objectives

- Discuss the history of the development of policy instruments of access and benefit sharing of plant genetic resources for food and agriculture
- International quarantine regulations better understood
- Discuss the salient provisions of the ITPGRFA and the SMTA.

### Description of lessons covered

The lecture will discuss the development of the present regimes for access and benefit sharing of PGRFA and the status of ITPGRFA implementation (2004-2009).

### Lessons to learn

- Facilitated access and benefit sharing of PGRFA listed in Annex 1 of ITPGRFA can only be realized if Contracting Parties nominate their Annex 1 germplasm materials in the MLS.
- Contracting Parties should establish the legal mechanism for the implementation of the ITPGRFA.
- Need to evolve similar guidelines for exchange for non Annex1 crops

### Germplasm exchange

Over the last 20 years or so, technological advances particularly in molecular biology and genetic engineering, have given rise to a growing appreciation of the monetary and non-monetary value of genetic resources. This in turn has spurred increasing conflict over rights and responsibilities for these resources. The current international debate on legal regimes for plant genetic resources has its origins in the late 1970s and early 1980s when developing countries became concerned over actions by the plant breeding industry in industrialized countries to extend intellectual property rights over 'improved' varieties. The concern focused in particular on the inequity of continuing the free flow of germplasm, which was seen as being a flow of resources from developing countries to industrialized countries.

During this same period, efforts to collect and conserve plant genetic resources in genebanks heightened and the International Union for the Protection of New Varieties of Plants (UPOV) Convention was amended to admit non-European members.

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This led to expanded international cooperation in the recognition of plant-related intellectual property rights, which resulted in even greater attention being paid to questions over plant genetic resources ownership in various fora.

In the International Undertaking on Plant Genetic Resources (IU), Member States recognized that “plant genetic resources are a heritage of mankind to be preserved, and to be freely available for use, for the benefit of present and future generations.” The IU made clear that this open availability was to apply to all plant genetic resources, including “special genetic stocks.”

The Convention on Biological Diversity (CBD) reaffirmed the sovereign rights of countries over their own biological resources and established that States have the authority to determine access to genetic resources under their jurisdiction. The assertions of sovereignty are most visible in Article 15, Access to Genetic Resources. Article 15 ultimately became a balancing act between traditional notions of sovereignty and the desire to ensure that access does not become so cumbersome as to make it effectively impossible. Despite its recognition elsewhere of a common concern for biodiversity loss, the Convention’s provisions on access to genetic resources reaffirm national sovereignty over these natural resources and hence national authority to regulate access to genetic resources under a State’s jurisdiction. The International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) was negotiated to address the outstanding issues not resolved by the CBD. The ITPGRFA entered into force on June 29, 2004 after ratification by 40 countries. A Governing Body, composed of all Contracting Parties to the Treaty, will be convened. Until then, the FAO Commission on Genetic Resources for Food and Agriculture will act as the Interim Committee for the Treaty, and will oversee a number of tasks to be undertaken in the interim period.

The salient features of the ITPGR are:

- It is at the crossroads between agriculture, trade and the environment. It provides agriculture with a new, legally binding instrument that is at par with trade and environmental instruments, and promotes harmony and synergy across the sectors.
- It covers all plant genetic resources that are relevant to food and agriculture. Its objectives are the conservation and sustainable use of plant genetic resources and the fair and equitable benefits arising out of their use, in harmony with the Convention on Biological Diversity, for sustainable agriculture and food security. It aims at ensuring that the inherited capital they represent is conserved, and continues to supply the flow of services on which food security and development depend.
- It establishes a Multilateral System of Access and Benefit-sharing for plant genetic resources, for an agreed list of crops, established on the basis of interdependence and food security. The list currently covers 35 food crops, and 29 forage genera, representing more than 80% of the world’s calorie intake. The genetic resources of these crops are pooled. The country of origin cannot therefore be the basis of benefit-sharing, which means that the benefits must also be shared on a multilateral, rather than on a bilateral, basis.
- It provides for benefit-sharing through information exchange, technology transfer, capacity-building, and the mandatory sharing of the monetary and other benefits of the commercialization of products incorporating material accessed from the Multilateral System. The primary focus is on farmers in the developing world, who conserve and sustainably utilize plant genetic resources for food and agriculture.
- It includes a Funding Strategy to mobilize funding for priority activities, plans and programmes, in particular in developing countries and countries with economies in transition, taking into account the Global Plan of Action adopted in Leipzig in 1996.

- It provides for the realization of Farmers' Rights by national governments through:
  - the protection of relevant traditional knowledge;
  - equitable participation in sharing benefits derived from the use of plant genetic resources for food and agriculture; and
  - participation in national decision-making related to their conservation and sustainable use.

The main instrument of the ITPGRFA for the implementation of its access and benefit sharing provisions is the Standard Material Transfer Agreement (SMTA). In broad terms, the SMTA stipulates the conditions under which a provider of germplasm materials listed in Annex 1 of the ITPGRFA grants access to a recipient. There are two options for the benefit sharing:

- 1.1% of the sales of a commercialized product which incorporates material from the Multilateral System, when there are restrictions, such as patents, that result in the product not being freely available to others for research and breeding; and
- A crop-based payment system, whereby user pays a lower rate, namely 0.5%, on all his commercialized products of a particular crop, regardless of whether material from the MLS is incorporated in those products, and regardless of whether or not they are freely available to others for research and breeding through the exercise of IPRs.

### **Legislation, import practices and plant quarantine**

Essentially, there are three main types of legislation to consider when importing or exporting plant material:

- Plant health legislation
- Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)
- Convention on Biological Diversity (CBD).

Plant health legislation and CITES regulations are enforceable by law, and institutions must comply with them. The CBD, while not yet legally enforced at border controls, has been ratified by the UK Government, and, as a result, institutions should ensure that they act in a manner consistent with the letter and spirit of the Convention.

Plant health import and export legislation is mainly to avoid the spread of damaging pests and diseases while exchanging germplasm material.

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) regulates the trade in approximately 25 000 species of plant, through a permit system. This guards against the export/import of threatened plants and animals.

Convention on Biological Diversity (CBD) has three principal objectives: the conservation of biological diversity, the sustainable use of biological resources and the fair and equitable sharing of benefits arising from the use of genetic resources. In contrast to CITES, which is very specific and species-oriented and has certain exemptions for artificially propagated plants, the CBD has a very broad scope, takes an ecosystem approach and is more concerned with *in situ* conservation, sustainable use and ethical benefit-sharing with countries of origin.

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## Policy, management, and legal issues specific the location of genebanks

**National, regional and international collections:** Conservation of genetic resources is a long-term responsibility and requires long-term commitment of institutions and governments. It is for this reason that any conservation effort preferably be conducted within the framework of a national programme, that clear institutional responsibilities are assigned as part of a National mandate and that a reliable budget-line is established for continuous funding. At the regional and international level, the situation is different since it is not easy to assign clear mandates and responsibilities for the conservation of genetic resources of a specific gene pool agreed upon and implemented. The international germplasm collections held by the International Agricultural Research Centres are an exception as they have been placed under the auspices of FAO as part of the International Network of *Ex situ* Collections. Individual centres have accepted responsibility of conserving global genetic diversity for one or more specified gene pools as part of their broader mandate to deal with the improvement of the so-called mandate crops.

**Sustained commitment:** it is important to critically examine the existing arrangements with regard to mandate and responsibilities at both the national and regional level for genetic resource conservation in general and about the crop in question, in particular. An assessment of the level of governmental and institutional commitment (of the host country in the case of coconut regional field genebank) to the maintenance of collections in field genebanks is necessary. Only when an effective governmental commitment exists should the establishment or extension of a collection be considered. This is specially so for crops which need a relatively large land area in order to plant sufficient number of plants/trees necessary to represent the genetic diversity. Initial establishment costs, which in some cases can be very high, and recurring costs for maintenance of the collection should be considered at the planning stage and should be provided for. In many cases, the latter is ignored and the collections can run into problems within a few years of their establishment. Given this background, establishment and maintenance of FGBs, appears to be more easily organized at national level, as part of national PGR programmes, rather than at regional or international levels. In the case of regional or international efforts, it is essential to obtain the full support and commitment of the government of the country in which the FGB is to be set up and to obtain commitments from the individual member states of the network to financially support the effort. For any emergency situation, provisions have to be made as to how and where the collection can be duplicated, if so decided. The role of the co-operating international institutions needs to be defined as well.

**Legal issues:** Since the Convention on Biological Diversity (CBD) has come into force, countries now have the sovereign rights over the biological diversity present within their borders. Existing *ex situ* collections, established prior to the CBD, or germplasm acquired not in accordance with the CBD does not fall under the legal framework of the Convention and no obligation exists to share the benefits derived from their use. The FAO has been requested to resolve this latter issue as part of the harmonisation of the International Undertaking on PGR. In view of this, a clear consensus must be reached by all the member countries of a given crop genetic resources network with regard to access to sharing of the benefits derived from the germplasm conserved as well as on access conditions to the conserved germplasm and information related to it. The necessary agreements and mechanisms on access to and provision of accessions should be in place prior to the establishment of a regional or international genebank, be it seed genebank or a field genebank. Within these mechanisms, there might be a need to develop some form of material transfer agreement to accompany germplasm accessions being sent to researchers/breeders within and outside the network. Considering the current legal situation with regard to genetic resources, one of the options for regional or global PGR networks is to consider the possibility of placing their germplasm collections under the auspices of FAO, thus becoming part of the FAO International Network of *Ex situ* Collections.

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In doing so, the host country which acts as trustee of the germplasm on behalf of all the member countries, agrees not to claim ownership over the germplasm and not to claim any form of intellectual property protection to the material or on any information related to it. The host country can also ensure that any further recipients of the germplasm are bound to the same conditions as mentioned above.

### **Further Reading**

Food and Agriculture Organization. (Homepage of FAO). (online) URL: <http://www.fao.org>

Long, H., F. Inches and K. Treseder. 2006. Guidelines on Legislation, Import Practices and Plant Quarantine for Botanic Gardens and Kindred Institutions. Edited by Judith Cheney. PlantNetwork: UK and Ireland.

<http://www.plantnetwork.org/downloads/planthealth.pdf>

Ramanatha Rao, V. and B.M.C. Reddy. 2010. Tropical Fruit Genetic Resources Conservation. Pp. 83-98 in Horticulture to Hort-Business (K.L. Chadha, A.K.Singh and V.B. Patel, eds.). Westville Publishing House, New Delhi.





## PART 2 Practicum





## PRACTICUM 1

### Tissue culture technologies for germplasm *in vitro* conservation – principles and exercise

#### Introduction

Germplasm can be acquired through donations, requests, exchange with another institution, and through field exploration and collecting in farmers' fields and natural habitats. *In vitro* techniques have been increasingly used for plant propagation and conservation in the past few decades. They consist in conserving parts of plants in flasks or tubes in artificial media, under controlled environments. *In vitro* conservation of plant genetic resources is also becoming a complementary approach to the conventional conservation methods. It offers many distinct advantages e.g. a tool for the elimination of pests and diseases, rapid plant propagation and for the exchange of clean germplasm internationally. For the management of clonal germplasm, factors that influence *in vitro* conservation will be discussed in the class.

#### Objectives

- Learn how to use *in vitro* techniques for PGR conservation
- Principles of *in vitro* conservation of clonal germplasm
- Discuss factors that affecting *in vitro* conservation
- Learn how to monitor the performance of tissue cultures in slow growth storage

#### Description of the topics covered

We will introduce the principles of *in vitro* conservation of clonal germplasm in the practicum and factors that influence *in vitro* conservation management. Participants will practice *in vitro* techniques through lab exercise using sweet potato material. Through demonstration and practice, this lesson will benefit participants customizing their own *in vitro* conservation system.

#### Lessons to learn

- Why using *in vitro* techniques for the conservation of clonal germplasm
- Principles of *in vitro* conservation
- Factors affecting *in vitro* conservation and practices and problems solutions
- Customized *in vitro* conservation system

#### Description of practicum

The advantages of *in vitro* preservation for clonal germplasm resources maintenance include small space for storage, pathogen-free from plants diseases, and convenience to international exchange of germplasm resources.

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The genebank has worked on backups of clonal germplasm conservation *in vitro* for the vegetatively reproduced crops. We will provide the introduction for the establishment of *in vitro* collection. We use sweet potato as TC material for demonstration and exercise *in vitro* conservation.

For the purpose of healthy management of clonal germplasm, the pathogen-free citrus will be used as an example for tissue culture and grafting seedlings.

## PRACTICUM 2

### Collecting and maintenance of fruit and vegetatively propagated vegetable germplasm in FTHERS field genebank

#### Introduction

Germplasm can be acquired through donations, requests, exchange with another institution, and through field exploration and collecting in farmers' fields and natural habitats. The types of germplasm materials that can be acquired include farmers' varieties, landraces and primitive cultivars, wild and weedy related species, and products of plant breeding, e.g. improved cultivars, obsolete varieties, mutants, genetic stocks, breeding lines etc. Careful planning is necessary in germplasm exploration and collecting in order to collect the maximum amount of genetic diversity in the target taxon. In addition to germplasm, information including indigenous and traditional knowledge associated with the germplasm should also be collected whenever practicable. When collecting germplasm, social and ethical considerations should also be given importance. Changes in planned itinerary can occur. Flexibility therefore should be practised to maximize the use of time and resources in a collection. Collected germplasm of fruit tree and vegetatively propagated plant is maintained in field genebanks.

#### Objectives

- Learn the good practices in collecting germplasm of fruit tree and vegetatively propagated vegetables, and associated knowledge;
- Learn how to gather and document passport, characterization, and image data which can be stored in database system;
- Collect and prepare germplasm materials for conservation in field genebank; and
- Learn management and maintenance for conservation of field genebank.

#### Description of the topics covered

The practicum will discuss the pre-planning necessary for germplasm collecting demonstrate the managing strategy (how to cultivate and maintain germplasm survival, considering the number of plants, number of sites); demonstrate the use of GIS and the good practices for initial processing of the collected materials, and demonstrate the procedure for gathering and documenting data during collection. Germplasm regeneration is the most critical technique in genebank management. In this practicum, we will introduce how to properly regenerate tropical fruit trees, including asexual propagation, grafting and cutting and sexual propagation by seeding.

#### Lessons to learn

- Regeneration/multiplication and maintenance of fruit tree and vegetatively propagated vegetables (such as grafting for citrus and *Cucurbitaceae*).
- Demonstration of good practice of field genebank *in vitro* conservation.
- The procedure for gathering and documenting data of *in vitro* collection.

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- Learn how to gather and handle papaya germplasm diversity from monitoring papaya seeds quantity and quality in order to maintain papaya seeds vigour.
  - Principles of propagation of leafy stem cuttings, techniques for propagating of tropical fruit tree vegetative reproduction

### **Description of practicum**

Some species of precious tropical fruit tree and cucurbitaceae are easy to lose survival during germplasm conservation, due to their short growing period, quick senescence, especially after fruit setting in the field. Furthermore, the survival of vegetatively propagated native varieties or wild plants becomes threatened, due to pest damage or abiotic stress. For germplasm *in vitro* conservation, some techniques are used so that genetic diversity can be maintained and avoid genetic resources loss. In the way, genetic resources can be maintained for further improvement and biotechnological studies.

## PRACTICUM 3

### Cryopreservation technologies for germplasm *in vitro* collections

#### Introduction

Cryopreservation is becoming a very important tool for the long-term storage of plant genetic resources for future generations, requiring only a minimum of space and maintenance. With increasing interest in the genetic engineering of plants, the preservation of cultured cells and somatic embryos with unique attributes is assuming greater importance. Recently, cryopreservation has been reported to offer real hope for enhancing the preservation of endangered and rare plants.

#### Objectives

- Learn the principles of cryopreservation
- Learn the procedure of vitrification method
- Learn how to increase the survival rates after cryopreservation

#### Lesson to learn:

- Vitrification method using wasabi shoot tips

#### Items needed

Day 1: Dissecting shoot tips and preculture

- Tools: microscope, forceps, scalpels, etc.
- Solidified 1/2MS (half strength of  $\text{KNO}_3$  and  $\text{NH}_4\text{NO}_3$ ) with 0.3 M sucrose for preculture (25 mL in a plastic Petri dish).

Day 2: Vitrification and Recovery

- Sterile pasture pipette for removing solutions
- LS solution (2 M glycerol + 0.4 M sucrose in liquid MS medium)
- Cryotubes (about 2 mL)
- PVS2 solution: 30%W/V glycerol + 15%W/V EG + 15%W/V DMSO + 0.4 M sucrose in liquid MS medium for dehydration
- Liquid nitrogen
- 1.2 M sucrose solution in liquid MS medium for unloading
- Medium for recovery (solidified 1/2MS medium with 3% sucrose and BA 0.1 mg/L)
- Sterile filter papers about 70 mm in diameter
- Tools and sterile Petri dishes

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- Water bath or hot water at about 40°C

## Procedure

### Day 1: Dissecting shoot tips and preculture

- Shoot tips (about 1 mm x 1 mm) derived from 30 to 40 days old in vitro plantlets (about 30 mm long) preculture on solidified MS supplemented with 0.3 M sucrose and maintained under continuous light for 16 hr at 20°C.

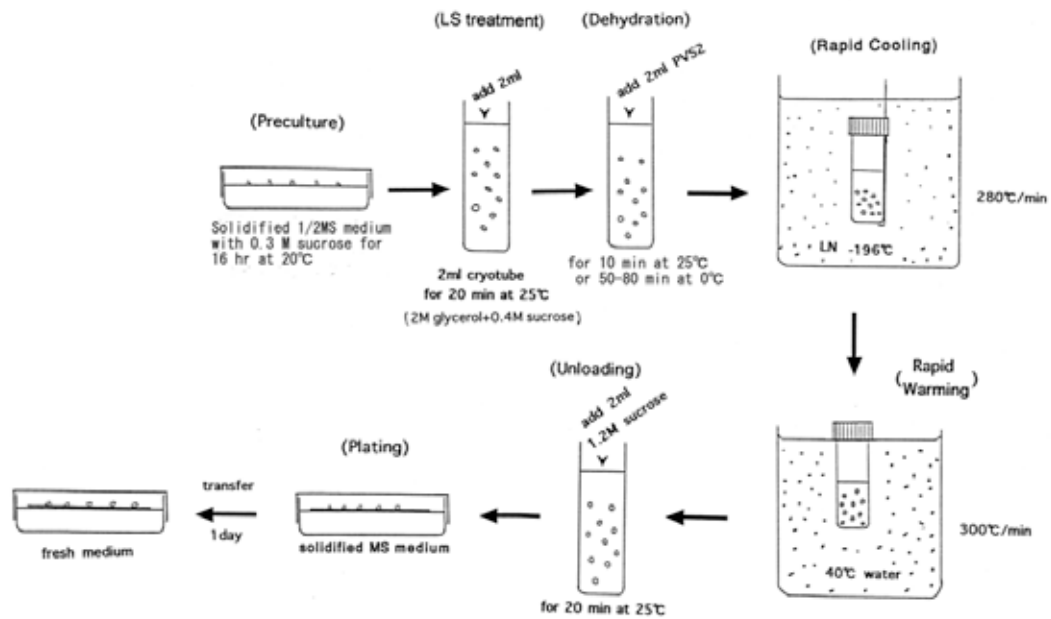
### Day 2: Vitrification and recovery

- Place 10 precultured shoot tips in a cryotube.
- Treat with a mixture of 2 M glycerol + 0.4 M sucrose (LS solution) for 20 min at 25°C.
- Drain LS solution using a pasture pipette. (You need to be carefully not to injure the shoot tips!)
- Dehydrate with PVS2 solution for 10 min at 25 °C or 50 to 80 min at 0 °C (in crashed ice) prior to a plunge into LN using a pasture pipette (You need careful treatment not to injure the shoot tips!).
- Drain PVS2 solution and add fresh PVS2 solution after 5 min at 25 °C or 30 min at 0 °C using a pasture pipette (You need careful treatment not to injure the shoot tips!).
- Exchange fresh PVS2 solution before 1 min of the end of PVS2 dehydration treatment (You need careful treatment not to injure the shoot tips!).
- After PVS2 treatment, immerse cryotubes into liquid nitrogen and hold there for more than 1 hr.
- Warm the cryotubes in 40 °C water for 1 min with stirring. Then, drain PVS2 solution immediately and carefully, and add 1.2 M sucrose solution for 20 min at 25 °C for unloading (You need careful treatment not to injure the shoot tips!).
- Transfer meristems onto two sterilized filter paper discs over recovery medium and culture on the recovery medium under white fluorescent light (about 50  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ) with a 16 hr photoperiod at 20°C.
- After one day, transfer the meristems onto two fresh paper discs over the same medium and culture under the same condition above.

### Possible Problems:

- During draining and adding solutions using pasture pipette, meristems in cryotube are easy to be injured. You need careful technique.
- PVS2 solution is harmful for plant material, especially at 25°C. You need to finish the PVS2 treatment in time.





### PROCEDURE FOR VITRIFICATION OF WASABI SHOOT TIPS

#### Reference

- T. Matsumoto. 2008. Cryopreservation of monocots, cryopreservation of herbaceous dicots, cryopreservation of dormant buds. *Plant cryopreservation. A Practical Guide* (B. M. Reed, ed), Springer, pp.325-326.

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