General Comments

Germplasm banks play a crucial role in the conservation and use of biodiversity. They are important institutions, not just for the preservation of germplasm but also for its sustainable use. Germplasm banks are also expected to generate and provide new scientific knowledge and information on ecosystems, species, and genes.

The reports presented at the International Technical Conference on Plant Genetic Resources in Leipzig indicated that the number of germplasm banks had grown rapidly since the early 1970s when there were fewer than 10, holding perhaps half a million accessions. At this Conference, 1300 germplasm banks were identified as holding about 6.1 million accessions, of which about 10% were conserved in field collections (FAO 1997).

Few African countries have national germplasm banks for agricultural crop species, and the few that do exist are not adequately equipped and organized to attain the continent's goals (African Ministerial Council on Science and Technology [AMCOST]). Even so, *ex situ* conservation for forest genetic resources, in the form of field germplasm banks, is practised for most exotic plantation species in Malawi, South Africa, Tanzania, Zambia, and Zimbabwe. In Malawi, field germplasm banks of important seed sources of indigenous species (e.g., *Afzelia quanzensis*, *Khaya anthotheca*, and *Pterocarpus angolensis*) were established. The SADC Regional Gene Bank is also located in Lusaka, Zambia. It stores duplicate samples of germplasm of national institutions (FAO 2003).

The information provided by countries of the Andean Region shows that a significant number of germplasm banks house genetic resources of great importance agriculturally, socio-economically, and in terms of food security. In all, 88 active banks are reported in the Region, of which 60% are managed by public institutions. A further two are national base collection banks (Colombia and Ecuador) and another is being planned (Venezuela). Most of the accessions conserved in the banks correspond to species belonging to Andean flora such as tubers and roots (17,289 accessions), cereals (27,839), vegetables (6415), fruit trees (6331), forest flora (2866), legumes (11,064), forages (426), plants for industry (14,945), and ornamentals (1679) (Comunidad Andina 2002).

The conservation of plant genetic resources (PGRs) is not limited to attaining and physically possessing the materials (collection and storage) but also includes ensuring the existence of these under viable conditions and with their original genetic characteristics intact. This is achieved, in the case of seeds or *in vitro* materials, by controlling storage conditions so that they inhibit or reduce the samples' metabolism; and, in the case of vegetative planting materials, by maintaining them under optimal agronomic and cropping conditions (Jaramillo and Baena 2000).

Information on the Module

This module contains five submodules, each of which contains two lessons.

Objectives

When you have completed the entire module, you should be able to, with regard to plant germplasm:

- Describe multiplication and regeneration
- Describe the procedures for harvesting, conditioning, and quantification
- Describe the procedures for verifying the germplasm's biological status
- Describe what constitutes plant health quality and the verification procedures used
- Describe the alternatives for storing and conserving seeds and propagules in the short and long term

Lessons

The lessons for Module 3 on *Germplasm Conservation* are as follows:

Submodule	***Lessons***
A. Multiplication and regeneration	• Multiplication • Regeneration
B. Harvesting, conditioning, and quantification	• Harvesting • Conditioning and quantification
C. Verifying the biological quality of germplasm	• Basic concepts • Verification procedures
D. Verifying phytosanitary quality	• Basic concepts of phytosanitary quality • Procedures for verifying phytosanitary quality
E. Storing germplasm	Basic concepts of storage, an essential component of the *ex situ* conservation of germplasm

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Throughout this module, a bibliography is provided for each section, that is, the *General Comments* and each *Lesson*. The bibliographies follow a format of two parts:

1. *Literature cited*, which includes those references cited in the text itself. Some of these citations were used to develop the original Spanish-language course on *ex situ* conservation and may therefore appear in Spanish or Portuguese. However, where practical, references to the English versions of the original Spanish-language documents are provided.
2. *Further reading*, which is a list of suggested readings in the English language, with few exceptions in French.

A list of *Acronyms used in the bibliographies* is also given. The idea is to save space by not having to spell out each institution's full name each time it appears in the references.

Acronyms used in the bibliographies

ACIAR	Australian Centre for International Agricultural Research
AMCOST	African Ministerial Council on Science and Technology
AOSA	Association of Official Seed Analysts

APS	American Phytopathological Society
AVRDC	Asian Vegetable Research and Development Center
CESAF	Centro de Semillas y Árboles Forestales
COSAVE	Comité de Sanidad Vegetal del Cono Sur
CSSA	Crop Science Society of America
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization of the United Nations
IBPGR	International Board for Plant Genetic Resources
ICA	Instituto Colombiano Agropecuario
ICAR	Indian Council of Agricultural Research
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics
IPGRI	International Plant Genetic Resources Institute
IPGRI–APO	IPGRI Regional Office for Asia, the Pacific and Oceania
IPPC	International Plant Protection Convention
IRRI	International Rice Research Institute
ISTA	International Seed Testing Association
JIRCAS	Japan International Research Center for Agricultural Sciences
OIRSA	Organismo Internacional Regional de Sanidad Agropecuaria
OSU	Ohio State University
SCBD	Secretariat of the Convention on Biological Diversity
USAID	United States Agency for International Development

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Next Lesson

In the next lesson, you will learn about germplasm multiplication.

Lesson 1 Multiplication

Objectives

- To define germplasm multiplication
- To review the requirements and procedures for multiplication

Introduction

Once germplasm has been acquired and introduced, it must be stored (temporarily) to preserve its essential characteristics (the reason for conservation), that is, its physiological and physical qualities, genetic identity, and plant health quality. The temporarily stored germplasm may undergo other important stages as according to the germplasm bank's goals. These stages would form part of the monitoring needed to conserve the germplasm for the pre-established term.

After introducing the germplasm, an essential activity in *ex situ* conservation of PGRs is preliminary multiplication, or initial increase, of the acquired materials. However, PGRs can be conserved without going through this stage, especially if sufficient conservable material (i.e., highly viable and healthy) had already been acquired. Likewise, instead of conducting a preliminary multiplication, a definitive multiplication programme can be developed according to the germplasm bank's goals. For *in vitro* conservation, initial multiplication is probably not necessary.

Initial multiplication is key to successful conservation, as acquisition rarely provides security of status of plant health (despite what is expressed in the pertinent certificates) or of the material's viability.

In many cases, the preliminary multiplication of PGRs is carried out as botanical seed because the germplasm bank received very little material and has to increase it to meet a series of requisites for conservation. One requisite is the periodic verification of viability, involving a consumption of seeds from the sample. The idea of requisites leads to an important concept: that the germplasm bank must specify its needs according to its mandate.

Because one goal of conservation is to maintain the germplasm's essential characteristics, five major risks should be avoided during multiplication or regeneration. Best known as the 'capital sins of conservation' (Daniel Debouck 2004, personal information), these are:

1. Mechanical mixture with contaminants (other seeds or any material alien to the samples)
2. Infection by pathogens—whether of quarantine importance or not—that can affect the material's viability
3. Genetic contamination through uncontrolled hybridization; a common event because plants may have received residual cross-pollination, even if they are autogamous
4. Genetic erosion, a common phenomenon in germplasm banks; it occurs when making repeated samplings for different events of increase, permitting the effective multiplication of only a few seeds

5. Genetic drift, which is a more subtle effect, where materials produce variable numbers of propagules and, hence, change the gene frequencies that were originally found in the samples on collection

Over the time it is conserved, germplasm can decline in quantity (number of seeds) and quality (viability). Sample size shrinks with use and distribution, while viability declines over time, even if the germplasm has been stored under optimal conditions (FAO 1996; Sackville Hamilton and Chorlton 1997). When this happens, the germplasm must be multiplied or regenerated.

If the objective is to **recover viability,** one speaks about **regeneration or rejuvenescence**; if it is to **bring the samples** to an **optimal size (quantity),** then one speaks of **multiplication.**

Increase, multiplication, and regeneration are activities that embrace the same principle: to obtain a given quantity of viable propagules, free of pathogens, and genetically identical to the original. Hence, similar methodologies are applied. As we present this theme we will always refer to the essence of the three activities as multiplication but, for convenience in developing the theme, we will refer to multiplication on the one hand and regeneration on the other.

Undoubtedly, when dealing with procedures for germplasm multiplication, we must take into account the two basic strategies of plant reproduction. The first strategy is that of sexual reproduction, which uses the seed as the building block. A seed consists of an embryo, its stored food reserves, and the surrounding protective coats. The other reproductive strategy is asexual or vegetative reproduction, which generates new individuals directly from pieces of mother plants or specialized organs. Thus, in terms of germplasm multiplication, one always refers to the two strategies: reproduction by seed or vegetative reproduction.

The Concept of Initial Multiplication

Initial multiplication (also known as *preliminary multiplication* or *initial increase*) is the increase of introduced germplasm. It is carried out under optimal agronomic conditions to guarantee sufficient viable samples that maintain the original genetic identity. The multiplied material will permit storage, conservation, and distribution of the targeted species, and the establishment of representative populations for characterization and evaluation. Initial multiplication is almost always necessary, as samples obtained through donation, exchange, or field collection have small numbers of seeds and a usually irregular percentage of viability.

When a germplasm sample is imported from another country, under normal conditions, before starting initial multiplication, certain plant health transactions should have been fulfilled, as described in *Module 2: Germplasm Acquisitions and Introduction (Seeds and Asexual Propagules*) (Lesson 3, Figures 4 and 5). The germplasm is then taken to the place of conservation where the samples are verified as being sufficient and viable for conservation.

As a safety measure and according to established agreements, initial multiplication is conducted under quarantine and supervised either by the national institutions responsible

for plant health such as the ICA Office for the Prevention of Plant Health Risks for Colombia or according to regional agreements such as the Inter-African Phytosanitary Council (IAPSC) in terms of the stipulations of the International Plant Protection Convention (FAO 1997). These stipulations include inspection to detect pests and pathogens, treating or cleaning samples, and certification and release if no danger exists or the material's destruction if it is highly contaminated or no technology is available to clean it.

Monitoring the need for multiplication

As with other conservation activities, multiplication starts with monitoring the samples. It is governed by standards and procedures that specify the quality and quantity of the required material, the number of plants, and the environment (FAO and IPGRI 1994; Sackville Hamilton and Chorlton 1997).

A sample is in optimal condition when it is viable and present in sufficient numbers. If, on monitoring, a sample does not fulfil either requirement, it should be multiplied. Size is determined by counting the number of available seeds or propagules per accession. If the sample consists of seeds, the permissible minimum size indicated by the *Genebank Standards* (FAO and IPGRI 1994) is 1500 to 2000 seeds. No standards exist for the sample size of vegetative propagules conserved in the field or *in vitro* but between 3 and 20 replications are usually kept per accession, taking into account the number of propagules initially received. Viability is established through observation or testing, depending on sample type.

The viability of vegetative material (plants in the field or in *in vitro* slow growth) is systematically established by observing the health, development, and conditions under which the material is being conserved. If any one of the criteria listed is not met, then the material should be regenerated. If the conserved material is seed, viability is analyzed through germination tests that involve germinating a selection of seeds and evaluating how many (%) germinate. Of those that did not germinate, then observations must be made to determine if they had died or were dormant. Findings are then compared with the initial viability, which had been determined before preliminary multiplication. If viability has declined to 85% or less, then the sample must be regenerated.

Germination tests are carried out on a minimum sample of 200 seeds taken at random (FAO and IPGRI 1994). Seeds are placed on paper (towels or rolls) or a substratum (sand or soil) and, depending on the species, incubated at different temperatures until they germinate. The tests should follow the standards indicated in the *International Rules for Seed Testing* (ISTA 1993). If the germination tests do not give satisfactory results, then complementary tests such as that of tetrazolium and X-rays can be carried out to determine if the embryo is dead, dormant, or non-existent. Once these tests are applied, the material cannot be used later, which means, in practical terms, that sufficient seed must be available to do these tests. Likewise, in practice, even if seed is insufficient, a smaller quantity can be used, for example, two independent replications of 50 seeds each or, if preferred, the necessary minimum quantity for the test can be determined by statistical analysis.

To decide when to multiply, one should not wait until the sample reaches minimum levels of size and viability, but neither should multiplication be done frequently, as it is expensive and endangers the germplasm's genetic integrity. Furthermore, viability should take priority over size; thus, multiplying a large sample with low viability is more urgent than multiplying a small sample whose viability is optimal.

Requisites for multiplying germplasm

Establishing and managing multiplication procedures require prior understanding of propagation techniques and when to use conventional versus biotechnological ones. For vegetative samples, the use of the simplest or most complex techniques as according to the totipotency of cells and/or tissues can strengthen the process. Germplasm multiplication usually requires:

- Knowledge of the characteristics of reproduction and biological cycle of the species being multiplied (e.g., whether they reproduce sexually by seed or asexually by stems, cuttings, roots, bulbs, buds, meristems, or stolons; allogamous or autogamous species; and annuals or perennials)
- To prevent drift, knowledge of the geographical and ecological origins of the material is needed to programme its multiplication under conditions similar to those of the acquisition site
- Knowledge of the germplasm's characteristics of adaptation so it can be multiplied in:
  - The field (site, soils, environmental and biological conditions)
  - Greenhouses and mesh houses (site, airtightness, temperature, light, humidity)
  - Growth chambers (place, temperature, light, humidity)
  - *In vitro* laboratory (infrastructure, light, temperature, safety)
- Knowledge of the initial viability and quantity of available material
- An estimate of yield, for example, g/plant, kg/ha, kg planting material/area, and number of stakes/plant
- Clarity on multiplication rates, for example, kg planted/kg harvested, area established/area multiplied, and area established/kg planting material
- Knowledge of the likely users of the materials produced such as germplasm banks, research institutions, seed companies, and individuals
- Resources, whether physical, financial, human, or infrastructure
- Selection of sites, multiplication methods, and area size.
- Establishment of the size of area needed for multiplication as according to information on yield and requirements of seed or planting materials.

How to Increase or Multiply?

Procedures

- Adequate preparation of the sites selected for increase (e.g., infrastructure, procedures, machinery, equipment, soil, culture media, and propagation containers)
- Creation of lists of the species to multiply and preparation of propagules (seeds and/or plant parts)
  - Seeds (e.g., treatment, scarification, and pregermination)
  - Plant parts (treatment according to the method to be used, e.g., thermotherapy for *in vitro* propagation)

- Transplanting or sowing of materials in containers with substrata or media suitable for seedling development, previously placed in the sites for increase (e.g., greenhouse, mesh house, or laboratory)
- Care during growth
- Plant health inspections, sampling, and analysis to certify health and 'release' of the introduced materials
- Hardening or conditioning of the seedlings for transplanting to the final sites for increase
- Transplanting to the final sites for increase, according to predefined criteria on location and requisites of the species (e.g., greenhouses, mesh houses, laboratory, growth rooms or chambers, and the field)
- Agronomic attention and care to ensure successful increase
- Acquisition of samples for the herbarium, as according to species
- Harvesting, collecting, conditioning, and temporary storage according to methods as defined by species and material type
- Documentation of the general multiplication plan

Identifying samples

In multiplication, as in all germplasm conservation activities, the samples must be precisely identified to prevent mixtures that cause confusion or loss. The location of plots, furrows, and plants undergoing multiplication should be stated on a map and, at the site, with their accession numbers, using weatherproof materials. In cases of doubt, the identification of the plant materials can be confirmed by comparing them with herbarium samples or against available data such as passport data, or from characterization and evaluation. Currently, digital imaging can be used to make photographic records of the materials before their multiplication, so that they may serve as reference to prevent mechanical mixtures or accidental hybridizations.

Establishing the germplasm

Orthodox seeds can be multiplied in the field, but their multiplication is better done in the greenhouse to prevent genetic recombination and the presence of pests and diseases. Before multiplying the samples, size and viability should be confirmed. The sample's initial viability will serve as a basis for later monitoring.

Plant species that propagate asexually have long growing periods or produce short-lived seeds (i.e., recalcitrant) are normally left in the field. Strategies and procedures for establishing and maintaining collections must be practical, rational, economical, and scientifically well grounded (Engelmann 1999).

Planting materials multiplied in the field or greenhouse, using propagules (e.g., stakes or bulbs), are first sterilized or grown *in vitro* through buds or meristems taken from the original samples. Recalcitrant and intermediate seeds are planted in the field or greenhouse to obtain complete plants, from which buds or meristems are taken to multiply *in vitro*. The new plants can also be left in the field in the hope that they will produce seeds and thus be multiplied in the field.

Once the decision has been made to multiply a sample, plants are established in the multiplication site under optimal conditions of development. The resulting sample of

germplasm should be viable, healthy, in sufficient quantity for storage, and genetically equal to the original. The type of reproduction a species has will determine those conditions.

Species that do not require control over pollination such as those with asexual reproduction or are autogamous are multiplied in the field or mesh houses. If they are multiplied in the field, the germplasm is planted in relatively small plots and in large populations. Those with vegetative reproduction are multiplied through sterilized samples such as stakes, layering, and grafts.

Species with sexual reproduction that do need control over pollination (allogamous) are preferably multiplied in greenhouses and mesh houses. They can be multiplied in the field, provided that the area is isolated and pollination is strictly controlled. If the accessions are wild species, they can be multiplied in furrows or plots in the field, mesh houses, or greenhouses, depending on the quantity of available seed and of the species' requirements. Certain wild species (e.g., *Lycopersicon peruvianum*) need special environmental conditions to reproduce.

Multiplication of germplasm in the field or mesh houses requires space, time, and great quantities of materials and resources. For species multiplied by *in vitro* tissue culture, not much time or space is needed, permitting work with various sample types and thereby offering the possibility of multiplying a variety of species. It can also ensure healthy samples that are genetically identical to the original. Tissue culture consists of micropropagating apices of axillary buds and meristems until entire plants are obtained.

Controlling multiplication conditions in the field

Obtaining a sample of good physiological quality (i.e., viable, vigorous, and healthy vegetative propagules or seeds) and identical to the original genotype requires strict control of the environment. The physiological quality of the germplasm depends on its genetic characteristics and on the environment in which it develops. As it can be affected during the growing cycle by adverse environmental factors, any biotic or abiotic stress must therefore be prevented.

The selected site should have fertile soil with sufficient water to supply the species' requirements. Preferably, the site should also be isolated to prevent attacks from pests and pathogens or have facilities for controlling them should they appear. Uniform distances between furrows and between plants should be established, and the agronomic tasks necessary for the given species carried out. Seeds and propagules should be harvested when they are physiologically mature and healthy, taking care to prevent mechanical damage.

Controlling the environment to maintain the original genotype consists of preventing accessions from becoming contaminated through pollen exchange (allogamous) or mechanical mixing (autogamous and asexual reproduction). Populations can be isolated in the field or in mesh houses. If they are planted in the field, the area should be isolated, separating the accessions by suitable distances, and submitting them to thinning and pruning to prevent overlapping of plants and mixing of fruits and seeds. Furthermore, allogamous species require strict control over pollination, which is achieved by bagging the reproductive structures and managing populations of insect pollinators. Using individual mesh houses for each accession eliminates risks of contamination but increases costs.

Conditioning and propagating planting materials

The plant parts collected are washed and disinfected before propagating and taking them to the conservation site. Disinfection may be carried out with bactericides, fungicides (bulbs and rhizomes), or thermotherapy (stakes). Once they have been disinfected, the planting materials may be propagated in the field, greenhouse, or *in vitro*. In the field and greenhouse, samples are planted in seedbeds or flower pots and left to grow until plants are obtained from which new samples can be collected. The procedure is repeated until there are enough plants to establish the collection in the definitive site.

If propagation is to be *in vitro*, samples are planted in greenhouses, in soils of optimal nutritional quality. From the resulting plants—preferably the younger ones—explants are extracted and micropropagated *in vitro* to obtain complete plants that are also taken to the greenhouse. These are planted into sterilized soil and, 2 or 3 weeks later, are transferred to their definitive site in the field. For cassava, micropropagation consists of (a) disinfecting the explants in a solution of sodium or calcium hypochlorite, mercury bichloride, or ethanol; (b) planting them in an *in vitro* culture medium until new shoots develop; and (c) rooting the shoots until complete plants are obtained (Frison 1994; George 1996; George and Sherrington 1984; Roca and Mroginski 1991).

When using seeds, propagation in the field and greenhouse is simple but requires time and space. Nor can it guarantee that the plants obtained will be healthy and genetically identical to the originals. *In vitro* propagation solves these problems and can be used to propagate many species, even those that reproduce by seed. It is, therefore, more convenient. However, this type of propagation has limitations such as cost, the need for skilled personnel, and the risk of induced somaclonal variation, especially if artificially synthesized hormones are used (e.g., auxins or 2,4-D).

Selecting and preparing the site

The site selected to conserve the material in the field should be safe and favour plant development. It should be isolated to prevent pest attacks and diseases but easy to access for management tasks. The physical and chemical preparation of the planting site depends on the species' requirements and on the number of accessions that is expected to be planted in the field.

Planting vegetative materials in the field

If vigorous plants are taken to the field in a number that represents the genetic variability of the accessions, then the continuity of the conserved materials will be ensured. Plants should be arranged in the field in such a way that no risk of pollen exchange exists, thereby preventing the populations from losing their original genotype. The exact site where each accession is planted should be recorded on a map, with the accessions being identified both in the field and on the plants.

Evaluating the Lesson

With this lesson, you should have understood the concept of germplasm multiplication; and have generally reviewed the procedures for multiplication, and their requirements and conditions.

Before going on to the next lesson, prepare a well-based plan for germplasm multiplication. Take as an example, the germplasm with which you currently work or, in its absence, with which you are most familiar.

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Next Lesson

In the next lesson, you will study major aspects of germplasm regeneration.

Lesson 2 Regeneration

Objectives

- To define what constitutes germplasm regeneration and understand its purposes
- To describe the criteria and limitations in establishing regeneration protocols
- To describe the monitoring procedures for decision making on regeneration

Introduction

Over the time germplasm is kept conserved its viability can diminish, even if the germplasm has been stored under optimal conditions. When this happens, the germplasm must be regenerated or rejuvenated. Regeneration is carried out as a result of information obtained during seed control and usually occurs when the viability of a lot is below an acceptable level, often 85%. This percentage, however, corresponds to the lower limit of acceptability of loss of a sample's internal variability. Regeneration is also carried out when factors inducing genetic drift or erosion appear, jeopardizing the existence of alleles in the original accession and possibly causing their elimination from the next generation.

Regeneration is based on the same principle as multiplication: to obtain a given quantity of viable propagules, free of pathogens, and genetically identical to the original. As a result, similar methodologies are applied.

Planning regeneration also requires passport and other data on the accession. Information (when this is adequate) is requested on the number of plants, distances between plots, crop improvement system, any isolation, and pollination method. Most likely, the germplasm bank already has these data recorded as part of its standard practices. Hence, obtaining such data for each accession will not usually be needed before regeneration.

If regenerating the accessions is possible in more than one site, then the 'preferred regeneration site' should be listed in the inventory file. This will help by eliminating the step of having to consult the passport data file whenever regenerating an accession is planned, thereby simplifying activities. Preferentially, to prevent to the utmost genetic drift, only the most suitable site should be selected.

Types of Collections Held in Germplasm Banks

To better understand the regeneration protocols, we need to know the types of germplasm collections that are held by germplasm banks around the world. Collections differ according to the purposes of those holding the germplasm. Briefly, four types of collections exist; these are base, active, working, and core. The base collection is for long-term conservation. It comprises that set of accessions, each of which is distinct and, in terms of genetic integrity, as close as possible to the sample provided originally. Active collections comprise accessions that are immediately available for multiplication and distribution. Working collections are those held by breeders, and core collections are a small representation of a larger collection (FAO and IPGRI 1994).

Germplasm Regeneration and Its Purposes

'Regeneration' is that process that identifies in time those accessions, already introduced and recorded in the germplasm bank, whose seed (sexual or asexual) needs renewing. It also includes the establishment of protocols or optimal procedures for such renewal. Its essential goal is to maintain optimal quality and the genetic integrity of each accession and minimize the costs of carrying out these protocols.

For species with sexual seed, accessions that need to renew their seed are identified basically by monitoring the minimum factors of seed quality (viability and health) or quantity against values or standards previously established for the species. Standards are essential for providing targets that institutes can aim for. However, the problems inherent in setting standards should be considered, including that where some institutes may have difficulties meeting specified standards. In view of these problems, for these cases, two sets of standards are specified: (1) acceptable, that is, minimal but considered adequate, at least for the short term; and (2) preferred, that is, higher and thus safer standards. For most criteria, good scientific reasons exist for meeting the 'preferred standards'. Efforts should therefore be made to attain them (FAO and IPGRI 1994).

For species with asexual or vegetative seed, accessions are conserved continuously as live field collections or *in vitro*. The factors requiring close attention are those related to quality because, usually, the accessions are clones whose number of individuals remains constant from harvest to harvest. It should be remembered that, with these materials, the biggest risks are confusion of identity (or loss of this) and infection of materials by pathogens, especially those of quarantine interest. When collections are kept *in vitro*, these risks are not so significant.

The ideal goal of regeneration would be to produce seed whose viability is kept at 100% over long periods and whose accessions do not lose their original genetic composition. However, this ideal is difficult to achieve under real-life circumstances. Hence, the following practical goals are suggested for the curator of a collection that is conserved *ex situ*:

- Optimize the quality of seed produced
- Maximize the quantity of seed produced
- Wherever possible, maintain the genetic identity of each accession
- Maximize the cost-to-benefit ratio for each regeneration, that is, minimize costs and efficiently use equipment and resources without sacrificing the three previous objectives

Maximizing seed quality

'Seed quality' is understood as the maximum quality of an accession that is economically obtainable in terms of its plant health status, viability, and capacity to remain viable over time under storage. This means that, when it is regenerated, an accession should be, as far possible, free of pests or diseases. Moreover, its initial viability after regeneration should be 95% or higher.

Viability can be affected by different factors. Hence, to regenerate an accession in a suitable environment, the curator needs to know the optimal physiological state for harvesting, use handling procedures that will not damage the seed, know how to induce germination, and know if some type of dormancy exists (see *Module 3, Submodule C*). Thus, the curator must

know the species' biology, this knowledge being more critical for wild species and forms for which the level of knowledge is not usually as high as for cultivated forms.

To maintain the initial viability of a given accession for as long as possible, the seeds of that accession should be harvested when physiologically mature, but as young as possible. The earliest possible processing will ensure that the seed possesses optimal moisture content, which is about 5%, depending on the species, according to *Genebank Standards* (FAO and IPGRI 1994). These standards also indicate that minimum viability should be 85% for most seeds and 75% for some vegetable and forest species.

Optimizing the quantity of seed produced

To optimize the quantity of seed produced means that the cost-to-efficiency ratio for regeneration is maximized when the seed produced is sufficient to supply needs for use, before viability falls below the pre-established minimum. If the seed produced is not sufficient, then it must be regenerated more frequently, which is not desirable. In short, the quantities of seed expected from regeneration will depend on four factors:

1. The species' reproduction system (autogamous or allogamous)
2. Seed size
3. Adequate selection of the regeneration site to prevent problems of fertilization and/or flower abortion and fruit filling
4. Types or categories of conservation that each bank uses

With respect to seeds, the 'acceptable' standard for base collections, that is, the absolute minimum, is 1000 viable seeds for the accession being conserved; although, of course, any single number is arbitrary. In cases where fewer than 1000 seeds are available, the accession may nevertheless be kept under good storage conditions until such time as further collection or regeneration is possible. For active collections, the 'preferred' standard is 1500–2000 viable seeds (FAO and IPGRI 1994).

The material's regenerative capacity versus the time invested in the process should also be taken into account. For example, a tree may take several years before producing major volumes of seed. Thus, the curator must be careful when defining needs for seed for that material (e.g., bank's mandate), and will verify the germinating quality of the harvested seed before eliminating such a material from the production site. For example, if the interval between one set of seeds to the next is 7 years, then, in reality, the interval could be 14 years if something went wrong in the first cycle. Sackville Hamilton and Chorlton (1997) cited ranges, established by several germplasm banks, of seed quantities for species type (Table 1).

Table 1. Ranges of seed quantities for regeneration as per species type.

Type of species	Number of seeds
Autogamous	1,500–6,000
Allogamous	4,000–50,000
Large-seeded species	1,500–4,000
Small-seeded species	2,000–50,000

SOURCE: Sackville Hamilton and Chorlton (1997).

Maintaining genetic identity

Ideally, the objective of keeping the original accession's genetic identity is to maintain jointly the frequency of all alleles of all genes (loci). However, various limitations of a practical order make meeting this objective almost impossible. The two most important are ignorance of the integral genetic composition of the individuals forming the original collected accession, and the physical impossibility implied in regenerating each seed of a conserved accession. Nevertheless, one useful exercise is to minimize the number of regenerations by increasing the number of years between each one, using best practices of conservation, for example, improved drying activities. Another useful exercise is to increase the size of the sample to be regenerated, for example, using 100 plants instead of the 2 or 3, as is normally done, and harvesting the same number of propagules planted per plant planted and harvested.

During regeneration or multiplication, certain risks exist that should be avoided as far as possible to maintain the germplasm's genetic identity. One is the mistaken identification of samples, a situation that, in practical terms, leads to the material's loss of identity.

Another risk is contamination by exotic genes. These genes may come from (1) foreign plants being mixed in during seed preparation, planting, or harvesting; (2) seeds of the same species that come either from previous plantings in the regeneration lot or from nearby plantings; and (3) pollen from other accessions of the same lot or from nearby plantings of the same species or other related species.

A third major risk involves genetic drift and selection processes. Changes in gene frequencies occur at random (drift), or are generated by the environment, or are a consequence of management by man (selection). The results are changes in gene frequencies and thus loss of the original genetic composition. Gene frequencies change mainly because:

- The sample being regenerated does not represent the original genetic composition
- A percentage of the initial seeds planted do not germinate, plants are dead or had not matured
- The contributions of feminine and masculine gametes are different
- The genetic composition of pollen and ovules differs from that of the original population
- Manual pollination had favoured certain phenotypes

Criteria and Limitations for Establishing Regeneration Protocols

The optimal protocols for regenerating accessions depend on:

- The characteristics of the species concerned, particularly its reproduction biology, the accession's physiological condition and original genetic composition, the use it will have, and its value to the collection
- Documentation on previous multiplications (degree of success or failure)
- Availability of human resources, infrastructure, and budget

Establishing adequate regeneration protocols for most species presents limitations because of poor knowledge of the structure of the original populations. Consequently, these protocols should be flexible so that they can conform with the needs of each bank in terms of categories or types of conservation (short or long term) and research objectives.

The need to regenerate depends on how and why the accessions concerned are being conserved. The answers relate to each germplasm bank's conservation objectives.

Sexual seed. Regeneration is necessary when the germplasm's viability has declined to below established limits, or the quantity of sexual seed existing in the bank is less than the minimum number of seeds established by the bank holding the collection. Such a minimum may be established by using the standards recommended by FAO and IPGRI (1994). These standards are designed to maintain the original genetic composition of each accession, and depend on whether the species is autogamous or allogamous.

Asexual seed. For species that propagate vegetatively, collections are kept as field collections and the number of plants will depend heavily on the species' type of propagule, and the availability of land and resources for maintaining the collection in the field. Usually, the number of plants is relatively low because of the high genetic homogeneity of individuals of an accession, as, in most cases, the plants are clones of the same progenitor.

Monitoring the Need for Regeneration

Determining the need to regenerate or refresh the germplasm starts with monitoring the viability of the samples conserved according to standards and procedures that define the quantity and quality of the material to use, the number of plants, and the environment (Box 1).

According to the recommendations offered in paragraph 30 under *Viability Monitoring* from *Genebank Standards* (FAO and IPGRI 1994), the purpose of conducting control tests on viability is to determine if regeneration is needed. To save seeds, between 50 and 100 units of the entry can be chosen at random for each control test. The simplest method to ascertain if a substantial loss of viability is occurring (ruling out possible fluctuations in results largely attributable to sampling errors) consists of representing graphically the results of successive control tests against storage period and observing if a gradual loss of viability is occurring. If it is, and seeds are sufficient, another sample of 100 seeds should be extracted at random and tested again for viability. This sampling will guard against unnecessary regeneration. If the decision is made to regenerate, the allotment of seeds for the germination tests will be cancelled, thereby saving seeds, which, under these circumstances, will be more valuable.

No standards exist for sample size of plant propagules conserved in the field or *in vitro* but, usually, 3 to 20 replications are kept per accession. Viability is established through observations or tests, depending on the type of sample. Viability of plant material (plants in the field or *in vitro* slow growth) is systematically established by observing its health and development, and the conditions under which it is being conserved. If any one of the prior criteria is not being fulfilled, then the material should be regenerated. If the conserved material is seed, then viability is analyzed by practising germination tests, which consist of germinating a sample of seeds to ascertain how many (%) germinate and, of those that do not germinate, determining which have died or are dormant. The findings are then compared with the initial viability—taken during preliminary multiplication—and if the current viability has declined to 85% or less, then the sample should be regenerated.

Procedures for determining viability as the key element of monitoring conserved materials are described in more detail in *Lesson 2* on verifying the biological status of germplasm (*Module 3, Submodule C*).

Box 1

Genebank Standards: Regeneration

35. Regeneration standards are needed to ensure that the seeds stored in base collections do not fall below acceptable levels of viability and yet minimize the number of regeneration cycles to ensure that the genetic integrity of accessions is maintained. The regeneration interval will depend on the longevity of the seed in storage and demand for the accession (if seeds are not available from an active collection).

36. Seeds which are produced for storage in base collections should, as far as possible, be of the highest possible viability and free of pests and diseases. Recognizing that the initial germination capacity will depend on the environment during production and processing, maturity and physiological state of the seeds at harvest and genetic differences between species, initial germination values should exceed 85% for most seeds, e.g. cereals, and 75% for some vegetables and even lower for some wild or forest species, which do not normally reach high levels of germination.

37. Regeneration should be undertaken when viability falls to 85% of the initial value. Regeneration methods should follow the standards for the crop, where available, and ensure that sufficient plants are used to maintain the genetic integrity of the accession. As far as possible all sources of selection pressure should be removed, the contribution of seeds from each plant should be equalized and all possible care taken to minimize genetic change.

38. It is desirable to use 100 plants or more for regeneration to avoid the probability of large losses of alleles. However, in wild species this may be limited by the total number of seeds available. Wild species may also vary in breeding system, storage behavior and germination from the related crop species. This should be taken into consideration when deciding when, and how to regenerate an accession.

39. In order to ensure that the genetic integrity is maintained and accessions are distinct, it is recommended that seeds used to plant material for regeneration should be as close as possible genetically to the original germplasm. It is recommended that for active collections, regeneration should be done from original seeds whenever possible or from its offsprings within two or three cycles of regeneration to ensure that genetic integrity is maintained. This implies that, assuming a 15 year storage cycle for the active collection, seeds for regeneration will need to be taken either from the base collection or other original seed in long-term storage once in 45 to 60 years, providing sufficient seeds are regenerated to meet demands on the active collection for distribution. Genebanks carrying out regeneration should also consider what methods they could use to monitor variation during regeneration to measure any changes in genetic constitution in accessions.

SOURCE: FAO and IPGRI (1994).

Regeneration Protocols

Regeneration protocols are essentially the same as those used to multiply germplasm; therefore, see the previous lesson (i.e., *Lesson 1, Submodule A*).

Evaluating the Lesson

With this lesson, you should have understood the concept of germplasm regeneration and its purposes, reviewed the criteria for establishing protocols, and learned how to monitor needs for regeneration.

This lesson finalizes *Submodule A* of *Module 3* but, before going on to the next lesson, prepare a well-based plan for germplasm regeneration. You may use, as an example, the germplasm with which you currently work or, in its absence, with that with which you are familiar.

If you are not directly familiar with the process, list and discuss the criteria that, in your opinion, should be taken into account when regenerating a given germplasm.

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Next Submodule

In the lessons of the next *Submodule B*, you will study the principal aspects of harvesting, conditioning, and quantifying the germplasm after its multiplication and regeneration.