HANDBOOK FOR GENEBANKS NO. 5.

Regeneration of accessions in seed collections: a decision guide

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Volume editor: J. Engels

International Plant Genetic Resources Institute
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Citation:
Sackville Hamilton, N.R. and K.H. Chorlton. 1997. Regeneration of accessions in seed collections: a decision guide. Handbook for Genebanks No. 5. International Plant Genetic Resources Institute, Rome, Italy.

ISBN 92-9043-319-1

IPGRI
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Preface

Ex situ conservation is vital for ensuring the long-term safety and continued availability of plant genetic resources for use by scientists and farmers in their collective efforts to achieve global food security. The storage of dry seed at low temperature is the most widely practised method of *ex situ* conservation.

Seed genebanks worldwide share at least two essential objectives, i.e. ensuring long-term conservation of the genetic diversity represented in the seed collections they hold, and maintaining an adequate stock of seed for distribution to users. The periodic regeneration of the seed accessions is necessary to maintain optimal seed viability over the long term as well to replenish the seed stock.

To conduct sound regeneration practices, species-specific and general information such as knowledge about the reproductive biology and the extent and distribution of the genetic diversity of the material to be conserved is needed. In addition, it is necessary to minimize genetic drift and genetic shift which might occur during the regeneration process. The mechanisms underlying these phenomena and their potential effects need to be quantified and methods developed to mitigate them. The effect of seedborne pathogens on the maintenance of genetic integrity of accessions is another, related aspect that requires further investigation. In the case of cross-pollinated species, questions remain regarding the most effective isolation techniques, pollination control procedures and mating methods.

Since regeneration has proven to be a relatively costly procedure requiring a significant amount of resources such as land and labour, adequate budget planning and overall cost-effectiveness of the procedure are important aspects.

In order to take stock of these research needs as well as to gather the wealth of knowledge and experience held by genebank curators and researchers worldwide, IPGRI, on behalf of the CGIAR System-wide Genetic Resources Programme (SGRP) and in association with the Food and Agriculture Organization of the United Nations (FAO), organized a consultation meeting on the regeneration of germplasm of seed crops and their wild relatives which was hosted by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru, India (4-7 December, 1995). The meeting served as an effective forum for bringing together the elements necessary to develop a strategy for the task at hand: experienced genebank curators and plant genetic resources experts; researchers with a range of complementary perspectives as well as the pertinent scientific and technical information. The consultation was successful both in identifying and articulating key constraints and problems and in outlining the various possibilities available for resolving them. It produced a framework for decision-making, covering the manifold issues and options to be considered in carrying out regeneration.

Providing curators with a 'decision guide' for selecting appropriate options, rather than prescribing procedures, allows for the specific requirements of different accessions and the varying circumstances of different genebanks to be taken into consideration throughout the decision-making process.

The development of this decision guide and its publication are particularly timely, in that they coincide with the preparations being made by countries to implement the Global Plan of Action (GPA) for the Conservation and Sustainable Use of Plant Genetic Resources for Food and Agriculture adopted at the International Technical Conference on Plant Genetic Resources, held at Leipzig, Germany in June 1996. The GPA underscores the urgent need for

devising methods to sustain existing *ex situ* conservation efforts worldwide through, *inter alia*, the regeneration of seed accessions which may be at risk of losing viability. It is hoped that this decision guide will contribute to efforts toward this end.

Many of the considerations involved in seed regeneration also relate to the overall management of seed collections in genebanks. By calling attention to such aspects, it is hoped that this decision guide will go beyond its primary objective, i.e. to serve as a tool for genebank managers and personnel in planning and implementing the systematic regeneration procedure, to serve as a contribution to germplasm management efforts aimed at promoting the overall efficiency and cost-effectiveness of genebank operations in order to ensure the long-term security of the valuable plant genetic diversity represented in *ex situ* seed collections worldwide.

A final draft of the manuscript was made available at the Seventh Session of the FAO Commission on Genetic Resources for Food and Agriculture (May 1997, Rome, Italy) for comment by members.

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Acknowledgements

IPGRI highly appreciates the commendable work of the two authors: Dr N.R. Sackville Hamilton, Biodiversity Group Leader and Head, Genetic Resources Unit and Dr K.H. Chorlton, Genetic Resources Officer both from the Institute of Grassland and Environmental Research, Aberystwyth, Wales, UK. The Institute also wishes to acknowledge the valuable inputs of all the participants of the consultation meeting – the outcome of which provided the basis for developing this document. ICRISAT's assistance in hosting the meeting is also duly acknowledged. Special thanks go to the peer reviewers: Dr L.M. Engle, Head, Genetic Resources and Seed Unit, the Asian Vegetable Research and Development Centre, Taiwan; Dr P. Lawrence, Plant Genetic Resources Officer, Australian Tropical Crops Genetic Resource Centre, Biloela, Australia; Dr W. Salhuana, Research Fellow, Miami, USA; Dr H. Shands, Associate Deputy Administrator for Genetic Resources, USDA-ARS, Beltsville, USA; Dr E. Thoen, Director, Nordic Gene Bank, Alnarp, Sweden; Dr A.C. Valois, Head of CENARGEN-EMBRAPA, Brasilia, Brazil, and Dr M. Widrlechner, Horticulturist USDA-ARS, Iowa, USA. The valuable scientific and technical contributions of scientists at FAO and CGIAR centres is acknowledged with gratitude. These include Dr N.M. Anishetty, Senior Officer, Plant Genetic Resources and Dr K.L. Tao, Training Officer, Seed and Plant Genetic Resources Service at FAO, Dr J. Crossa, Biometrics, Head, CIMMYT, Mexico; Dr J.A. Hughes, Virologist, ICRISAT, Andhra Pradesh, India; Dr V.R. Rao, Genetic Diversity Conservation, IPGRI, Selangor Malaysia; Ms J. Toll, Coordinator, SGRP, IPGRI, Rome, Italy; Dr M.T. Jackson, Head, Genetic Resources, IRRI, Philippines; Dr R. Rellano, Senior Research Assistant, Genetic Resources Center, IRRI, Philippines, and Dr Z. Huaman, Germplasm Curator, Genetic Resources, CIP, Lima, Peru. Finally, the financial support provided by FAO, ICRISAT, the CGIAR System-wide Genetic Resources Programme (SGRP) and IPGRI is duly acknowledged.

Executive summary

This decision guide is intended to facilitate the development of optimum procedures for regeneration of seed germplasm. It deals with the timely identification of accessions with inadequate quality or quantity of seed. It also considers the regeneration of those accessions to produce new seed of maximum quality and optimum quantity, with minimum loss of genetic integrity and as cost-effectively as possible.

The optimal protocol for regeneration depends on the breeding system and seed storage characteristics of the species concerned, the physiological condition and genetic composition of the original sample, its expected usage and its perceived value within the collection, and operational constraints on genebank activities, such as funds, human resources and equipment. There is often insufficient knowledge about the species concerned to select the optimal regeneration protocol, and the effects of the various options on genetic population structure are often poorly known. Regeneration procedures must, therefore, be flexible, to enable them to meet the needs of different genebanks and accessions and to be responsive to research developments. This guide is intended to facilitate the decision-making process involved in developing appropriate protocols.

The guide discusses establishment of achievable targets for quality and quantity of seed produced, the maintenance of genetic integrity and minimizing the costs of regeneration. Calculations are based on the requirements of different units of usage – the distribution unit, test unit and base unit – together with a safety factor allowing for losses and a factor allowing for uncertainty of usage. A single case study is presented by way of illustration, and discusses the consequences of the various causes of loss of genetic integrity, by drift, selection and contamination.

The main body of the guide deals in detail with establishing the regeneration protocol. To maximize the practical value of the guide, subsections are presented in the same order as the practical activities involved in regeneration: selection of the site, accessions and seed for regeneration; preparation of the site and seed; crop management before, during and after anthesis, and harvesting and post-harvest management. Flow charts of the decision-making process are provided to assist understanding.

Two aspects of location for regeneration are considered. The choice of the overall site is jointly determined by policy considerations, adaptation of the crop to the regeneration environment, and the need for maintenance of genetic integrity. The types of location at a site include field, glasshouse or other facilities for better control of the environment.

Selection of accessions for regeneration requires the definition of threshold levels for seed quality and quantity, below which regeneration is required; a protocol for monitoring seed quality and quantity, and a protocol for prioritizing accessions when the number in need of regeneration exceeds genebank capacity. Highest priority is given to regenerating accessions that have seed of inadequate quality in the base collection. Separate thresholds and protocols are required for newly received seed and accessions already held in storage.

Selection of seed to be used to provide parental plants deals with the source of seed, the number of seeds to be used and their identity. Emphasis is placed on using seed held in the base collection to replenish seed stocks in the active collection to prevent cumulative degradation of genetic integrity. The number of seeds to be used is determined jointly by the number required for the satisfactory

maintenance of genetic integrity and the number of offspring seeds to be produced. Seed for use as parents is usually selected at random from the available seed; however, in a few cases, consideration should be given to selecting seed that more fully represent the genotypic composition of the original population sample.

The remainder of the regeneration protocol is heavily dependent on the agronomy of the species concerned. The guide focuses on issues that are of particular importance to regeneration and that, therefore, will not feature in standard agronomy texts. The importance of these issues and approaches to resolving them is again heavily dependent on the biology of the species concerned.

Ensuring accuracy and preventing contamination by alien plants, seed or pollen are key issues throughout, from preparation of regeneration plots to storing the harvested seed. Mechanization should be based on purpose-built machinery, since adequate cleanliness and accuracy are not usually achievable with commercial agricultural implements. Effective use of information technology is encouraged in combination with cross-checking procedures. Where possible, complete isolation from all sources of alien pollen is strongly recommended for all species except obligate inbreeders and obligate apomicts.

Maximizing uniformity among plants in their contribution of male and female gametes to the offspring generation is also a key issue throughout, although different measures are required at different stages. Pruning, manual pollination and balanced bulks are among the more labour-intensive measures that should be considered to increase uniformity where variation between plants is high.

Ensuring the highest possible health and viability of offspring seed is another key issue that becomes important from anthesis onwards. It depends on good disease control, appropriate harvesting and appropriate rapid post-harvest processing, particularly for seed-drying and threshing.

The decision guide also touches on broader issues of genebank management policy that have implications for regeneration strategy. However, while consideration of these broader issues is important for the development of an efficient regeneration programme, their interactions with other aspects of genebank management place them beyond the scope of this guide. Therefore, it is planned to deal with them more fully in a future work.

In conclusion, the guide aims to provide general considerations on how to improve the effectiveness of germplasm regeneration programmes. In addition, there will be a need to develop more detailed guidelines for individual crops or groups of crops, which could in many instances be done through the activities of the international crop genetic resources networks. There is also an urgent need for research to gain the crop-specific knowledge necessary to optimize regeneration protocols, and to quantify the consequences of the various options presented, in particular from a population genetic and economic point of view.

1 Introduction

"Timely regeneration must be a priority activity of all genebanks" (FAO 1996). Effective regeneration programmes are essential to maintain the viability and genetic integrity of *ex situ* seed collections of germplasm. Without such programmes, it will not be possible to realize the potential benefits of the substantial global investment in *ex situ* germplasm conservation. Yet the majority of the world's genebanks are experiencing a large backlog and continuing difficulties with regenerating their collections (FAO 1996).

This decision guide is intended to facilitate the implementation of efficient regeneration programmes, by presenting a range of options for regeneration and their applicability in different situations. The objective is to help curators reach logical decisions on the appropriate procedures, highlighting the questions that need to be addressed and the factors that influence each decision. The guide does not provide prescriptive guidelines.

2 Scope and structure of decision guide

The objectives of a regeneration programme, as addressed in this decision guide, are to:

1. ensure timely identification of accessions with inadequate quality or quantity of seed, and
2. at the earliest opportunity, produce a new seed sample that, as far as possible (subject to a wide range of constraints, e.g. resources, knowledge, biology, policy, etc.) has:
   - maximum quality
   - optimum quantity
   - the same genetic composition as the original
3. achieve the above as cost-effectively as possible without compromising the maintenance of quality, quantity and genetic integrity or the utilization of germplasm.

Identifying and regenerating accessions are only two components of an efficient regeneration strategy. Regeneration must be undertaken within the context of a strategy that minimizes the overall need for regeneration without reducing the efficacy of conservation or utilization. However, the issues involved in developing such a strategy are also central to broader issues of genebank management policy, and their implications for regeneration strategy form only one component of genebank management. Therefore, such strategic issues are deemed beyond the scope of this document even though they have to be considered by curators in establishing regeneration programmes. To facilitate consideration by curators without impinging on other aspects of genebank management, the issues are summarized in section 4, but without detailed discussion. Only the regeneration protocol itself will be discussed in detail. There is a need for future guidelines to cover overall genebank strategy including regeneration strategy.

The optimal protocol for regeneration depends on numerous factors:

- breeding system and seed storage characteristics of the species concerned
- the condition and genetic composition of the original sample
- its expected usage and its perceived value within the collection
- operational constraints on genebank activities, such as funds, labour and equipment.

It is therefore not possible to lay out a single uniquely optimal protocol. It is the responsibility of every curator to adopt appropriate genebank-specific procedures – procedures that need to be sufficiently flexible to accommodate accession-specific protocols. The objective of this document is to help a curator develop appropriate procedures.

In many cases there is insufficient knowledge about the species concerned to select the optimal regeneration protocol. Moreover, the effects of the various options on genetic population structure are also often not known. In the short term, the selected protocol must be based on whatever knowledge is available. In the longer term, there is a need for research to gain the necessary knowledge, for example on the population characteristics of the species/accessions concerned. Research needs in relation to regeneration are beyond the scope of this decision guide, but assessing these needs to be addressed more fully in the future.

The decision-making process may be viewed from two angles: (i) theoretical consideration of the biological and infrastructural issues that determine the optimal regeneration protocol, and (ii) the practical, chronological sequence of events involved in regeneration. Each theoretical issue may have consequences for several practical steps, and the optimal conduct for each practical step depends on several theoretical issues. For example, the need to eliminate contamination by alien pollen influences the choice of site, site preparation and crop management during anthesis.

Breese (1989) adopted the first, theoretical approach in reviewing regeneration theory. This document adopts the second approach to help fulfil its intended role as a practical guide to decision-making. To provide the necessary background, targets and prerequisite knowledge are summarized in sections 5 and 6, but without detailing their practical consequences for the regeneration protocol. Options for regeneration are then discussed in detail in section 7, where they are presented in chronological sequence from selecting locations and seed for regeneration to post-harvest procedures. Where an issue affects several steps in regeneration, the background to its consequences is discussed at the first relevant step in section 7; only specific additional details are covered at later relevant steps.

To facilitate the decision-making process further, it is also presented as a series of flow charts, in which decisions and activities are cross-referenced to the section of the report that presents the options and discusses the factors that influence the decision.

3 Types of collection

The need for regeneration depends on how and why accessions are stored. Three main conceptual categories of collection – base, active and safety duplicate – are recognized as serving different purposes (see Genebank Standards – FAO/IPGRI 1994). Additional categories include core and working collections, but these relate primarily to targeted utilization of selected accessions and will not be dealt with in this guide (but see section 4.4).

> A **base collection** is "a set of accessions, each of which should be distinct and, in terms of genetic integrity, as close as possible to the sample provided originally, which is preserved for the long-term future."

Emphasis is on **conservation**, and the genebank assumes responsibility for long-term conservation of the germplasm; accessions in the base collection should therefore be held in optimal conditions for long-term storage. The preferred standard for storage is "–18°C or cooler with 3-7% seed moisture content (depending upon species)" (FAO/IPGRI 1994). Preferred standards for quantity of seed vary with species (sections 5.2 and 7.2.2).

Strictly, to qualify for inclusion in the base collection, an accession should be genetically unique. However, there may be little or no information on genetic composition of an accession. In such cases, depending on the conservation policy of the genebank, it may be desirable to adopt a more pragmatic approach and accept uniqueness based on passport data as a sufficient criterion for distinctness (acknowledging that passport descriptors differ in their value for distinguishing accessions and that passport data may also be incomplete).

Some genebanks place samples of every accession in the base collection without regard for their uniqueness. This is in fact essential in order to comply with preferred and acceptable regeneration practice (FAO/IPGRI 1994) of using seed in the base collection to replenish seed stocks in the active collection, at least once in four regeneration cycles (see section 7.3.1.3). Use of the base collection in this way eliminates any requirement to demonstrate uniqueness.

It is necessary to distinguish between base collections defined by individual genebanks, and base collections established by formal agreements between genebanks in national and international networks. In the latter, one or more genebanks may be designated as holding the base collection for the entire network, in which case the holding genebank(s) is given broader responsibility for secure conservation but not necessarily for utilization and regeneration. This document is not concerned with such formal base collections, but rather with base collections defined by individual genebanks for their own benefit, particularly in relation to their role in regeneration.

> An **active collection** is a set of "accessions which are immediately available for ... use."

Emphasis is on **utilization**, not conservation: accessions in the active collection potentially have an important role in breeding and/or research by

genebank staff or by outside users of the genebank. That is, maintenance needs are defined in terms of the immediate user base of the collection, not in terms of conservation.

Storage conditions for an active collection are undefined except in functional terms: conditions should "ensure that accession viability remain above at least 65% for 10 to 20 years" (FAO/IPGRI 1994). The majority of genebanks maintain an active collection under less stringent conditions than the base: different genebanks maintain the active collection under conditions ranging from –10°C to 5°C and 15–50% relative humidity (ICRISAT 1995). As with the base collection, preferred standards for quantity of seed vary with species (sections 5.2 and 7.2.2).

> A **safety duplicate collection** is a duplicate copy of accessions, held at a distant site or series of sites and preserved for the long-term future, as an insurance measure guarding against accidental loss of germplasm through natural disaster or other hazards.

The safety duplicate collection should contain all accessions in the base collection. Like the base collection, it should be held in optimal conditions for long-term storage. In many cases, safety duplicates are held by a different genebank. In this case, the genebank responsible for storing it normally holds it on a 'black-box' basis, i.e. doing nothing but storing it optimally, with no responsibilities or rights for monitoring viability, regeneration or distribution, except by specific agreement with the base genebank.

4 Strategic issues

This section summarizes many of the strategic issues that the curator must consider in relation to regeneration. No attempt is made to provide answers or discuss consequences, because the issues are broadly relevant to all aspects of conservation and utilization of germplasm, not just to regeneration. As such, full discussion of the issues is beyond the scope of this document.

4.1 Ensuring that regeneration is part of appropriate conservation policy

Before attempting to establish an optimal regeneration strategy, it is of course necessary to ensure that conservation and regeneration are relevant.

- Is it appropriate to conserve a given genepool at all?
  - is it part of the mandate?
- If it should be conserved, is it necessary to conserve it *ex situ*? – either instead of or in addition to *in situ* conservation. Or should it be conserved exclusively *in situ*?
  - will an ex situ collection ever be used?
- If it should be conserved *ex situ*, should it be stored as a seed collection? Alternatives are conservation *in vitro* (e.g. as pollen or tissue culture), or as a living collection in the field or glasshouse.
- If it should be conserved *ex situ* as a seed collection, is regeneration an appropriate way of maintaining seed stocks? If a population still survives *in situ*, is it preferable to make repeat collections from the original site of collection instead of regenerating?

4.2 Ensuring adequate institutional capacity

Is capacity sufficient to maintain an effective seed collection of germplasm? In particular:

- Are current resources adequate?
  - facilities
    - seed storage
    - land and controlled environments
    - other general infrastructure
    - information management
  - financial resources
  - human resources
    - skills, knowledge, experience
    - quantity
  - institutional management policies.
- Is there potential to increase effective capacity through collaboration with other institutes?
- Is there potential to decrease the marginal costs (financial and labour) of regeneration by combining it with other genebank tasks, e.g. using regeneration plots for characterization?
- Can the institute adopt the necessary long-term outlook?
  - is current status secure?
  - can the genebank respond to and exploit new opportunities?

- Is there a quality assurance mechanism, to evaluate success of the genebank and ensure that it meets defined conservation priorities, regeneration targets, cost targets and the needs of its users?

4.3 Optimizing the size of the collection

Regeneration and other maintenance costs can be reduced by reducing the size of a collection, or at least by reducing its rate of increase in size. However, this may adversely affect conservation and utilization of germplasm in relation to national, regional and institutional policies, and limit the potential to meet the challenges the future holds. Optimizing the size of the collection requires consideration of the following issues and questions.

1. **General conservation priorities and policies.** Should a new sample be added to the *ex situ* collection?
   - Is it a priority in national, regional or institutional context?
   - Is genebank conservation capacity sufficient?
   - Are there opportunities for sharing responsibilities with sister genebanks in national or international networks?
   - Does the quality of the new sample and accompanying data meet genebank defined standards? If not, can anything be done to improve it, e.g. by an initial cycle of regeneration to improve seed quality and quantity, or by seeking better data?
   - Is a similar or identical sample already present in the collection?

2. **Priorities and policies for methods of acquiring new accessions**
   - Should they be acquired by new collecting expeditions or by introduction from other *ex situ* collections?
   - Why should they be acquired (genetic erosion, novel diversity and gap filling, satisfying specific breeding or research objectives)?
   - What should be the relative emphasis on wild relatives, landraces, primitive varieties, disused breeders' lines, current breeders' lines, modern varieties? Should breeders or genebank be responsible for holding, maintaining and distributing current breeders' lines and modern varieties?
   - Who determines acquisition policy (genebank and/or users)?

3. **Management of genetic variation within populations**
   - Maintain as a single variable accession or split into several more uniform accessions? (see section 7.2.3 for partial discussion)
   - If maintained as a single variable accession, should the component strains or subpopulations be stored in different containers for improved maintenance of genetic integrity during regeneration? (see section 7.3.3 for partial discussion of the kind of population structure that might merit this option, and of the need for integrating methodologies for collection, regeneration and storage).

4. **Management of redundant genetic variation**
   - Can biologically duplicate accessions (as distinct from historically duplicate accessions with a common origin) be identified and combined or eliminated?
   - Is it possible to devise a strategy for eliminating duplication that is economically justifiable, i.e. where the savings in maintenance costs exceed the costs of identifying biological duplicates?

4.4 Minimizing the regeneration requirement of each accession

Maintenance costs of a collection can be reduced by minimizing the regeneration requirement of each accession. Most aspects of this are central to this decision guide, e.g. optimizing storage conditions, optimizing quantity, maximizing quality, etc., and will be discussed in later sections. In addition there are some more strategic issues that lie partly or wholly outside the scope of this decision guide. These are:

1. Policy on initial regeneration, i.e. on regenerating seed when a sample is first received and before it is formally registered as an accession in the collection. Should initial regeneration be avoided where possible in order to retain maximum genetic integrity, or encouraged in order to maximize seed quality? (see partial discussion in section 7.2.1.2).

2. If policy is to avoid initial regeneration, to what extent should policy on acquiring new accessions be modified to reduce the need for it? For example, the need for initial regeneration can be reduced by imposing the following restrictions:
   - do not collect vegetative samples
   - do not collect where only small seed samples are available
   - do not accept seed donations comprising few or poor-quality seed.

   Are any of these acceptable restrictions?

3. Allocate samples to which collection(s) – active, base, safety duplicate, core, working, other? The active collection is more costly to maintain, as it requires more frequent regeneration and more active management than the base, and accessions in the active collection deteriorate more rapidly in genetic integrity. On the other hand, the active collection is the one for which there is demand.
   - Is there scope for reducing regeneration costs by targeting utilization at a working active subset of accessions (e.g. working and/or core collections)? Restricting utilization of the remainder would allow them to be maintained more cheaply (through use of better storage conditions, less regeneration, less quality testing, less information management, etc.).
   - Can this be done at least without adversely affecting utilization?
   - Is it possible, through interacting with users in defining the optimal subset of accessions for utilization, actually to increase the amount and effectiveness of utilization at the same time as reducing regeneration costs?

4. What should be done with the original seed sample?
   - Should it be stored in only the base collection, keeping only seed derived by regeneration for the active collection? This would optimize long-term maintenance of genetic integrity. By using only the original sample in the base collection for replenishing seed stocks in the active collection, it would optimize the long-term genetic quality of seed available to users.
   - Should it be kept in the active collection? This would give the user community access to the best possible sample in the short term, but would have adverse long-term consequences for seed supplies after the original sample is used up.

5. Are the existing concepts of base, active and safety duplicate collections adequate, or is there a need to revise concepts and procedures?
   - For example, is there scope for reducing the frequency of regeneration by holding all seed in long-term storage conditions like the base collection, increasing the number of seed stored for each accession, and distributing seed direct from the base collection? Would the impact on maintenance of genetic integrity be acceptable? Would the impact on accessibility be acceptable?

5 Targets for regeneration

This section discusses targets for regeneration. It many cases the ideal target is not achievable (e.g. 100% germination rate and zero genetic change on regeneration both represent the ideal but neither of them is achievable), so achievable targets are considered. Determining how to achieve these targets is the subject of section 7.

The curator should consider establishing two distinct sets of targets: high-stringency targets for regenerating the base and safety duplicate collections; and cheaper, lower-stringency targets for regenerating the active collection. Genebank Standards (FAO/IPGRI 1994) recommend reducing the cumulative loss of genetic integrity in the active collection by replenishing its seed stocks from seed in the base collection (see section 7.3.1.3). Losses of genetic integrity in the active collection are then only short-term losses and relatively low-grade regeneration conditions may in some cases be considered acceptable. In contrast, **regenerating the base and safety duplicate collections represents critical regeneration cycles for the long-term maintenance of genetic integrity of all collections.** It is therefore important to make these highly controlled regenerations to create samples for future regeneration.

The general target objective is to maximize the cost-efficiency of each regeneration event. This means to:

- maximize quality of seed produced
- optimize quantity of seed produced, and
- as far as possible, maintain genetic integrity of the accession, while
- minimizing costs, making efficient use of available equipment and resources, without sacrificing quality of regeneration as defined by the above three criteria.

5.1 Maximizing seed quality

1. Seed quality should have the maximum economically achievable quality, as defined by its health, viability and ability to remain viable in storage.

2. It should be as far as possible free of any pathogen or pest.

3. It should have the maximum initial viability that can be achieved for the species. Maximum achieved viability should typically be 95% germination rate or better for most crops. It may be less for some species, if:
   - the growing conditions required to produce good-quality seed are not known for the species
   - the stage at which seed reach physiological maturity optimal for harvesting is not known for the species
   - seed-cleaning procedures are not appropriate for the species and so fail to eliminate aborted seeds from the sample (see section 7.9.4), or
   - the germination requirements of the species are inadequately known
   - the species produces dormant seed – germination rates are then low immediately after harvesting, but this does not indicate low viability, and germination rate increases with time in storage.

Thus difficulty in achieving good germination for a species may indicate a need for further research on its biology in order to identify optimal procedures.

This tends to be more often the case for wild species than for crops, as comprehensive seed biology research has focused mainly on crops.

For species that produce dormant seed, the resulting low initial germination rate may be desirable. Attempting to increase germination rate by breaking dormancy before storage (by means that depend on the mechanism of dormancy, e.g. vernalization, scarification or allowing a period of after-ripening) may reduce seed longevity in storage.

In all other cases, accepting lower germination as the maximum achievable target should generally be only a temporary pragmatic measure pending research to develop superior protocols. The requisite research may not be cost-effective for wild and weedy species represented by only a few accessions in a collection, if they show complex and variable dormancy and morphology. However, in many cases it will enable more efficient, cheaper regeneration, which will recoup research costs. In such cases the research will be highly cost-effective and is strongly recommended.

Seed should be of an age (i.e. young but physiologically mature) and condition (harvested at or just prior to physiological maturity and processed without delay to reach optimal moisture content and physiological status) that ensures maximum longevity in optimal storage conditions (see section 7.9.2). Optimal moisture content for stored seed depends on seed characteristics and on the temperature used for storage (Vertucci-Walters and Roos 1990; Vertucci-Walters *et al.* 1994). Factors affecting the vigour of viable young mature healthy seed have received little attention to date. Following further research this may become an important part of future protocols for evaluating and maximizing seed vitality.

Further details are given in Genebank Standards (FAO/IPGRI 1994) and other IBPGR publications.

5.2 Optimizing seed quantity

5.2.1 Relationship to seed usage

Cost-efficiency of regeneration is maximized when seed quantity is just sufficient to provide enough for use before viability drops below threshold. Regenerating fewer seeds than this raises costs by necessitating more frequent regeneration. Regenerating more incurs greater costs by producing and storing seed that is never used.

However, it is not possible to achieve this maximum cost-efficiency because it is impossible to predict exactly how much seed will be used. Genebanks will inevitably expend resources on regenerating and storing seed that is never used for accessions that are used less than expected. At the same time they will also inevitably expend resources on more frequent regeneration of accessions that are used more than expected.

Optimizing seed quantity therefore requires an assessment of how far actual seed usage may differ from expected usage, and of the relative costs of (i) producing and storing too many seeds, and (ii) regenerating more frequently when too few are produced. Where producing too many seeds is preferable to regenerating more frequently, the curator should overestimate expected usage of seed and so regenerate more seed than necessary for most accessions, and *vice versa*. The optimum magnitude of over- or underestimation will increase with the uncertainty of usage and with the difference in costs of over- and underproduction. This is termed the **uncertainty factor**. Target seed quantity is then expected usage multiplied by the uncertainty factor.

The curator will need to consider not just financial costs, but other costs or impacts on genebank functionality, such as consequences for maintenance of genetic integrity. The definition and calculation of costs, and therefore the value of the uncertainty factor, will be different for base and active collections (see sections 5.2.3 and 5.2.5). In most cases, because of the economies of scale in seed production, it will be more economical to produce and store excess seed, implying an uncertainty factor greater than 1.

5.2.2 *Definition of units*

Since targets for seed production vary with usage of an accession, calculation of targets requires definition of the units of seed usage as follows.

- The **distribution unit** is the number of seed distributed with each request. This must be sufficient to provide the user with a sample that adequately represents the genotypic composition of the accession. For highly variable accessions that need large sample sizes for adequate representation of the range of variation, some users' objectives may be satisfied with smaller samples that may be less representative. Different users require different quantities of seed of each accession; the genebank should be flexible in meeting users' quantity requirements. The curator will then need to estimate a mean size of the distribution unit, but should also set a maximum number of seed that can be distributed to meet a request for an accession.

- The **test unit** is the number of seed required to test seed quality and viability.

- The **base unit** for regeneration is the number of seed needed to ensure the successful accomplishment of the regeneration of a representative sample of the original accession, with genetic integrity maintained intact, as far as possible, factoring in all causes of seed losses. It is "an accession-specific population size, reflecting the effective population size given a certain mating system, needed to preserve diversity under certain assumptions" (ICRISAT 1995). These assumptions are as follows.
  a) A defined target quantity of offspring seed is required.
  b) Effective fecundity[1], the number of useable offspring seed produced per parent plant, cannot exceed a certain value that depends on the species and regeneration conditions.
  c) The genetic structure of population is to be conserved to a defined extent, with particular reference to conserving the frequency of rarest alleles, and taking into account the number of loci to be considered (Crossa 1989; Crossa *et al.* 1993).
  d) Parental seed viability is less than 100%: allow for germination rate below the threshold for regeneration (section 7.2.2), typically 50%.
  e) A certain proportion of plants and seed will be lost during normal crop management and post-harvest procedures.
  f) The regeneration process may fail, with a certain probability. Allowance must be made for the possibility of at least one crop failure, so that the

[1] In some cases, for example if the new seed sample is formed by taking a balanced bulk (see section 7.9.7), not all the seed produced in a regeneration plot are used to form the new seed sample. Effective fecundity, the number of useable offspring seed produced per parent plant, may therefore be less than actual fecundity, the total number of offspring seed produced per parent plant.

estimate of base unit size must be at least double the size calculated from the previous assumptions. In some cases it may be necessary to allow for several crop failures, such as exotic species with unknown characteristics and requirements in the regeneration environment, and in species where viability drops very fast once it has reached threshold.

Thus in essence, the base unit is the number of parental plants that must be used to produce seed for the next generation, multiplied by a safety factor[2] that makes due allowance for stored seed failing to produce offspring seed that can be used to form the next generation. The number of parental plants is jointly determined by target seed quantity and genetic integrity considerations as follows:

a) The number required to produce the target quantity of seed equals the target quantity divided by effective fecundity.
b) The number required to maintain genetic integrity depends on the genetic composition of an accession, together with any measures adopted to control genetic changes.

The number of parental plants is then the larger of 1 and 2, and the base unit is this number multiplied by safety factors for items d) to f) above.

5.2.3 *Base collection*

Seed in the base collection is not used for distribution. The theoretical target quantity after regeneration is enough for:

- viability monitoring
  - quantity required is the test unit multiplied by the number of times the tests are likely to be applied during the life-span of the seed in the base collection.
- replenishment of stocks in the base collection
  - quantity required is, by definition, one base unit.
- replenishment of stocks in the active collection
  - quantity required is the base unit multiplied by the number of times the active collection is likely to have to be re-established from the base collection during the lifespan of the seed in the base collection (see section 7.3.1.3).

Uncertainty factor: the base collection exists to conserve germplasm and the genetic integrity of each accession (section 3). The 'cost' of compromising genetic integrity during regeneration overrides all other cost factors. The curator should aim to regenerate only when viability falls below threshold, and as far as possible avoid having to regenerate because of insufficient seed quantity. This requires a large uncertainty factor, with a target quantity typically 3–4 times the above expected usage.

The preferred minimum accession size for base collections is given as 1000 viable seeds in Genebank Standards (FAO/IPGRI 1994). It will often need to be higher, especially for small-seeded species, for highly variable populations and where seed stored in the base collection are used to replenish seed stocks in the active collection (for an example, see section 5.2.6).

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[2] Note that the **safety factor**, allowing for losses, is distinct from the **uncertainty factor**, allowing for uncertainty of usage.

5.2.4 Safety duplicate collection

Target seed quantity is one base unit. The uncertainty factor is 1.

If possible, all accessions in the base collection should also be held in a safety duplicate collection. Accessions in the safety duplicate collection should be held in optimal conditions for long-term storage, at least as good as those used for the base collection (see section 3). Accessions in base and safety duplicate collections should therefore lose viability at similar rates. The base collection should need to be regenerated only when seed viability drops below threshold (see previous section). Therefore, with good genebank practice, the safety duplicate collection should need regeneration at about the same time as the base collection. In the absence of specific alternative agreements, responsibility for regenerating the safety duplicate collection lies with the genebank holding the base collection, not with the genebank holding the safety duplicates.

Recommended practice is therefore that the base genebank should add the target quantity for regenerating the safety duplicate collection to that for regenerating the base collection, and regenerate sufficient seed for both in the same regeneration plot. In the rest of this document it is assumed that both will be regenerated simultaneously.

5.2.5 Active collection

The theoretical target quantity after regeneration is enough for:

- viability monitoring
  - quantity required is the test unit multiplied by the number of times the tests are likely to be applied during the life-span of the seed in the active collection.
- regeneration
  - quantity required is either zero or one base unit, depending on whether the next regeneration should be undertaken from the base collection or from residual seed in the active collection (section 7.3.1.3).
- expected usage of accession, by external users and by genebank personnel
  - quantity required is the distribution unit multiplied by the expected number of times that seed of the accession will be requested before the planned date of the next regeneration.

Uncertainty factor: no general solution can be given. The optimal uncertainty factor is necessarily genebank-specific and may even be accession-specific. Estimation of the uncertainty factor will rely on the curator's experience of the pattern of demand for seed and the uncertainty of that demand. In most cases, costs are defined primarily in financial terms: the curator must estimate the additional marginal costs and resources to produce and store more seed of an accession at each regeneration when too many seed are produced, relative to the costs and resources for more frequent regeneration when too few are produced.

Non-financial considerations may also affect the value of the uncertainty factor. Where regeneration may have relatively high impacts on genetic integrity, the curator should consider increasing the uncertainty factor to reduce the number of regeneration events. If there is a large backlog of accessions awaiting regeneration, it may be possible to increase the number of accessions that can be regenerated by reducing the uncertainty factor.

For some types of regeneration, marginal costs may increase in a stepwise fashion with the number of seed produced. For example, regenerating within isolation chambers of fixed size sets an upper limit to the amount of seed that can

be produced at one time in one chamber. Costs will then increase relatively little up to that maximum, but beyond that maximum will jump to prohibitively high levels because of the need to allocate an additional chamber. In such situations, the target quantity will often be the maximum number of seed that can be produced within one chamber.

Typical target quantities of seed per accession in the active collection, as adopted by a range of genebanks, are as follows (ICRISAT 1995).

Crop category	Target number of seeds per accession
inbreeders	1500 – 6 000
outbreeders	4000 – 50 000
large-seeded species	1500 – 4 000
small-seeded species	2000 – 50 000

Close adherence to target quantities is rarely critical for genebank function. Quantity is immediately critical if it is less than the threshold for regeneration (section 5.2.5). Above this minimum acceptable quantity the primary effect of variation in seed quantity is on the cost-efficiency of regeneration.

5.2.6 A case study

To illustrate the above principles, we include here a specific example of the necessary calculations. Background details that influence the calculations are as follows.

Genebank	Genetic Resources Unit, Institute of Grassland and Environmental Research, United Kingdom
Storage conditions	Silica gel dried seed, sealed in foil packs Active collection: –2°C Base collection: –20°C
Species	*Lolium perenne* (Gramineae: perennial ryegrass)
Expected seed longevity in storage	Active collection: 25 years Base collection: 100 years
Life history characteristics	Long-lived perennial Obligate outbreeder Natural clonal propagation by tillers
Population characteristics	Wild populations High genetic variation within populations
1000-seed weight	1.8 – 2.2 g
Effective fecundity	200–5000 progeny seeds per parent seed†
Regeneration constraints	Use fixed isolation chambers (glass quarantine house) Maximum 50 parent plants per chamber Maximum output 50 x 200–5000 = 10 000–250 000 seeds per chamber
Storage options	Form a balanced bulk for storage in the active collection Use a separate container for the seed of each parent plant for storage in the base and safety duplicate collections (see section 7.9.7)

† Such high variability in first-year reproduction of a long-lived perennial that is able to propagate clonally as an alternative to sexual reproduction, is typical of species with this life history, in addition to high variation in adaptation.

The relevant estimates and calculations are as follows.

Unit sizes	
Test unit	100 seeds
Distribution unit	250 seeds
Base unit:	
Number of parent plants	50
Safety factors:	
Germination rate	x 2
Seed losses	x 2
Crop failure	x 2
Total base unit size	50 x 2 x 2 x 2 = 400 seeds
Active collection	
Requirements for viability monitoring:	
Number of tests during life time of seed sample	5
Total number of seed required	100 x 5 = 500 seeds
Number of seed required for regeneration	0 (regenerate using seed from base)
Requirements for seed distribution:	
Average number of requests during lifetime	10 (range 1–50)
Uncertainty factor	x 5[†]
Total number of seed required	250 x 10 x 5 = 12 500 seeds
Total number of seed required for active collection	13 000
Base and safety duplicate collections	
Requirements for viability monitoring:	
Number of tests during lifetime of seed sample	10
Total number of seed required	100 x 10 = 1000 seeds
Number of seed required for regeneration:	
Replenishment of stocks in base and safety duplicate collections	400 + 400 = 800 seeds
Replenishment of stocks in active collection	
Expected number of times	5
Uncertainty factor	x 4
Number of seed required	400 x 5 x 4 = 8000 seeds
Total number of seed required for base and safety duplicate collections	9800
Total number of seed required	**22 800**

[†] This uncertainty factor reflects high uncertainty of usage, high financial costs of each regeneration event, and potentially high negative impact of each regeneration on genetic integrity, against the low additional costs of producing and storing excess seed up to the maximum output from one isolation chamber.

Note the correspondence between the maximum effective seed output per isolation chamber and the total number of seed required for simultaneous replenishment of stocks in base, safety duplicate and active collections. This reflects optimality in the design of the isolation chambers, in that for most accessions the size of the regeneration chamber is sufficient for production of the optimal number of seed.

The wide range of variation in effective seed output per isolation chamber is an inevitable consequence of the wide genetic diversity between accessions. It

has to be accepted pragmatically, so that the number of seed actually placed in storage for each accession varies between accessions according to their fecundity. Where the number of seed produced significantly exceeds the calculated optimum, excess seed is made available for field evaluation in plots that require considerably more than the normal distribution unit of 250 seed.

5.3 Maintaining genetic integrity

Maintenance of genetic integrity involves maintaining the joint frequency distribution of all alleles at all loci. The ideal, although usually unachievable target, is to maintain the joint frequency distribution constant. This section deals with setting achievable targets, which requires consideration of the various processes and mechanisms of change and their consequences for genetic integrity.

5.3.1 Accession identity

Human error introduces the possibility that samples might be incorrectly placed or labelled at any step, resulting in a seed sample being incorrectly ascribed to the wrong accession. The target is a zero misidentification rate (see sections 7.5.1, 7.6.3, 7.9.1.3).

5.3.2 Contamination with alien genes

Alien genes may be accidentally introduced as plants, seed or pollen, at several stages.

1. Inadequate attention to cleanliness, for example use of machinery that cannot be adequately cleaned of seed internally, will cause seed to be mixed with other accessions or other sources of seed. In addition to general cleanliness and the risk of contamination with seed from other sources, there is a particular risk of carry-over of seed from one sample to the next:
   - during seed preparation
   - during sowing
   - during harvesting
   - during all post-harvest seed handling through to seed storage.

2. The regeneration plot may be contaminated with alien seed, from previous crops or wild, naturalized or feral populations previously growing in or near the plot, and leaving seed in the soil seedbank. The risk of such contamination depends on the species biology (e.g. seed longevity in the soil, seed dormancy, seed dispersal characteristics, distribution of naturalized populations) as well as recent cultivation history of the plots and surrounding fields.

3. The regeneration plot may be contaminated with alien pollen, from
   - other accessions being regenerated nearby
   - other material (crops, wild or naturalized populations) in the vicinity.

The preferred standard is zero contamination by any of the above routes. In some cases genebank policy may accept a low level of contamination with alien plants or alien pollen, but this is not recommended because of the resulting loss of diversity in the collection (section 5.3.3.2).

5.3.3 Other changes in genotypic composition

Even with zero contamination by alien genes, accessions may change in their genotypic composition. The changes can occur through several processes:

- differential loss of viability during storage
- mutation
- genetic composition of the seed subsample used for regeneration may differ from that of the original accession, e.g. by sampling error from inadequate mixing or small sample sizes
- some plants of an accession in a regeneration plot may die or may not mature before harvest
- surviving plants may contribute unequal numbers of female and/or male gametes to the next generation of seed – this may include plants that never flower during the regeneration cycle or flower but produce no ripe seed and/or pollen
- genetic composition of the pollen population contributed by each parent plant may differ from that of the parent
- genetic composition of the ovule population contributed by each parent plant may differ from that of the parent
- the pollen source for each zygote may result in loss of certain genotypes and/or production of novel recombinant genotypes and/or inappropriate (high or low) levels of heterozygosity.

All eight processes except the first, i.e. differential loss of viability during storage, occur during regeneration. All except the second, i.e. mutation, apply only to genetically variable accessions and not to inbred pure lines.

The mechanisms of change fall into two broad categories: drift and selection. All eight processes listed above are subject to drift. All except the second, i.e. mutation, are subject also to selection.

Drift refers to random changes caused by chance factors, such as sampling error and the effects of uncontrolled microenvironmental variation on growth, survival and reproduction. Selection refers to non-random changes, which occur whenever genetic variation within an accession is expressed in the regeneration environment as phenotypic variation among plants for any component of evolutionary fitness. These changes can be brought about by unconscious selection, rogueing or differential reactions of plants to the regeneration environment. The curator should avoid imposing artificial selection pressures, and needs to consider methods for minimizing the response to natural selection. Some examples of unconscious selection by the curator include:

- pollinating only the first flowering plants, or some other portion of the population not balanced throughout the total duration of flowering
- pollinating only easily reachable flowers, e.g. omitting 3.5 m tall
- not harvesting the late-maturing plants
- failing to break dormancy of all seed in species showing polymorphism for seed dormancy.

The variation to be maintained in a collection must be considered at two levels: within accessions and among accessions. For example, an allele may be rare at the accession level (present at low frequency in an accession) or at the collection level (present in only a few accessions). An allele that is present at low frequency in most accessions is rare at the accession level but more common at the collection level. Conversely, an allele may be present at high frequency in only a few accessions but absent from most, i.e. locally common at accession level but rare at the collection level.

5.3.3.1 Drift and bottlenecks

Random changes in allelic frequency may result in complete extinction or fixation of an allele from an accession, or smaller changes without extinction (Chakraborty and Nei 1977; Maruyama and Fuerst 1984, 1985; McCommas and Bryant 1990). Losses of alleles by drift accumulate over successive cycles of regeneration. Smaller changes in allele frequency by drift are not necessarily cumulative, since they are by definition random and therefore may be in opposite directions in successive regeneration cycles.

The probability P of losing an allele from an accession during regeneration depends on its initial frequency f and the number of parents N used for regeneration. In a neutral gene model with random mating and diploid inheritance, the probability is:

$$P = (1 - f)^{2N}$$

Thus the probability of losing an allele is highest for rare alleles, but can be reduced by using more parents for regeneration. Likewise, the expected magnitude of all random changes, whether or not they involve allele extinction, can be decreased by using more parents.

The equation needs minor modification for polyploidy and non-random mating. For autopolyploids, the number 2 is replaced with the ploidy level. For other polyploids, the number depends on the extent of allele-sharing between homoeologous loci. A major cause of non-random mating is self-compatibility. For such species, the number 2 is reduced, reaching a minimum value of 1 in the extreme case of 100% selfing in a population of homozygous plants.

Targets must include a critical value of N, say N_c, that gives an acceptable compromise between minimizing allele losses and achieving cost-effective regeneration (Crossa *et al.* 1992). A bottleneck is considered to have taken place if the number of parent plants that produce seed during a cycle of regeneration is less than N_c, resulting in an unacceptably high rate of loss of alleles. This may occur at the point of collecting or seed introduction for new accessions. A bottleneck may also occur during regeneration of an old accession if the regeneration programme failed to process the accession in time to avoid the bottleneck.

A bottleneck not only increases the expected magnitude of change by drift, but also reduces genetic variation within accessions of outbreeding species, by increasing inbreeding and by increasing the risk of allele extinction.

In contrast to its detrimental effect on genetic diversity within accessions, drift (and its magnified effects at a bottleneck) has relatively little adverse effect on diversity among accessions, for the following reasons.

1. The risk of loss of alleles from the entire collection increases only for alleles that are rare at both accession level and collection level, i.e. that are present at low frequency in only a few accessions and absent from the majority of accessions.

2. Drift increases the expected genetic variance among accessions. Statistically, variances of independent processes are additive. That is, provided the direction of genetic change in an accession is independent of its initial genotypic composition, expected genetic variance among accessions after a cycle of regeneration will equal the sum of (i) genetic variance among accessions prior to regeneration and (ii) genetic variance due to drift.

3. Inbreeding further increases the expressed genetic variance among accessions by uncovering the effects of recessive alleles.

4. Inbreeding further increases the genetic distances and the statistical significance of differences among accessions, by reducing genetic variance within accessions and so increasing the ratio of genetic variance among accessions to that within accessions.

5.3.3.2 Selection

In contrast to drift which affects all polymorphic loci, selection affects only traits for which there is genetic variation associated in some way with differential survival or reproduction in the environments used for storage and regeneration. Because of their association with survival and reproduction, such traits are likely to be of high interest and value for breeding and research. Natural selection[3] will change:

- allele frequency at a locus if the phenotypic effects of those alleles have differential consequences for survival or reproduction
- allele frequency at a locus that is genetically linked to the locus in 1
- expression of characters that are pleiotropic expressions of loci controlling the characters that directly affect survival or reproduction.

Regeneration is undertaken in an environment that may be very different from the environment(s) from which the original population sample was taken. Also, compared with the highly variable environments occupied by natural populations and primitive landraces, the regeneration environment is relatively very uniform, even for regeneration plots in the field and notwithstanding year-to-year fluctuations in climate. The result is likely to be relatively strong, uniform, directional selection pressure that:

- favours a single genotype or group of genotypes, which may not be the predominant genotype in the original population
- progressively eliminates other genotypes
- reduces genetic variance within accessions
- progressively changes mean phenotype away from the original population.

Such selective changes can accumulate over successive cycles of regeneration.

It should be stressed that adaptation to spatial and temporal variation of the environment is only one of the classes of evolutionary mechanism that maintain genetic diversity within populations. Other mechanisms include neutral genes, heterozygote advantage and frequency-dependent selection. Environmental uniformity will therefore not totally eliminate genetic diversity. In addition, most regeneration plots do not completely eliminate environmental diversity, particularly climatic variability, and will not eliminate genetic diversity associated with this.

Moreover, it is possible with care to create a uniform environment that imposes weak or no selection pressure even on genes that are selectively not neutral in other environments. Genes for stress tolerance (e.g. herbicide tolerance) provide common examples: they are selected for in the presence of the stress, but in some cases may carry a physiological cost that causes them to be

[3] This selection is considered 'natural' in the sense that, although the regeneration environment is not natural, the selection pressure itself is not a deliberately imposed artificial selection, but rather unintentional selection involving natural evolutionary processes occurring in the chosen regeneration environment.

selected against in the absence of the stress. A mild level of stress may be required to achieve selective neutrality for such genes. A simple balance of selection pressures like this is not sufficient to maintain a balanced polymorphism in a population, but can considerably reduce the rate of fixation of alleles.

Thus the statements above on loss of diversity are not absolute. They are merely comparative with the large amount of genetic diversity within and between populations that results from adaptation to natural or agricultural environments that are qualitatively and quantitatively far more variable than regeneration environments. The same applies to the rest of this section.

Regenerating accessions in a common environment is also likely to impose convergent selection pressure. That is, in a single uniform environment there is likely to be a single assemblage of genotypes with higher fitness than all others in that environment, and therefore a tendency for all accessions to change their genetic composition by natural selection towards that assemblage. They will thus naturally tend to converge towards a common endpoint, reducing genetic diversity among accessions. The rate and extent of convergence depend on genetic variance within and among populations, the potential for transgressive segregation through recombination and new mutation, and the number of regeneration cycles.

Introgression between accessions during seed multiplication occurs if the accessions are not fully isolated, enabling geneflow to occur by pollen transfer (section 5.3.2). This reduces genetic variance between accessions by increasing the sharing of genes. Ultimately, with unlimited introgression among all accessions, the stable endpoint would be that at which all accessions are genetically identical. Introgression does not *per se* reduce overall genetic variance in the collection, as the reduction in between-accession variance is offset by an increase in within-accession variance. However, the combination of introgression with convergent selection has a more detrimental effect than either alone: introgression both increases the rate of convergence and removes any limit to the extent of convergence.

In summary, compared with the effects of drift, selection:

- affects fewer loci
- affects only non-neutral loci, which are more likely than neutral loci to be of agronomic significance
- potentially has more adverse effects on conservation of diversity both within and among accessions.

The curator should therefore seek to counteract the effects of selection during regeneration.

5.3.3.3 Targets and priorities

As indicated above, the ideal target, zero genetic change, is not generally achievable. It is also costly and labourious to measure the extent of genetic change and its component processes such as pollen flow. It is not appropriate to specify here a particular target level of change, although it may be possible to agree on crop-specific target levels. The following observations and recommendations will suffice here.

Coordinated planning of the entire regeneration procedure (see section 7) is necessary to minimize changes by all the processes and mechanisms described in this section. Since the expected magnitude of change by drift and by selection is proportional to genetic variance within accessions, the stringency of

precautionary measures must increase with the amount of genetic variation occurring within accessions.

Highest priority is often given to preventing the loss of rare alleles. Crossa (1989) and Crossa *et al.* (1993) present an analysis of targets in relation to minimizing drift and the chance loss of rare alleles.

Increased priority should be given to minimizing non-random changes by selection, since these are more detrimental than drift in terms of losing agronomically significant diversity from the entire collection.

There may be a need to preserve diversity intact at accession level as well as at the entire collection level. This is the case particularly with well-characterized collections where the data available on each accession add significantly to its value in the collection; any genetic change will then reduce the value of an accession by making the associated characterization data less accurate. In such cases, any genetic change is undesirable, and avoidance of drift and selection must be given equal priority.

6 Relevant knowledge base

A large amount of background knowledge is required to make sound curatorial decisions. This section summarizes the relevant knowledge base without discussing consequences or clarifying why the knowledge is important. Implications of this knowledge for regeneration will be considered later, in section 7.

In many cases, the curator may not have all necessary or desirable specific information. Decisions will then have to be made with an understanding that they may not be optimal, and the curator will then rely more on general experience. It is usually preferable to compensate for unknown information by increasing the stringency of regeneration conditions.

6.1 Biology of the species/accession

Genetic variation within species includes variation in the characteristics that influence optimal regeneration procedures. The following factors need to be considered at accession level, not just at species level.

1. Adaptation to abiotic environmental conditions (climate, soil, photoperiod). For information, refer to floras, the Internet[4], libraries, bibliographic databases; also to breeders, crop networks, farmers, research scientists and other users and providers of germplasm and other collaborators with the genebank.
   - Prevailing conditions in the original collecting site can give a good, but not always reliable, indication of probable adaptation. For information, use passport data, ecogeographic databases, meteorological data.
   - General ideal conditions for growth and seed production.
   - Specific environmental triggers for each step in phenology such as germination and floral initiation. For example, many temperate species require vernalization (a period of cold treatment) to trigger germination, whereas other species may require heat treatment; depending on the species, the onset of flowering may be triggered by long days, short days, low temperature, a combination of high day and low night temperatures, etc.

2. Seed physiology
   - storage characteristics: effects of moisture content and temperature on expected seed longevity
   - dormancy and germination: how to break dormancy and maximize germination.

3. Growth morphology

[4] Information on the World Wide Web changes rapidly. It is therefore not feasible to present a definitive list of addresses here and interested users will need to conduct their own search for relevant information. The home pages of FAO (**http://www.fao.org/**) and IPGRI (**http://www.cgiar.org/ipgri/**) provide suitable starting points for some types of information. UNEP GRID (Global Resource Information Database) provides conditional access to numerous large relevant datasets through a series of sites (e.g. **http://www.grida.no/** and **http://www.grid.unep.ch/**).

4. Biotic environments
   - symbiotic associations: mycorrhizal fungi, *Rhizobium* and other bacteria, animal pollinators (insects, birds), endophytes
   - significant stresses: diseases and pests of plants and seeds – methods required for detection and control

5. Genetic structure
   - breeding system
   - fecundity
   - dispersal systems for pollen, seed and vegetative propagules, e.g. explosive pods, brittle rachises
   - effective population size of populations *in situ*

6. Farmer management (for cultivated taxa)

7. Risk assessment
   - weediness
   - other geneflow problems
   - toxic and allergenic phytochemicals in relation to humans handling seed and plants.

6.2 Accession history prior to current regeneration cycle

- Degree of genetic heterogeneity and how it has been managed
  - collecting techniques
  - number of times regenerated
  - locations and methods used for previous regenerations
  - previous occurrence of bottlenecks, if any
  - splitting variable accessions into two or more distinct accessions
  - combining duplicates.
- Seed status
  - storage: under what conditions and for how long
  - health/quarantine
  - percentage viability
  - seed purity
  - actual number of live seeds (total seed number multiplied by percentage viability).

6.3 Infrastructural considerations

- Regeneration environment
  - access to desirable sites
  - access to controlled environments
  - possibility of collaboration if appropriate environments are not available.
- Seed health (exchange/quarantine context) – linkage to acquisition policy and distribution policy
  - access to current international regulations – linkage to collaboration with plant health agencies
  - internal standards/self-regulation.

7 Establishment of regeneration protocol

This section presents the decisions that a curator should make to establish an optimal regeneration protocol. The order of the subsections is the same as the order of the practical activities involved in regeneration: selection of the site, accessions and parental material for regeneration; preparation of the site and parental material; crop management during growth; pollination control; harvesting and post-harvest management.

In a number of cases, the curator will be in a suboptimal 'no choice' situation, such as having access to only one site for regeneration, only one or two seeds to regenerate, or not being able to isolate accessions from each other. It is then necessary to establish whether the impact is critical in terms of dropping below minimum acceptable standards (FAO/IPGRI 1994), and how much policy renegotiation is necessary to achieve the minimum acceptable standard or to get closer to the preferred standard. For example, having only one seed left of an inbreeder may under exceptional circumstances be acceptable, albeit far from ideal. A curator should do everything possible to avoid such a situation while accepting it where it arises. In contrast, **regenerating outbreeders in an open field with no control of pollen contamination is unacceptable; a curator presented with that as the only option cannot manage a genebank adequately.**

7.1 Selection of location for regeneration

See flow chart 7.1.

The location for regeneration must be considered at two scales: the overall site, and the type of location chosen at a site, e.g. in the field or in a glasshouse or other facilities for more careful control of environment and genetic changes. A primary requirement is for proximity and controlled accessibility, so that the chosen location can be easily patrolled and monitored on a regular basis.

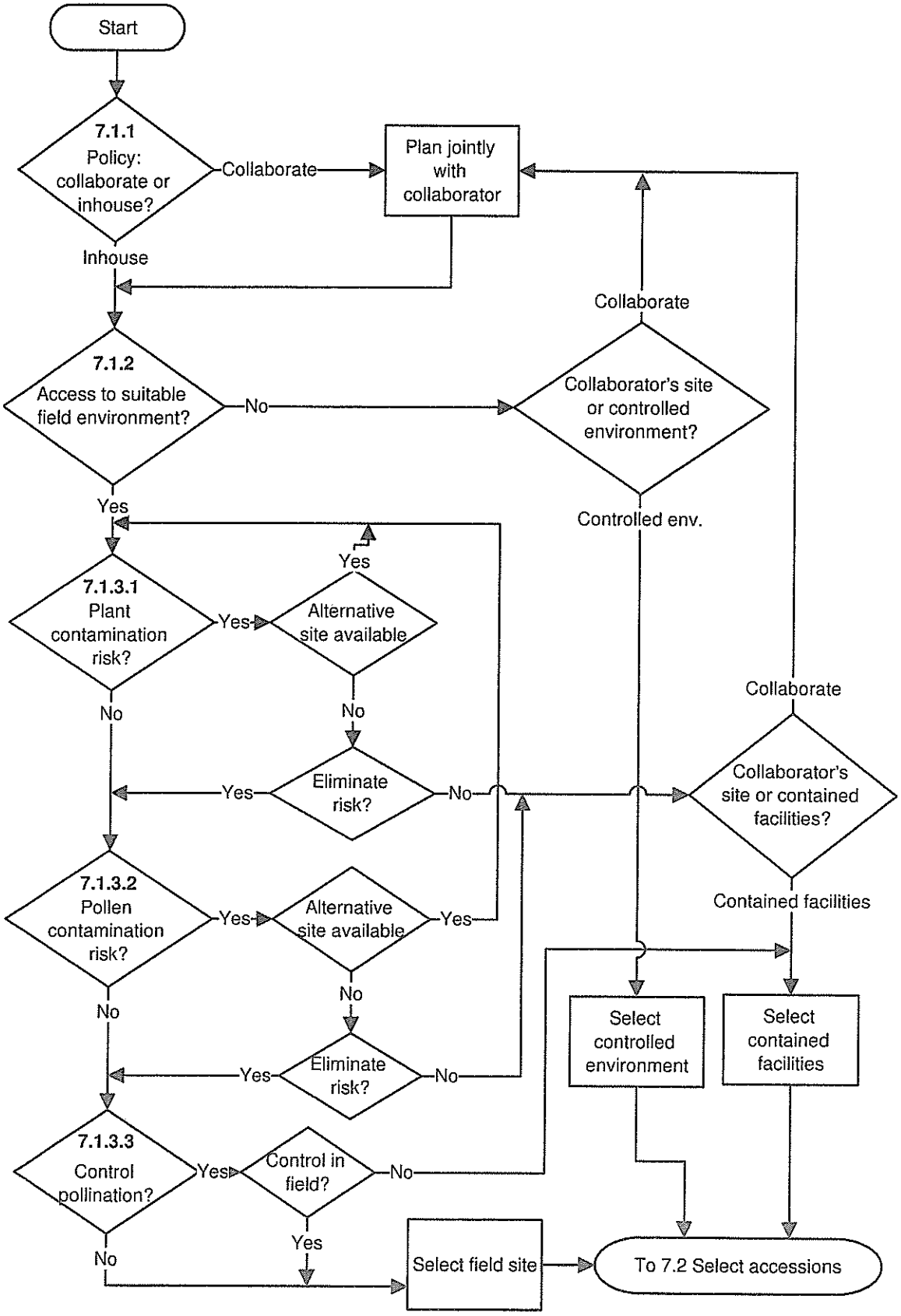
7.1.1 Policy considerations

National, international or institutional genebank policy will to some extent influence a decision on whether regeneration should be undertaken purely in-house or in collaboration with another institute. Policy considerations are beyond the scope of this document, but cannot be ignored.

If regeneration is undertaken in collaboration, regeneration must be planned jointly between the collaborating bodies, to take full advantage of complementary facilities, environments and expertise. International collaboration will necessitate consideration of additional factors, such as political or trade agreements and quarantine regulations for germplasm exchange.

7.1.2 Environmental considerations

Many accessions are 'exotics' maintained well away from their site of origin. They may be poorly adapted to the biotic and abiotic environment where they are maintained. Care should be taken to ensure that this does not have an unacceptable impact on regeneration; there can be adverse impacts on germination, yield, genetic integrity, and quality (health, longevity in storage) of the resulting seed (see e.g. Rao and Jackson 1996a, 1996b). Curators of genebanks containing accessions differing widely in ecological adaptation should consider maintaining a network of sites in different agro-ecological zones, possibly through collaboration with other genebanks, and select the appropriate site for regeneration of each accession.



Flow chart 7.1. Selection of location for regeneration.

This is not always a straightforward task. Usually, although not always, the environment of origin provides a good guide to the optimal environment for regeneration. Sometimes preliminary small trials may be desirable to determine the best regeneration environment. The abiotic and biotic environments are considered separately below.

7.1.2.1 Abiotic environment

The abiotic environment – climate, photoperiod and edaphic conditions – must be suitable for regenerating the accessions. It must provide optimal general conditions for good and reliable plant growth and seed production. It must also provide all environmental stimuli needed to trigger particular stages in plant development, such as germination and flowering. For example, depending on the species and genotype, initiation of flowering may be triggered by low, fluctuating or high temperatures, short, long, shortening or lengthening days, drought or high moisture supply, or some combination of these. There may be only certain critical stages in plant development at which the plant is able to respond to these stimuli, so the environment must provide the right stimuli at the right stage.

It should be noted that 'optimal' general conditions do not necessarily imply free of all stresses. In many species, certain stresses, such as cold, drought or low nitrogen, may promote a uniform switch from vegetative to reproductive growth if applied at key stages. The nature of such stresses may be poorly defined. For example, it has been observed that in the UK, alien populations of *Lolium perenne* often produce more reliable, uniform seed crops during regeneration than do native UK-adapted populations. This may be attributable to the alien populations being under more stress in an environment to which they are not adapted.

Many species, especially those with a wide ecogeographic distribution, show considerable genetic variation in environmental adaptation, both in general growth responses and in requirements for environmental stimuli to trigger specific phenological events. In these cases, a curator may need access to several regeneration environments, and make accession-level decisions on which to use.

Many species, especially outbreeders, are genetically variable within populations in environmental adaptation, both in general growth responses and in responses to specific triggers. The curator should seek a location that does not select some genotypes in preference to others in a population. As far as possible, the environment should be suitable for reliable flowering and seed production by all genotypes in the population.

If no suitable site can be located, the curator is left with two options: either to seek collaboration with a new institute that can provide a suitable site (with appropriate policy changes if the new collaboration is to continue long term), or to regenerate in a more highly controlled environment. Sophisticated controlled environment facilities will in most cases be too expensive: glasshouses, screen cages or simple shading facilities often provide sufficient control.

7.1.2.2 Biotic environment

The biotic environment at given locations and growing seasons must be examined in the context of *a priori* information about the plants and past experience, and in the context of the cost, efficacy and consequences of control measures. An inappropriate biotic environment can have highly detrimental effects on plants and seed quality, and on the genetic integrity of an accession through differential effects on different plants.

Important components of the biotic environment include the following.

1. **Pathogens and pests**
   Good control is essential. Cultivation of large populations in monoculture can cause serious insect and disease problems. The problem of monocultures is well known in crops. However, it can also be an acute problem in many wild species, especially those that occur naturally mainly in small populations as rare components of the plant community. In these species, protection against pathogens and pests in nature appears to result from the small population size and low density within a relatively dense community. The resulting scarcity of resistance genes appears to make them more sensitive to epidemics in monoculture.
   Pathogens or pests commonly have highly localized effects. This may arise by genetic variation in tolerance in the host plant, resulting in selective change. It may also occur at random in relation to host genotype: where initial infection of a plant is followed by an epidemic phase with limited dispersal, a plant may be severely affected before the pathogen or pest disperses to a new plant – an example is the rapid multiplication of *Aphis* on single plants. In either case, genetic integrity of the accession is compromised.
   Avoidance is often preferable to control, especially where detection and/or control are difficult. For example, a species that is readily infected with seedborne viruses should not be regenerated in an area where the vectors are common and the surrounding area may contain infected host plants.

2. **Symbionts** – mycorrhizae and, for legumes, *Rhizobium*
   Mycorrhizae generally have a broad host range. *A priori* knowledge of the plant species is required on whether it has an obligate requirement. If it has, then the curator will need either to find a field containing the required mycorrhizal fungus, or to inoculate the regeneration plots.
   Rhizobium strains often have a very narrow host range, even showing within-population variation in specificity, within both *Rhizobium* and legume populations. This specificity can generate strong selection pressures within accessions in favour of genotypes that are effectively nodulated by the available inoculum – whether the latter is present naturally in the soil or added as an artificial inoculant. *A priori* knowledge of the legume and its optimal symbiont *Rhizobium* is needed to evaluate the potential of a site. Options to be considered by the curator are:
   - inoculation – if so, with what (for example, if the legume was originally collected together with its root nodules and the original *Rhizobium* strain was isolated and remains available, consider using it)
   - use of mineral nitrogen fertilizer – useful for eliminating variation caused by specificity of association with *Rhizobium*
   - no additional fertilizer or inoculum – useful where the soil already has an acceptable nitrogen status or all genotypes show similar reactions to the resident *Rhizobium* population.

3. **Pollinators** (animal-pollinated plants only)
   If the curator relies on natural pollination, suitable pollinators must be present during anthesis, in sufficient numbers to ensure good pollination (see sections 7.1.3.3 and 7.7.4). The curator may need to provide pollinators.

7.1.2.3 Quarantine

A special case of control of the biotic environment is the imposition, by institute policy or national law, of a requirement to quarantine seed or plants newly imported from another country. Options for regeneration in this case include:

1. Comply with quarantine requirements before undertaking initial regeneration.
   - This is likely to be the preferred option in most cases. The curator can then continue with choice of an appropriate regeneration environment without regard for quarantine requirements.

2. Regenerate within approved contained quarantine facilities.
   - This option may be preferable when the priority is for rapid regeneration without the delay imposed by the quarantine period, e.g. when seed quality is poor. It may even be obligatory, for example if the imported material comprises living plants, and quarantine regulations specify destruction of those plants, but not their seed, while still in the quarantine facility.
   - This option will not always be feasible, for example in cases where quarantine is outside the responsibility of the genebank. It is most likely to be feasible if prevention of pollen contamination (section 7.1.3.2) necessitates use of contained facilities with the same quality of isolation required by quarantine regulations. The genebank will then be able (subject to approval by the quarantine authorities) to use its pollination control facilities for quarantine purposes and *vice versa*.

7.1.3 Genetic integrity considerations

7.1.3.1 Contamination by alien plants

Possible sources of such alien plants may be:

1. Carry-over of seed from one plot to the next in machinery, clothing etc. Scrupulous attention to cleanliness of all equipment is necessary. If regeneration plots are sown mechanically, only purpose-built precision machinery should be used. Commercial agricultural machinery should not be used, as it is generally impossible to clean adequately inside the machine between plots.

2. Seed remaining in the soil from previous crops or regeneration plots on the site

3. Seed dispersed into the plot from nearby crops or regeneration plots

4. Seed dispersed into the plot from nearby feral or wild populations.

Assessment of 3 and 4 requires prior knowledge of characteristic and maximum seed dispersal distances and mechanisms. For example, dispersal distances for animal-dispersed seed vary with the vector (e.g. bird, mammal, ant) and mechanism (e.g. in mouth, on skin, in gut). Likewise, dispersal distances for wind-dispersed seed vary with aerodynamic properties of the dispersed propagule.

Options that may eliminate 2 to 4 include the following:

- geographical isolation: select a location that is not within dispersal distance of any crop field, previous regeneration plots, or feral or wild populations
- devise and follow a crop rotation to clean the soil
- pretreat the soil to kill seeds or seedlings, e.g. by a period of fallow/ploughing to promote germination of surviving seeds, followed by ploughing in.

In some cases, none of these options may be adequate, e.g. for widespread wild or naturalized populations with persistent seedbanks in the soil. **If complete cleanliness cannot be guaranteed, use of open field plots must be rejected as an option for regeneration.** The curator must then use some degree of containment that does permit sufficient control, e.g. by growing the plants in pots in a glasshouse.

7.1.3.2 Contamination by alien pollen

Sources of alien pollen are:

- adjacent regeneration plots
- nearby crops
- nearby feral or wild populations.

A priori information on pollination behaviour and the degree of outcrossing in the site of regeneration is necessary to determine the need for pollination control and the efficacy of the alternative methods for control. In some cases, such as obligate apomicts, risks of contamination are zero regardless of the proximity of other plants of the same or similar compatible species. In most cases, however, it is necessary to ensure that no other viable pollen is able to pollinate receptive flowers in the regeneration plot.

Recommendations for commercial seed production may provide useful guides for developing a suitable isolation protocol. Options include the following.

1. Keep the regeneration area free of feral and wild populations, as in the previous section, to help eliminate them as a source of alien pollen. However, in most species pollen disperses much further than seed, and so a much larger area would have to be kept clean.

2. As an alternative to complete removal, unwanted genotypes may be repeatedly clipped to remove all their flowers.

3. Isolate by distance, growing each regeneration plot far from other such plots, crops or feral or wild populations. Sound judgement of what constitutes a sufficiently high distance requires good *a priori* knowledge of the pollen dispersal characteristics of the species concerned. Pollen dispersal is highly dependent on current wind conditions for wind-pollinated plants or the species and abundance of vectors for animal-pollinated plants. Nevertheless, pollen dispersal distances can be surprisingly great, and isolation purely by distance is probably rarely feasible for outbreeders.

4. Isolate by a combination of distance and partial barriers. This is normal practice in some genebanks, but cannot guarantee sufficient control in all cases. Possible partial barriers include tall intervening crops for wind-

pollinated species, or crops with flowers of a similar colour, morphology, scent and timing of anthesis for insect-pollinated species. The efficacy of the latter depends on the following two typical characteristics of many insect pollinators.

- Pollinators often show marked short-term preferences for one particular flower type. Thus sequential visits are often to flowers of the same type and flowers of different types may not form an effective barrier between two accessions.
- Insects can fly long distances to a flower, but many species tend to stop at the first flower they encounter of their preferred type (Goplen *et al.* 1972). Thus, cross-pollination between two accessions occurs with high probability if there are no suitable flowers between them, even if they are located far apart; but can be reduced to very low levels by the presence of such flowers.

  The best control in insect-pollinated species is likely to be achieved using a conspecific male-sterile genotype, provided that (i) the pollinator forages for nectar rather than pollen and (ii) the male-sterile genotype produces abundant flowers containing abundant nectar throughout the duration of anthesis of the accessions. However, use of an incompatible species with similar flowers is usually more feasible and is less dependent on insects foraging for nectar rather than pollen.

5. Isolate by time, regenerating different accessions at different times, and/or outside normal flowering time for other populations of the same species in the vicinity. The feasibility of, and procedures for, effective isolation by time requires good *a priori* knowledge of the species flowering characteristics and general adaptation to the local environment. For species with a determinate growth habit and short duration of flowering (e.g. *Phaseolus vulgaris*, *Triticum aestivum*), complete isolation may be achieved by sowing two accessions only a few weeks apart. If in addition the length of the growing season is long (even potentially 12 months/year in irrigated tropics), then a successional planting regime can enable many accessions to be regenerated alongside each other each year without risk of cross-contamination. At the other extreme, there is no potential for isolation by time in cases where the species has a long duration of flowering and the growing season is relatively short (e.g. *Phaseolus coccineus* in northern latitudes). Similarly, if feral or wild populations grow nearby, natural variation in germination time increases the duration of flowering in those populations, and it may be impossible to sow regeneration plots at a time such that it can complete its life cycle yet have no alien pollen present when its flowers are receptive.

6. Erect pollen-proof barriers (or pollinator-proof for insect-pollinated species) that provide complete isolation from all alien pollen. Options include:
   - (a) bags over individual flowers, inflorescences or plants
   - (b) temporary or permanent isolation chambers built over regeneration plots in the field, erected at least for the duration of anthesis
   - (c) permanent enclosed isolation chambers, with plants in pots that are moved into the chambers just prior to anthesis and removed afterwards
   - (d) permanent enclosed pollen-proof chambers containing regeneration plots throughout the regeneration cycle;

   Option (a) is likely to be used only in conjunction with manual pollination (section 7.7.4).

Option (b) is likely to be more economically achievable with insect-pollinated species than with wind-pollinated species. The relatively coarse filters that are a sufficient barrier to insects are easier to install without leaks. In addition, the fine pollen-proof filters needed for wind-pollinated species may restrict air flow too much for effective pollination, necessitating an active air-circulation system that is not readily installed over field plots.

Option (c) allows higher throughput capacity than (d) with a limited number of chambers. Option (d) will be preferred where other factors also require it, such as quarantine, pathogen control, control of temperature, light, moisture, etc.

All options above except (c) and (d) are feasible in field plots. The last two require specific permanent containment facilities which rule out the use of field plots.

7.1.3.3 Control of pollination

If there is a need to control pollination within accessions in order to improve maintenance of genetic integrity of highly variable accessions (see section 7.7), the curator must establish whether this is easier in the field or in more controlled conditions. The choice will depend on the species and the chosen method of control. Options for eliminating alien pollen may also enable control of pollination within accessions (e.g. bagged inflorescences).

Use of isolation chambers is particularly useful for achieving open-pollination within accessions. For wind-pollinated species, isolation chambers must contain an active filtered air-circulation system that promotes pollen dispersal within the chamber using air free of alien pollen. For insect-pollinated species, isolation chambers must be able to house an effective working population of pollinators for the duration of anthesis. Such an approach is feasible only in conjunction with a system for maintaining pollen-free populations of pollinators in a form where they can be introduced into isolation chambers as necessary. A separate working population must be maintained for each isolation chamber.

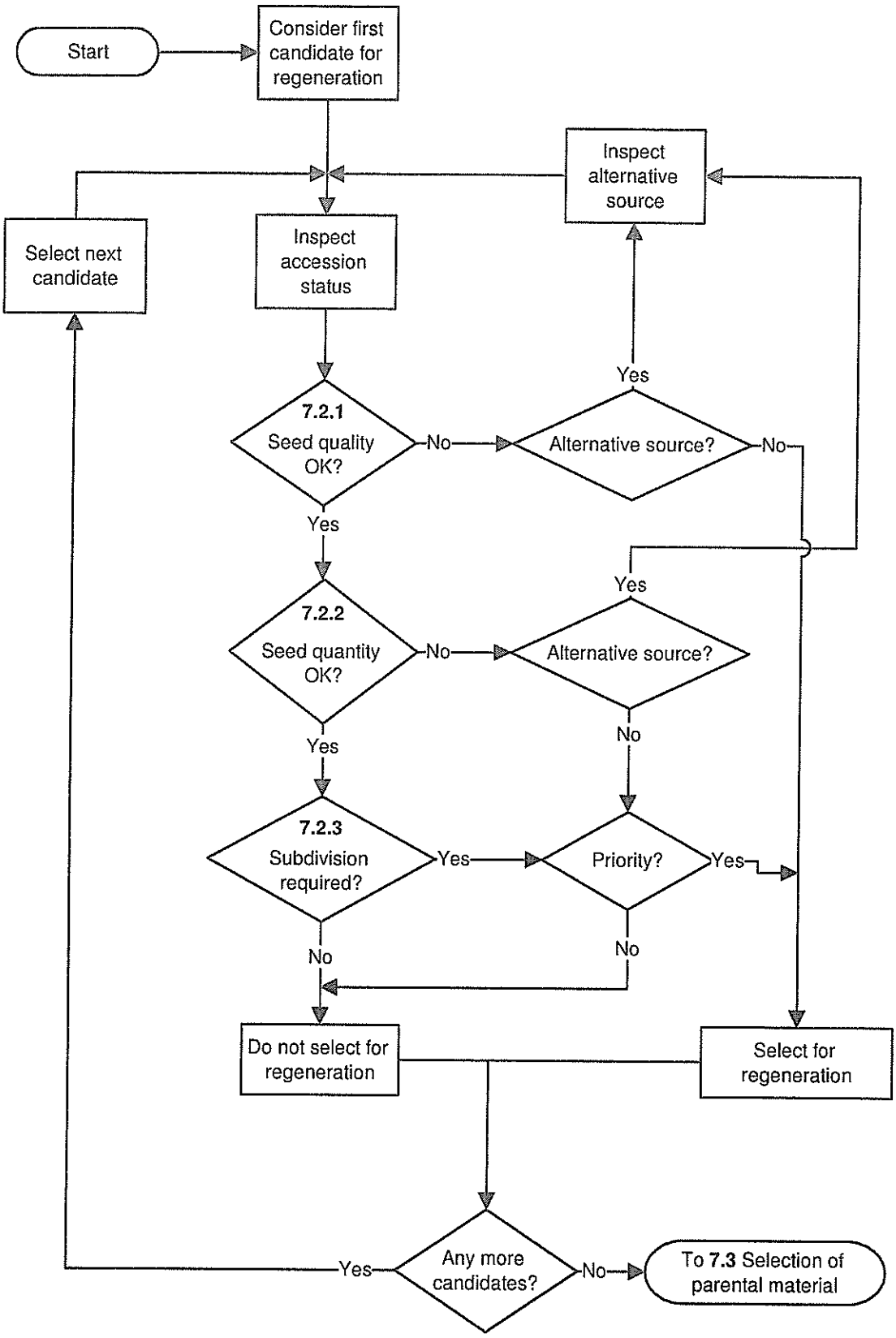
For hand-pollination, it is often as easy to achieve control in the field, e.g. by use of bagged inflorescences, as in contained facilities.

7.2 Selection of accessions

See flow chart 7.2.

As part of an overall planned programme of genebank maintenance, before each regeneration cycle the curator must identify accessions in need of regeneration. There are two principal reasons for regenerating: inadequate quality (section 7.2.1) and inadequate quantity (section 7.2.2) of seed. In some cases, depending on genebank policy and the genetic structure of accessions, a third possible reason can include the need to split some accessions into two or more genetically distinct subtypes (section 7.2.3).

Selection of accessions is preferably an information technology-assisted process. Before each cycle of regeneration, the genebank documentation system should be used to draw up a list of candidate accessions for possible regeneration. Final selection of accessions is made by the curator inspecting relevant data in relation to status of, and demand for, seed of each listed accession and, where necessary, undertaking additional tests. The need to reconsider the entire collection at every cycle is reflected in the outermost loop in flow chart 7.2, which shows the same decision process being applied to every accession.



Flow chart 7.2. Selection of accessions for regeneration.

Where the list of accessions in need of regeneration exceeds genebank capacity, the curator may have to resort to other criteria to prioritize accessions for regeneration. **Regenerating accessions that have seed of inadequate quality in the base collection should take priority over accessions represented by inadequate numbers of seed in the active collection.** There may also have to be subjective decisions on the scientific or conservation value of accessions, e.g. attaching highest priority to accessions known to be unique or whose original collecting site is known to have been destroyed.

7.2.1 Seed quality

A curator should establish appropriate seed quality controls, including a set of standards and procedures for monitoring and maintaining quality. The objectives of these controls are:

- to avoid irretrievable loss of quality by ensuring timely regeneration;
- to provide back-up procedures to minimize loss of quality in the event of a failure to regenerate in time.

Standards and procedures should include:

1. Definition of minimum standard for seed health: Seeds "should be as clean and free from weed seeds, pests and diseases as possible" (FAO/IPGRI 1994). No additional specific recommendations are given. In most species there is almost no information on the effect of seedborne pathogens on maintenance of seed viability in storage.

2. Definition of minimum standard for seed viability, as appropriate for the species concerned (see Genebank Standards, FAO/IPGRI 1994): Note that threshold quality standards for identifying old seed in need of regeneration – the topic of this section – are distinct from target quality standards for new seed produced by regeneration. The latter are dealt with in section 5.1.

3. A protocol for testing seed quality in relation to the above minimum standards

4. A set of protocols for procedures to follow when standards are not met: These should include placing accessions on hold, unavailable for distribution, until they have been regenerated. They should cover a range of eventualities relating to how far an accession falls below minimum standards. For example:
   - normal priority for regeneration during the next available cycle where seed quality is marginal
     (The curator should ensure that this is the normal route to regeneration for at least 95% of accessions losing quality. The remaining procedures in this list should be regarded as back-up mechanisms, to be followed when the viability monitoring programme fails to detect loss of seed quality in time.)
   - high priority for immediate regeneration where quality is significantly below minimum
   - a 'rescue regeneration', marked as such in the genebank documentation system, when quality is so far below minimum that the normal number and condition of parental plants cannot be established

- resort to special technologies, such as embryo rescue, when quality is so low that normal procedures would result in zero germination. Where appropriate technologies are not available in-house, it may be desirable to explore the possibility of using networks or other means of gaining access to them when necessary.
  When seed of an accession is stored in more than one location (e.g. in long-term and medium-term storage), procedures may include reverting to other sources when seed in one location fail to meet quality standards – e.g. using seed in long-term storage to regenerate poor-quality seed in medium-term storage. The safety duplicate collection may be used if necessary, but only as a last resort, and if it is used, then the safety duplicate sample should be replaced as soon as possible.

Two different sets of quality standards and policies are necessary:

- threshold quality for regeneration of seed in storage (section 7.2.1.1)
- threshold quality for regeneration of seed prior to initial entry into a collection (section 7.2.1.2).

7.2.1.1 Maintenance of viability in storage

Seed quality of cleaned seed in storage is defined primarily in terms of effective viability, measured as the percentage of seeds that germinate in an appropriate controlled environment (germination rate). Genebank policy must include an appropriate monitoring programme to identify for regeneration those accessions that fall below threshold viability. The level of viability set as a threshold for regeneration is necessarily lower than that for target quality after regeneration and will depend on species. Typical threshold levels include the following.

- 85% of seed germinate in appropriate controlled conditions. This option is suitable for most well-studied species.
- Viability dropped by 15% below maximum viability observed for the species or accession (e.g. if maximum observed viability is 80% germination rate, threshold is 65%). This option is necessary for species or accessions where it is difficult to achieve high germination rates. As discussed in section 5.1, this situation applies mainly where there is inadequate knowledge of the species concerned in relation to conditions for growth and harvesting, germination requirements and/or efficient cleaning procedures that eliminate aborted seed. In many cases, therefore, adopting this option should be treated as a short-term pragmatic necessity pending acquisition of further knowledge on the biology of the species.

For maximum security, viability of all accessions should be tested regularly. The frequency of testing each accession will depend on prior knowledge of its seed longevity characteristics in storage. It could range from 1 to 10 years for the active collection or even longer for the base collection. Most efficient use of resources is often (although not always) made by spreading viability testing equally across years and working cyclically through the collection. If, for example, a curator chooses to test each accession once every 10 years, then each year one-tenth of the accessions should be tested. Individual accessions would need to be tested more frequently if the results of one test show they are beginning to lose viability and are likely to fall below threshold before the next cycle of tests.

Where a genebank has insufficient resources for such a comprehensive viability monitoring programme, it is acceptable to establish a monitoring strategy that needs far less viability testing with only little loss of security. Such a strategy would include the following.

- Use the documentation system to identify accessions 'at risk' of falling below threshold viability. An accession is 'at risk' if its predicted viability is below a certain predefined level. Predictions are made on the basis of:
  - prior knowledge and experience of seed longevity characteristics of the species/accession
  - storage conditions
  - either the condition of the seed on entry into storage and the current age of the seed, or the results of the last viability test and the time elapsed since then.

  For most species, prior knowledge is not sufficient to permit accurate predictions. The viability at which accessions are considered to be 'at risk' should then be set higher than the threshold for regeneration. This reduces the possibility of erroneously classifying accessions as 'not at risk' when in fact true viability is below the threshold.

  Defining two or more 'at risk' categories – e.g. high risk and medium risk – should be considered as an aid to strategic viability testing (see below).
- Optionally test viability of some or all accessions predicted to be 'at risk'. Options represent a range of compromises between minimizing resources required for viability testing and minimizing risk of loss of accessions, and depend on the probable accuracy of predictions. They include:
  - testing a random subsample of accessions 'at risk'
  - testing a structured, non-random sample of accessions 'at risk'
  - testing a sample of one or more accessions from each batch 'at risk'.
  - The latter is most effective for batches of accessions previously regenerated at the same time, stored in the same conditions, and possessing similar seed longevity characteristics. Batches of newly donated or collected accessions may show significant variation among accessions from the same batch, so that testing only a subsample may not be effective.
  - use a different sampling strategy for different 'at risk' categories, e.g. testing all accessions at high risk, and a sample of those at low risk.
- The results of viability tests should be compared with predictions, and used to improve future identification of accessions 'at risk'.
  - If knowledge of seed longevity characteristics is poor, a small number of selected accessions should be tested at frequent intervals to improve accuracy of predictions. The effort expended in so doing should be cost-effective, in that ultimately it should reduce the level of human resources put into viability monitoring. It should therefore be a high priority.
- Selection of accessions for regeneration will be based on a combination of theoretical predictions of those accessions at risk and actual viability tests. Options available include:
  - regenerate, without further viability testing, all accessions that are predicted to be at risk
  - strategically test viability of some of the accessions at risk, and regenerate all that are below threshold and all those that, by interpolation of tests, also may be expected to be below threshold

* test viability of a subset of accessions in each batch of seed, and regenerate all accessions of all batches with a tested accession below threshold
* test viability of all accessions at risk, and regenerate all with germination rate below the set standard.

Where different 'at risk' categories are recognized, different options for regeneration may be applied to different risk categories. For example, all high-risk accessions may be regenerated without further viability testing, while medium-risk accessions are tested and regenerated only if the test results indicate a need for regeneration.

7.2.1.2 Health and viability on initial entry into collection

Genebank policy must include:

- standards for quality of material to be included in a collection
  These should include standards for quality of passport and other data associated with the seed, in addition to standards for seed health and viability.
- tests for assessing health and viability of incoming seed
- procedures to follow when standards are not met.
  In addition to the procedures outlined above (7.2.1), these procedures could optionally also include rejecting a donated accession if quality is considered unacceptably low, or seeking additional data if data quality is inadequate.

In addition, international introductions require quarantine. Quarantine regulations vary with country. They may lie within the responsibility of the genebank or may have to be complied with at a distant plant introduction office, so that material arriving at the genebank may already have passed through quarantine. Depending on national quarantine regulations, genebank policy may have to include quarantine controls.

If incoming seed is of inadequate quality, an initial, possibly immediate, cycle of regeneration is needed. Options for deciding what threshold quality standards to set for incoming seed, and therefore whether to undertake an initial regeneration, include the following.

1. Set threshold quality equal to that for regenerating seed already held in storage (section 7.2.1.1), and regenerate only if tests show health and viability are below this threshold. This option is likely to be preferred where the genotypic composition and breeding system of the accession make it difficult to maintain genetic integrity satisfactorily, so that regeneration should be undertaken only as a last resort. It should not be chosen solely on the grounds of reducing regeneration costs.

2. Regenerate only if tests show health and viability to be significantly below the target quality for newly regenerated seed. This option gives generally higher quality seed than the first, but at the expense of additional deterioration of genetic integrity. It is likely to be the preferred option for more uniform accessions, whose genetic integrity can be maintained more satisfactorily.

3. Always regenerate, regardless of any test results. This option guarantees maximum seed quality and has the added advantage of eliminating the

need for initial viability tests and their associated costs and delays. However, it involves the greatest risk for degrading genetic integrity. It is likely to be preferred where maintenance of genetic integrity is not difficult and where a significant decline in seed longevity may not be detectable by viability tests. This latter is a common feature of most species: stored seed typically show little or no apparent decline in viability for a number of years, followed by a progressively more rapid decline. Thus viability tests cannot reliably show the remaining longevity of seed with high viability.

Different options above may be adopted for the different types of collection. In many cases it is preferable to adopt option 1 for seed to be stored in the base and safety duplicate collections, and option 3 for the active collection. By this means, the original sample is retained intact for conservation for as long as possible, while high-quality seed is made available for distribution with minimal impact on the original sample.

7.2.2 Seed quantity

This section, like the previous one, deals with threshold levels for regeneration, not with target levels following regeneration. Different threshold levels must be set for accessions held in base and active collections. Quantity becomes critically low if it is impossible to establish the number of parental plants required for maintenance of genetic integrity (sections 5.3.3.1, 7.3.2). If quantity does become critically low, a bottleneck must be recorded in the documentation system.

Quantity is ultimately defined in terms of numbers of seed, although weight of seed may be a more practical basis for definition, especially for small-seeded species and species showing little variation in 1000-seed weight.

7.2.2.1 Threshold quantity for base collection

Threshold quantity for regeneration of the base collection is the sum of:

- one test unit (section 5.2)
- one base unit for regeneration of the base collection, and
- one additional base unit if there is an imminent need to regenerate the active collection from the base collection (section 7.3.1.3).

7.2.2.2 Threshold quantity for active collection

Threshold quantity for regeneration of the active collection is the sum of:

- one test unit
- one base unit if the next regeneration cycle is to use residual seed from the active collection, which will be the case only if the genebank has adopted the accepted, rather than preferred, standard for selection of parental material (see section 7.3.1.3), and
- the distribution unit multiplied by the expected number of times seed of the accession will be requested before the next possible regeneration cycle.

7.2.2.3 Quantity available on initial entry into collection

Genebank policy must include standards for minimum quantity of seed to be included in a collection, and a procedure for prioritizing the necessary initial cycle of regeneration when the initial number of seed is less than the minimum.

In the case of accessions that are normally stored as seed but were collected or introduced as vegetative material, the initial number of seed introduced can be

zero. In this case, immediate regeneration should be top priority, unless the plants are long-lived and easy and economical to keep as a vegetative accession.

7.2.2.4 Maintenance of sufficient quantity in storage

Options for monitoring remaining quantity in storage are as follows.

- As a minimum, quantity may be estimated visually each time seed is removed from an accession, and the accession marked for regeneration when quantity drops below threshold.
- The preferred standard, if genebank capacity and expertise permits, is to operate a more comprehensive and automated information technology-assisted system for monitoring seed movements and remaining seed quantities of each accession. If average seed weight is known, then weighing the remaining seed after each transaction can be used to trigger flags in the documentation system and place the accession in a regeneration queue. Such a system can be used to improve matching of supply and demand, with a regeneration protocol optimized to produce seed in appropriate quantity to satisfy demand. It also has benefits in genebank management beyond regeneration, for example in facilitating tracking of the destinations of seed used.

A protocol should be established for handling accessions that fall below threshold quantity, to include:

- normal priority regeneration for accessions at or marginally below threshold
- high priority regeneration for accessions well below threshold but still above critically low quantity
- high priority regeneration for accessions below critical quantity, together with marking a bottleneck in the documentation system.

As with seed quality, a curator should ensure that at least 95% of accessions are regenerated through the first, normal priority route. If possible, none should be allowed to pass through a bottleneck.

All accessions falling below threshold should be placed on hold, i.e. made unavailable for distribution, until they have been regenerated. In general, falling below threshold quantity should attract a lower priority than falling below threshold quality, because an accession with few but high-quality seed can safely be placed on hold and remaining seed stored viable until they can be regenerated.

7.2.3 Subdividing accessions

Subdividing an accession into two or more genetically distinct subtypes is an option for maintaining genetic diversity within accessions. Two main reasons are cited for subdividing a variable accession:

- to separate an allele known to be of particular interest to users
- to facilitate maintenance of genetic integrity of a highly variable accession by creating from it two or more new accessions that are distinct and relatively uniform.

Subdivision for the second reason is not generally recommended. Its primary use should be as a tool facilitating utilization of germplasm by separating known alleles, but this should usually be done in addition to, not instead of, conserving the original accession intact.

It is beyond the scope of this document to provide full discussion of the advantages and disadvantages of subdividing for improving conservation of

genetic resources. Briefly, it will help conserve specific alleles and combinations of alleles, and more specifically help conserve rare alleles, if the resulting divided accessions are highly distinct and have significantly lower diversity within accessions than the original variable accession. This will be the case if the original accession comprised clearly distinct subtypes. Subdivision will not be effective in this respect if the original accession shows continuous unstructured genetic variation.

However, even if subdivision is effective in reducing diversity within accessions, the increased ease of maintenance of diversity enabled by subdivision must be offset against the higher costs of maintaining a larger number of accessions.

Moreover, the very process of subdivision itself may destroy the genetic integrity of the original population. For example, a subdivided landrace cannot usually be reconstituted with genetic integrity intact simply by remixing the subdivided components. (There are exceptions to this rule: for example some landraces of *Sorghum* and *Phaseolus vulgaris* are deliberately maintained as mixtures by farmers subdividing a landrace into its components at each harvest and remixing for the next growing season. In such cases subdivision is more justifiable.) Subdivision must therefore be undertaken only with extreme caution. In the same way as with subdividing for the purpose of facilitating utilization, subdividing for conservation should generally be undertaken only in addition to, rather than instead of, retaining the original accession intact.

If an accession is to be divided into genetically distinct subtypes, and if these subtypes are apparent in the seed phenotype, the accession can be subdivided directly on the basis of seed phenotype. Regeneration is then necessary only if there are too few seed of one or more of the subtypes. If the genetic subtypes are apparent only in the growing plant, a specially tailored regeneration cycle will be necessary. Plants will have to be grown to identify the subtypes, in sufficient numbers to provide enough parents of each subtype, and in a way that allows appropriate pollination control, avoiding cross-pollination between subtypes.

7.3 Selection of parental material

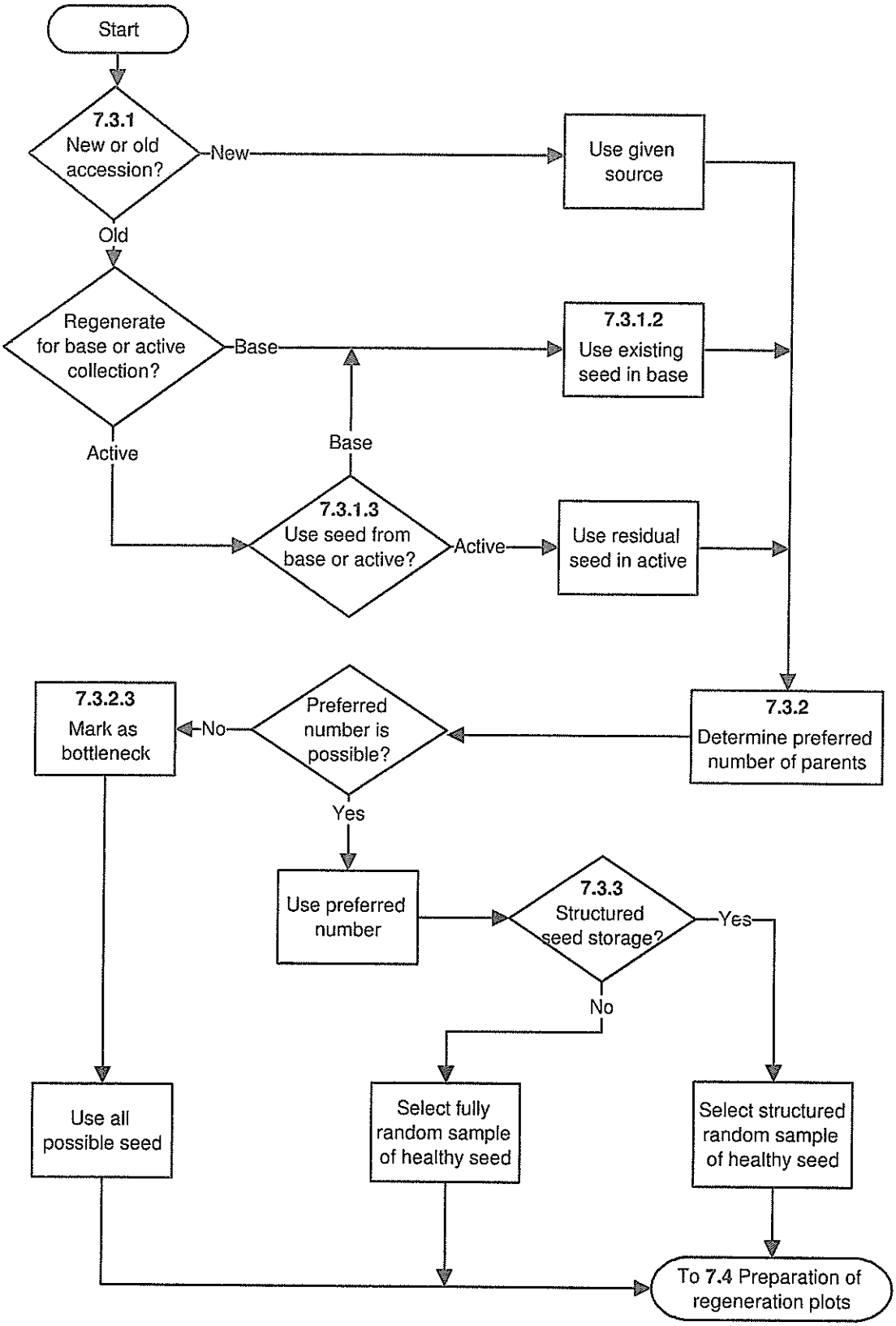
See flow chart 7.3. Parental material refers to seeds or plants that are to be grown to produce offspring seed to be used as the next generation of the accession, and thus to become the parents of the next generation.

7.3.1 Source

Possible sources of parental material include:

- living plants from a new vegetative plant-collecting expedition
- seed from a new seed-collecting expedition
- donated seed
- long-term storage
- medium-term storage
- safety duplicate collection.

When a new accession is entered into a collection for the first time, there is usually only one possible source of parental material (one of 1–3 above, although in some cases both a vegetative and a seed sample of a population may be taken). An accession already in a collection may be represented by several samples in different locations (4–6 above), and it is necessary to choose which to use.



Flow chart 7.3. Selection of parental material.

7.3.1.1 Safety duplicate collection

Samples in the safety duplicate collection should not be used except as a last resort, to regenerate an accession that would otherwise be unavoidably lost or suffer an unacceptable loss of genetic integrity.

7.3.1.2 Base collection

A sample held in the base collection in long-term storage should normally be regenerated using only residual seed remaining in that same sample.

7.3.1.3 Active collection

Options to be considered for an accession in the active collection are to regenerate from residual seed in the active collection or from original seed in the base collection. Factors to be considered are as follows.

- Changes in genotypic composition of an accession are cumulative. Each time an accession is regenerated from residual seed in the active collection, new genetic changes are usually added to old ones (but see section 5.3.3.1). The importance of this increases with the magnitude of genetic variation within accessions and decreases with the stringency of procedures adopted to minimize genetic changes.
- Use of the base collection to regenerate a sample for active use increases the rate at which the original accession is used, thus increasing the amount of seed that must be held in the base collection, and possibly increasing the frequency of regenerating the base collection itself. The latter possibility must be avoided.

The preferred standard (FAO/IPGRI 1994) is always to regenerate from the original seed in the base collection. This is particularly important for outbreeding species where:

- within-population genetic variance is high
- combined genetic changes over multiple regeneration cycles cannot be controlled with the desired precision
- the size of the sample in the base collection has been previously designed to accommodate the higher demand this option places on it
- the sample in the base collection is stored in a structured manner specifically to improve maintenance of genetic integrity during regeneration (section 7.3.3).

Genebank Standards (FAO/IPGRI 1994) also allow as an acceptable standard, alternation between base and active, regenerating from offspring generations in the active collection for up to three regeneration cycles before returning to the original seed. This allows limited but only temporary accumulation of genetic changes, with cyclical reversion to the original type. This option is preferred by some genebanks, especially for inbreeding species.

In some circumstances, such as with inbred lines and obligate apomicts, regenerating only from residual seed in the active collection may be an acceptable option. In most cases this is not acceptable genebank practice.

The above standards may be altered if seed from the alternative sources differs significantly in seed quality, including health, viability, genetic integrity and identity verification. Where the number of seed stored in the base collection is not enough to permit its frequent use for regeneration, the above acceptable standard (i.e. regenerating an accession in the active collection from remnant seed in the active collection) becomes the preferred standard. The reason is that

in such cases the normal preferred standard will result in unnecessarily frequent regeneration of the base collection itself, which should be avoided.

In addition, standards are based on the assumption that seed in the base collection is equal or superior to remnant seed in the active collection. This is normally true because the base collection is held purely for conservation purposes with higher priority attached to long-term maintenance of quality and genetic integrity. However, the possibility cannot be ruled out that sometimes the active collection will have superior seed, and the curator should use the active collection in such cases.

7.3.2 Number of parental plants

As discussed in section 5.2.2, the ideal number of parental plants is the larger of (i) the number required to produce the target quantity of offspring seed and (ii) the number required to maintain the genetic integrity of the accession. Genebank Standards (FAO/IPGRI 1994) recommend 100 plants or more. Crossa *et al.* (1993) recommend 150–220 on the basis of minimizing random loss of rare alleles. Many genebanks use less (ICRISAT 1995), typically 30–100. This may indicate a need to review regeneration protocols at some genebanks, although the case study in section 5.2.6 shows that other factors may also influence the ideal number of parents.

A bottleneck occurs, and should be noted in the genebank documentation system, if the number of parental plants available is less than the number required for maintenance of genetic integrity (section 5.3.3.1). If the number of parental plants exceeds the number required for maintenance of genetic integrity but is less than the number required to produce the target quantity of offspring seed, then the cost-efficiency of regeneration is reduced but genetic integrity is not compromised; this is not regarded as a bottleneck.

7.3.2.1 Prior bottlenecks

In addition to increasing losses of rare alleles (section 5.3.3.1), passing through a bottleneck also increases the minimum expected frequency of any rare alleles that remain (see Box 1). The effect of this is to reduce the probability of losing the rarest remaining alleles during a repeat regeneration. Conversely, to achieve a particular success rate in preventing loss of the remaining rarest alleles, the number of parents required for regeneration can be reduced following a bottleneck of N_b parents. Continuing to use N_b parents for subsequent regenerations after a bottleneck is sufficient to continue the same rate of degradation of genetic integrity (as measured by rate of loss of the rarest alleles) as would have occurred using N_c parents before the bottleneck. However, it is recommended to compensate for the rapid degradation at the initial bottleneck by reducing the probability of further allele losses, by using more than N_b parents for subsequent regenerations. Reverting immediately to the normal N_c parents provides maximum compensation.

Box 1. Effects of prior bottlenecks

Suppose N_c is considered the critical minimum number of parent plants to regenerate without unacceptable loss of rare alleles. Alleles that remain present in the offspring generation must have been represented by at least one copy in the parent generation. On a neutral gene model with random mating and diploid inheritance, the minimum expected frequency f_c of alleles surviving to the offspring generation is then

$$f_c = \frac{1}{2N_c}$$

with the same modifications for ploidy level and selfing rate as described in section 5.3.3.1. On a second cycle of regeneration, using N_c parents selected at random from the progeny of the first cycle, the probability P_c of losing one of these remaining rarest alleles is

$$P_c = \left(1 - f_c\right)^{2N_c}$$

Now suppose instead that a bottleneck occurs by using N_b plants as parents instead of N_c ($N_b = \alpha N_c$, $\alpha < 1$). The subsequent minimum expected frequency f_b of alleles surviving to the offspring generation is then

$$f_b = \frac{1}{2N_c\alpha}$$
$$= \frac{f_c}{\alpha}$$

That is, a bottleneck increases the minimum frequency of surviving rare alleles in direct proportion to the severity of the bottleneck. If N_c parent plants are used in a subsequent regeneration after the bottleneck, there will be a corresponding reduction in the probability of losing these remaining rarest alleles, to

$$P_b = \left(1 - \frac{f_c}{\alpha}\right)^{2N_c}$$

Now suppose that instead of using N_c parents and thereby reducing the probability of further allele loss, it is considered desirable to continue the normal targeted probability P_c of losing one of the remaining rarest alleles. The number of parents, N_r, required to achieve this target probability P_c is then

$$N_r = N_c \frac{\log\left(1 - \frac{1}{2N_c}\right)}{\log\left(1 - \frac{1}{2N_c\alpha}\right)}$$
$$\approx N_c\alpha$$
$$= N_b$$

As an example of the calculations shown in Box 1, suppose that regeneration is normally undertaken using N_e=100 parental plants. Expected frequency of the rarest possible alleles is then 0.005, and the probability of losing one such allele during a normal regeneration is 0.37. Now suppose that a bottleneck of 10 plants is experienced by one accession. Following the bottleneck, minimum expected allele frequency is 0.05. If subsequent regeneration is undertaken using the normal 100 parental plants, the probability of loss of one of these rarest alleles is reduced to 0.000035. However, if the bottleneck continues to be applied during subsequent regenerations, the probability of loss of the same rarest alleles is 0.36. This is almost the same as the rate of loss of rarest alleles (with expected frequency 0.005) in the absence of any bottleneck.

7.3.3 Identity of parent plants

Seeds and plants are usually collected without recording their position within a population or the specific characteristics of their immediate microenvironment, and seed of an accession is stored in one container without regard for its parentage within the original population. The curator then has no choice but to select a subsample at random for use as parents (unless there are so few plants remaining in the sample that they must all be used).

This is standard and preferred practice for the majority of crops, particularly for advanced annual crops and species with low genetic variance within accessions. The rest of this section is not relevant in these cases.

For many species, particularly wild species, curators may need to consider instead selecting parents non-randomly to improve the correspondence between genetic composition of parent plants and that of the original population, thus reducing genetic changes and improving maintenance of genetic integrity. This is generally justifiable only for highly variable populations that are spatially structured in their genotypic composition.

It is possible, but not desirable for PGR conservation, to devote considerable resources to maintaining the genetic integrity of such populations. This section will present only a simple extension to normal regeneration protocol that is only marginally more expensive than taking balanced bulks (section 7.9.7). If additional care is considered necessary, the curator should perhaps consider alternatives such as *in situ* conservation or tissue culture.

Implementation of procedures for non-random selection of parent plants requires coordinated subsequent planning of strategies and procedures for collecting and storing as well as regeneration. They represent a special case such that, although they are not required for most domesticated species, the rest of this section will be devoted to considering the type of population that merits such attention, and how collecting and storage procedures must be modified to enable non-random selection of parents.

7.3.3.1 Occurrence of substructured populations

Wild populations typically show very fine-scale substructuring into genetically distinct subpopulations. The effective genetic population area defines the smallest scale at which a seed population can be substructured. It is often remarkably small – e.g. about 2 m^2 for the insect-pollinated obligate outbreeding perennial legume *Trifolium repens*, and about 8 m^2 for the wind-pollinated obligate outbreeding perennial grass *Lolium perenne* (Hayward and Sackville Hamilton 1997). If the adult plants are long-lived perennials, the vegetative population may be subdivided into genetically distinct subpopulations at an even smaller scale.

The genetic differentiation between subpopulations can arise either by differential drift and inbreeding or by natural selection and microscale adaptation to the local microenvironment. Drift and inbreeding can be large because of the small size of the effective genetic population; and differential selection can be large because of the high spatial heterogeneity of most natural ecosystems.

In such species what appears physically as a single continuous population is in fact genetically substructured with many overlapping subpopulations. Each subpopulation then has no unique physical identity distinct from other subpopulations, but is a purely conceptual entity, as each plant in it is also simultaneously a member of many other subpopulations overlapping the first. Conceptually there are as many subpopulations as plants, with each plant being at the centre of its subpopulation.

7.3.3.2 Integration with collecting and storage methodologies

Sometimes the physical population covers a number of distinct microenvironments that previous experience shows is related to adaptive genetic variation – e.g. on or off a footpath trampled through a pasture. In this event the collector should consider sampling plants from the different microenvironments as different populations, for inclusion as separate accessions in the collection.

Where no such clear microenvironmental heterogeneity is apparent, it is not justifiable for PGR purposes to collect and maintain different subpopulations as distinct accessions (although it is justifiable for other purposes, such as population genetics studies). However, combining all sampled subpopulations into one for maintenance as a single accession, as is most often done, has disadvantages. The resulting accession shows higher levels of heterozygosity than was present in the original subdivided population *in situ*; a greater diversity of novel recombinants will be produced during regeneration; the accession will have higher additive genetic variance and higher heritability than each of the original genetic populations, and it is therefore more responsive to selection, and more likely to change its genetic composition in response to the natural selection pressure imposed by the particular environment used for regeneration. In short, if the original structured population is treated as a single panmictic entity, its genetic integrity is compromised from the moment it is entered into the genebank, and will deteriorate still more rapidly with regeneration.

An economical alternative is to maintain the original population sample as one accession for the purposes of documentation and distribution, but to store different parts in different containers purely for the purposes of improving maintenance of genetic integrity on regeneration. In this case, multiple containers per accession are used for conservation in the base and safety duplicate collections, whereas seed samples for distribution from the active collection are formed by mixing seed (preferably as a balanced bulk) from all subpopulations into a single container.

Samples from each subpopulation should then be kept separate. All containers for one accession should themselves be securely contained together, within a single, larger labelled container. For PGR conservation purposes it is not usually economically viable to document and label each subpopulation separately. It may be noted that this requires post-harvest management procedures almost identical to those for taking a balanced bulk, with the exception that for storage in the base collection the final stage of forming a balanced bulk is replaced by separately bagging up seed from each subpopulation. As such, minimal extra cost is incurred.

7.3.3.3 Selecting parental plants

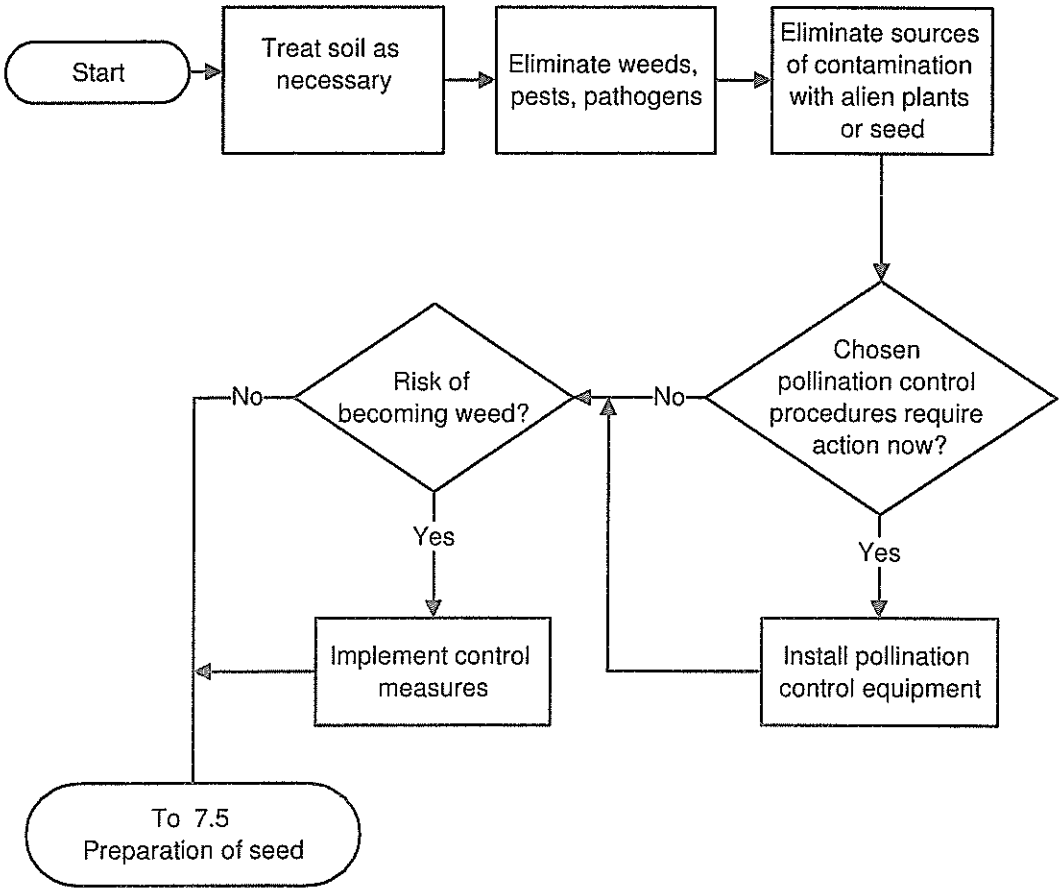
To regenerate the accession, an equal number of seed is sampled at random from each container, in order to make up the required total number of parent plants (section 7.3.2). This improves initial genetic composition of parental plants.

7.4 Preparation of regeneration plots

See flow chart 7.4. All procedures are species-specific, requiring prior knowledge of optimal requirements. Most of the decisions to be made are of an agronomic or horticultural nature and can be made by reference to crop-specific textbooks, although such texts are often not available for wild and weedy germplasm or even landraces. Therefore no specific guidelines can be given here. It is not even feasible to present a comprehensive range of options for consideration. This section will simply present the general areas that need to be considered, highlighting aspects, such as the need for uniformity and absolute cleanliness, that are of particular importance to regeneration and that therefore will not feature in agronomy texts.

- **Soil.** The regeneration plot must be as uniform as possible throughout the area of the plot so that each plot has equal space, light, nutrients, soil structure, physical and chemical composition. Consider the need to examine the soil, possibly including physical and chemical analysis. If necessary, apply soil treatments as appropriate for the crop and site (e.g. fertilizers, lime, drainage, irrigation, ploughing, soil structuring, preheating).
- **Weeds, pests and pathogens.** By inspection and prior experience, determine what weeds, pests and pathogens are currently, or may potentially become, a problem. Determine whether such problems can be reduced during preparation of regeneration plots by the application of appropriate treatments for elimination of weeds, pests and pathogens. Ensure that any treatment selected does not adversely affect seed production.
- **Cleanliness (1).** Regeneration plots must be kept scrupulously clear of alien seed and plants. Alien plants within a plot may be difficult to distinguish visually, and may produce seed contaminating the accession seed harvest. Determine whether this might be a problem. If so, consider measures to control, and preferably eliminate, such aliens during plot preparation before sowing the accession. Examples of possible control measures include spraying plants, sterilizing soil, using sterile compost and ploughing to encourage germination, followed by spraying or deep ploughing to kill emerging seedlings.
- **Cleanliness (2).** Consider also whether there is a risk of contamination with alien pollen (section 7.1.3.2). Alien seeds and plants nearby as well as within the plots are potential sources of alien pollen. If there is a contamination risk, consider appropriate measures to reduce it during plot preparation, for example by chemical sprays, mechanical cultivation or hand-weeding. Consider recommendations for reducing contamination in commercial seed production.
- **Cleanliness (3).** Consider whether the accession itself will require special containment to prevent it from becoming established as a weed in the field.
- Ensure that the method of plot preparation is appropriate for the chosen method of establishing plants, e.g. direct-seeded or transplanted as seedlings (section 7.5).

- Consider whether there is a need to coordinate plot preparation with installation and preparation of equipment for pollination control (section 7.7).
- Prepare bed for seed or transplants as appropriate. Size and shape will depend on:
  * size and shape of regeneration site
  * number of accessions to be regenerated
  * number of plants per accession
  * spacing of plants
  * requirement for ancillary services, e.g. pollination cages, or drainage or irrigation channels
  * requirement for machine access.
- The method of preparation will depend on:
  * availability of labour and machinery
  * soil structure, e.g. heavier soil may need more powerful equipment than light soil
  * species to be sown or transplanted and its cultural requirements
  * whether there is a need for plant supports, e.g. for climbing plants such as *Phaseolus coccineus*.



Flow chart 7.4. Preparation of regeneration plots.

7.5 Preparation of seed

7.5.1 Initial checks and seed selection

See flow chart 7.5. If necessary, check amount, condition, moisture content, health and viability. Dry, thresh, clean and reject poor-quality seed, as necessary. This is usually necessary only for newly donated or collected seed, as seed already stored should have been checked and prepared.

The curator should establish and follow a system for bagging, labelling and transporting seed that has a built-in cross-checking mechanism, to ensure 100% accuracy in the identification of accessions. The genebank documentation system should be used to print labels. Bar codes are recommended to avoid transcription errors.

Scrupulous attention should be paid to cleanliness, to guarantee zero contamination of seed samples with seed of other accessions. Where threshing and cleaning are mechanized, use only precision purpose-built machinery that enables absolute cleanliness. Commercial agricultural implements should not be used, as they cannot be adequately cleaned internally between accessions and will result in cross-contamination with seed from the wrong accessions.

Options for seed selection in relation to germination test are:

- use seedlings germinated during the test to form the parent plants for regeneration, or
- use two independent seed samples, one for the germination test and another for regeneration.

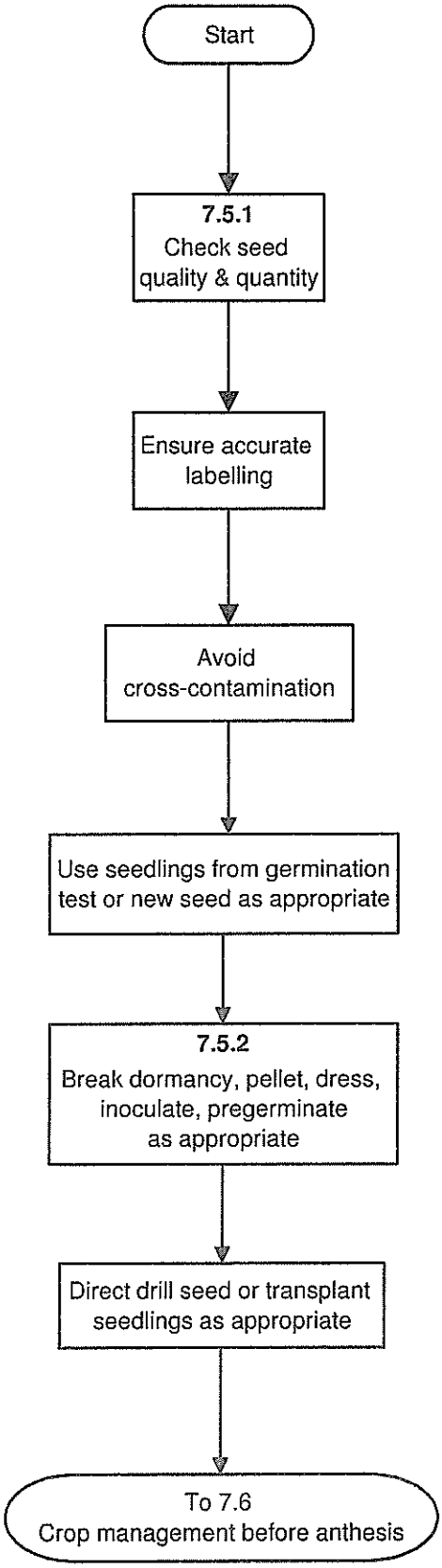
The first option requires that sufficient seeds are used in the germination test to produce at least enough plants for regeneration, and that seed for the germination test was from the source required for regeneration. Thus it is not appropriate when using seed from the base collection to replenish stocks in the active collection. It is obligatory when there are too few seeds to allow for the second option.

The second option may in some cases be necessary, for example if an initial germination test fails to produce sufficient seedlings for regeneration. The results of the germination test should then be used to obtain a better estimate of the number of seed needed to produce the required number of parent plants for regeneration.

7.5.2 Seed pretreatments and seedling management

In some cases, it may be sufficient to sow seed directly into regeneration plots. Depending on individual species requirements, specific pretreatments may be desirable to improve seed germination and establishment. Each of the following should be considered.

- Break dormancy if appropriate for the species or accession (e.g. by light, photoperiod, cold, alternating temperatures, leaching, stratification, scarifying, acid). It is important to break dormancy fully. Failure to do so may result in selection for genotypes with less stringent requirements for breaking dormancy, together with associated selection of characters that are pleiotropic expressions of the dormancy genes and of other genes that are linked to the dormancy genes.
- Pellet seeds, e.g. with dung, slow-release fertilizers, hygroscopic coatings, etc.
- Apply proprietary seed dressings to reduce disease or insect damage.



Flow chart 7.5. Preparation of seed.

- Inoculate with appropriate symbionts (*Rhizobium*, mycorrhizae), possibly as seed pellets.
- Undertake preliminary controlled rehydration of dried seed.
- Pregerminate in controlled conditions, e.g. in an incubator, agar, etc., followed by transplanting seedlings.
- Sow manually or by machine. If by machine, use only precision purpose-built machinery that can be completely cleared internally of all seed between plots.
- Grow young seedlings in pots or special seed beds (for transplanted crops).

7.6 Planting and crop management before anthesis

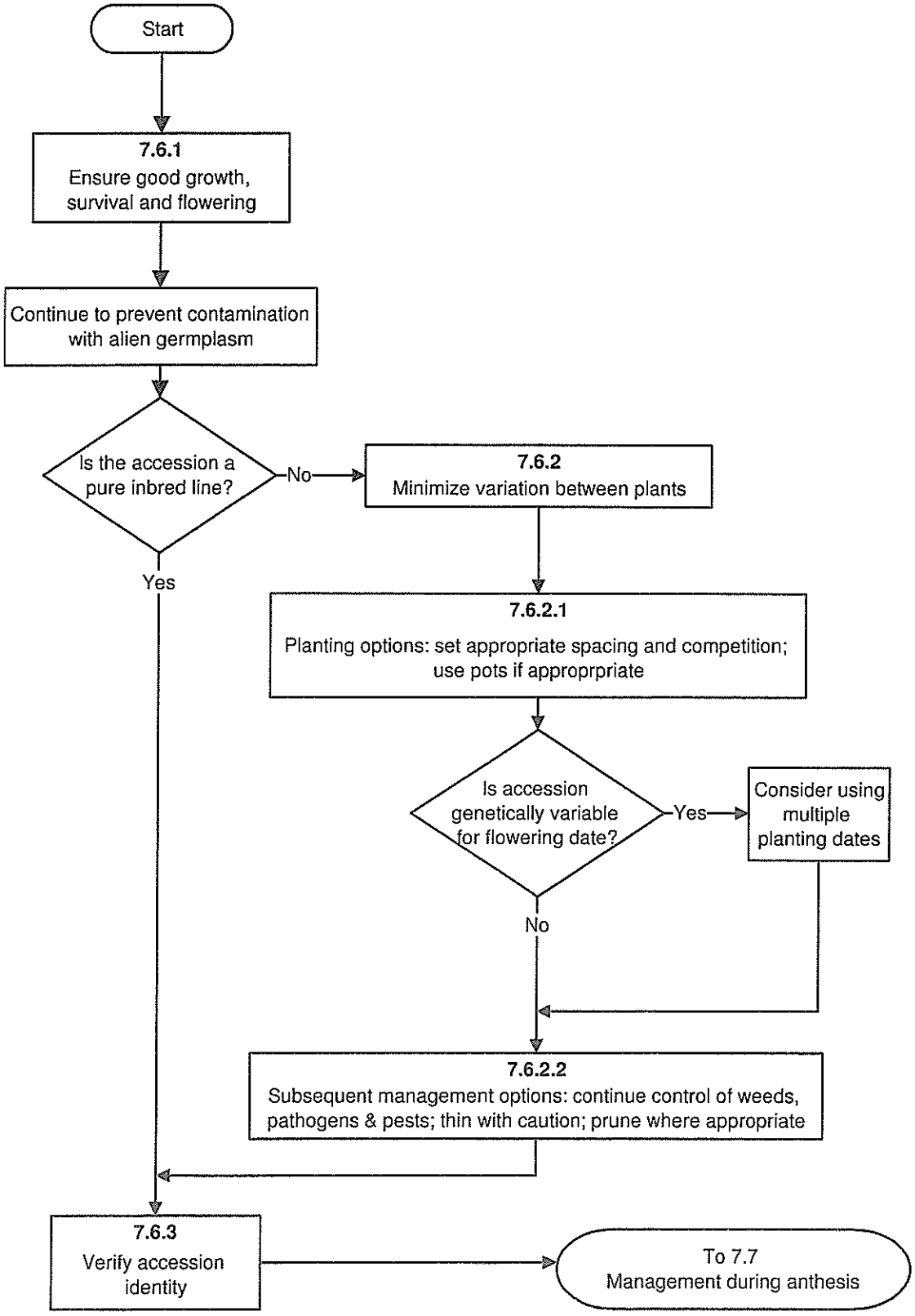
7.6.1 Management objectives

See flow chart 7.6. Before anthesis, the aim of crop management for regeneration is to produce plants that are in an optimal condition to maximize useable seed yield. Component objectives are to:

1. Provide suitable conditions for growth (conditions need not be optimal: restricting growth by using small pots or infertile soil can be a useful tool for reducing plot size and thereby reducing problems that would arise from limited pollen dispersal within the plot)
2. Provide suitable conditions to trigger abundant flowering so far as possible for the accession
3. Eliminate alien plants
4. Ensure the maximum possible survival of plants
5. Minimize variation between plants in growth rate
6. Minimize variation between plants in flowering and all its components: position and number of inflorescences, number of flowers per inflorescence, and seeds per flower/fruit.

Objectives 5 and 6 are not relevant for pure inbred lines. They are relevant wherever there is genetic variation within accessions, as an essential part of minimizing genetic change through differential contribution of both female and male gametes to the offspring seed population. With one exception (see below), reducing this component of genetic change to zero is neither feasible nor desirable. Complete uniformity between plants implies the elimination of genetic variation within accessions for growth and reproduction, which is incompatible with the objective of maintaining genetic integrity. The objective of crop management is to reduce to zero only the environmental, not the genetic, component of between-plant variation, and to apply management procedures that minimize expressed phenotypic variation attributable to genetic variation.

The exception to the above is pruning. Large plants may be pruned with the objective of eliminating variation in the amount and timing of pollen and seed production by reducing all plants to the same number of inflorescences and synchronizing flowering. Pruning may be undertaken at any stage, and may involve removal of any organ (growing points, leaves, branches, flower buds, roots) as appropriate for the species concerned. Usually it will be preferable to delay pruning to the last possible moment before anthesis, when the impact on number and developmental stage inflorescences can be best controlled. The particular advantage of this option is that it not only reduces environmental variance in pollen and seed production, but also masks genetic variance in the parent generation. By eliminating phenotypic expression of genetic variation, it



Flow chart 7.6. Crop management before anthesis.

causes the genetic variation to be transmitted intact to the offspring generation, thus maintaining genetic integrity.

Achievement of these aims depends in part on using an appropriate location for regeneration, which has been dealt with in section 7.1. This section deals only with additional issues related to crop management in the chosen location.

7.6.2 Management options

Regular inspection of plots and plants should be considered mandatory to monitor progress in achieving the above objectives. Achievement of the objectives requires good prior knowledge of the agronomic/horticultural requirements of the crop in relation to:

- soils (biotic and abiotic characteristics)
- fertility and water management
- weed, insect and disease control, including mulching
- timing of planting and harvest
- stand and plant density
  - thinning
  - plant habit management
- microclimate manipulation (windbreaks, shading, etc.).

To some extent, management procedures can be based on commercial and/or breeder practice. However, interplant variation is often not a primary consideration in normal commercial practice. Because of the need to minimize interplant variation, crop management for regeneration differs in certain key aspects from normal commercial practice as follows.

7.6.2.1 Planting

1. Planting patterns: sow or transplant in uniformly spaced rows and uniform spacing between plants within rows. This will require either manual planting or use of precision sowing machinery.
   There are two principal benefits. First, the uniform spacing helps reduces variation in growth rate between plants. Second, it facilitates identification of dropped seeds or volunteers as they are usually off-row or delayed in transplanted crops.
2. Use of pots can help increase uniformity among plants, by equalizing the volume of their root balls.
3. The mobility enabled by use of pots can be a valuable tool to increase flexibility of management, for example to:
   - temporarily move plants into a controlled environment at a critical phenological stage, to trigger uniform flowering
   - adjust plant spacing as plants grow, to minimize resource usage without adversely affecting uniformity
   - increase regeneration throughput capacity by restricting occupation of time-critical facilities to key stages in plant development (e.g. moving plants into pollen-proof chambers only during anthesis).
4. Planting dates: if there is much variation between plants in flowering dates, consider using two planting dates in each regeneration plot to obtain pollination between early and late genotypes of each accession.
5. Competition for light: avoid competition for light by sowing plants at wide spacing or limiting nutrient supply. Competition between plants for light increases both the coefficient of variation and the skewness of the size distribution of plants. At high density, there is a tendency for the majority

of the plants to be small and the plot to be dominated by a small proportion of large plants. The size inequality increases with time, as the small plants are, by virtue of their small size, progressively more shaded and therefore disproportionately further suppressed by the large plants. In extreme conditions, the smallest plants may show zero or negative growth rates and may fail to flower. The resulting genetic changes are likely to be high.

With competition for water and nutrients, small size does not *per se* lead to further suppression, and so does not necessarily generate such size inequality. In some species it is possible to obtain quite uniformly small plants by sowing at high density with relatively restricted water or nutrient supply. The curator may consider conducting experiments to determine the optimal spacing for a given regime of light, nutrient and moisture supply.

7.6.2.2 Subsequent management

1. As far as possible, ensure complete control of weeds throughout the regeneration cycle. The effects of competition from weeds on size distribution of the crop plants are similar to the effects of competition between crop plants.
2. As far as possible, ensure complete control of pathogens and pests. Infection is often restricted to some plants only and only rarely affects a whole plot uniformly, and so increases the magnitude of genetic changes.
3. Thinning should not normally be undertaken, even if it is part of normal agronomic practice, without careful consideration of the consequences on genetic change. There are, however, exceptions. In dioecious species it may be necessary to thin to leave equal numbers of male and female plants, as soon as the sexes can be recognized. If plants are thinned, care must be taken to thin at random and not, for example, preferentially remove the late-germinating plants or impose any other selection pressure.
4. Ensure continued absence of all alien plants throughout the regeneration cycle. Initial plot preparation (section 7.4) should be sufficient to guarantee permanent absence of such plants from the plot itself. However, depending on the procedure chosen for eliminating alien pollen, there may be a need to continue control measures against alien plants in the vicinity.
5. Promote the growth of small plants and prune large plants as and when appropriate, to decrease variation between plants.

7.6.3 Verifying accession identity

While the plants are growing, accession identity should be verified based on the phenotypes of plants in the accession. The phenotype should be cross-checked against:

- taxonomic descriptions
- its recorded phenotype in the genebank documentation system
- reference material retained for this purpose, e.g. original herbarium specimens, or seeds or infructescences from the first regeneration, catalogued in the genebank documentation system
- photographs of reference material, as hard copy or as computer images catalogued within the genebank documentation system.

Future use of fingerprints will help confirm accession identity.

This should be combined with a policy decision on procedures to adopt when the whole accession, or individual plants within the accession, do not conform to documented characteristics. Although such policy decisions are beyond the

scope of this document, caution must be urged in relation to practices such as rogueing to preserve genetic integrity, or splitting as a tool to preserve rare variants and to simplify evaluation (see section 7.2.3).

In general, rogueing should be avoided unless it is absolutely clear that rogue plants are genuine aliens. For many species, especially outbreeders, rogue plants occur as rare extremes of the normal population distribution. Such plants should not be eliminated, as doing so would destroy rather than preserve genetic integrity. Where plants are grown in rows, plants growing off-row may be eliminated.

7.7 Crop management during anthesis

See flow chart 7.7. The general principles of crop management for high uniform yield should be continued during anthesis. In addition, anthesis introduces the following two new factors.

1. Meiosis and pollination are often particularly sensitive stages in plant development. Additional care may need to be exercised to avoid any stresses such as high temperature or drought.
2. Unless the species is an obligate apomict or obligate inbreeder, appropriate pollination controls will need to be implemented.

This section is concerned mainly with pollination control, of which there are four components:

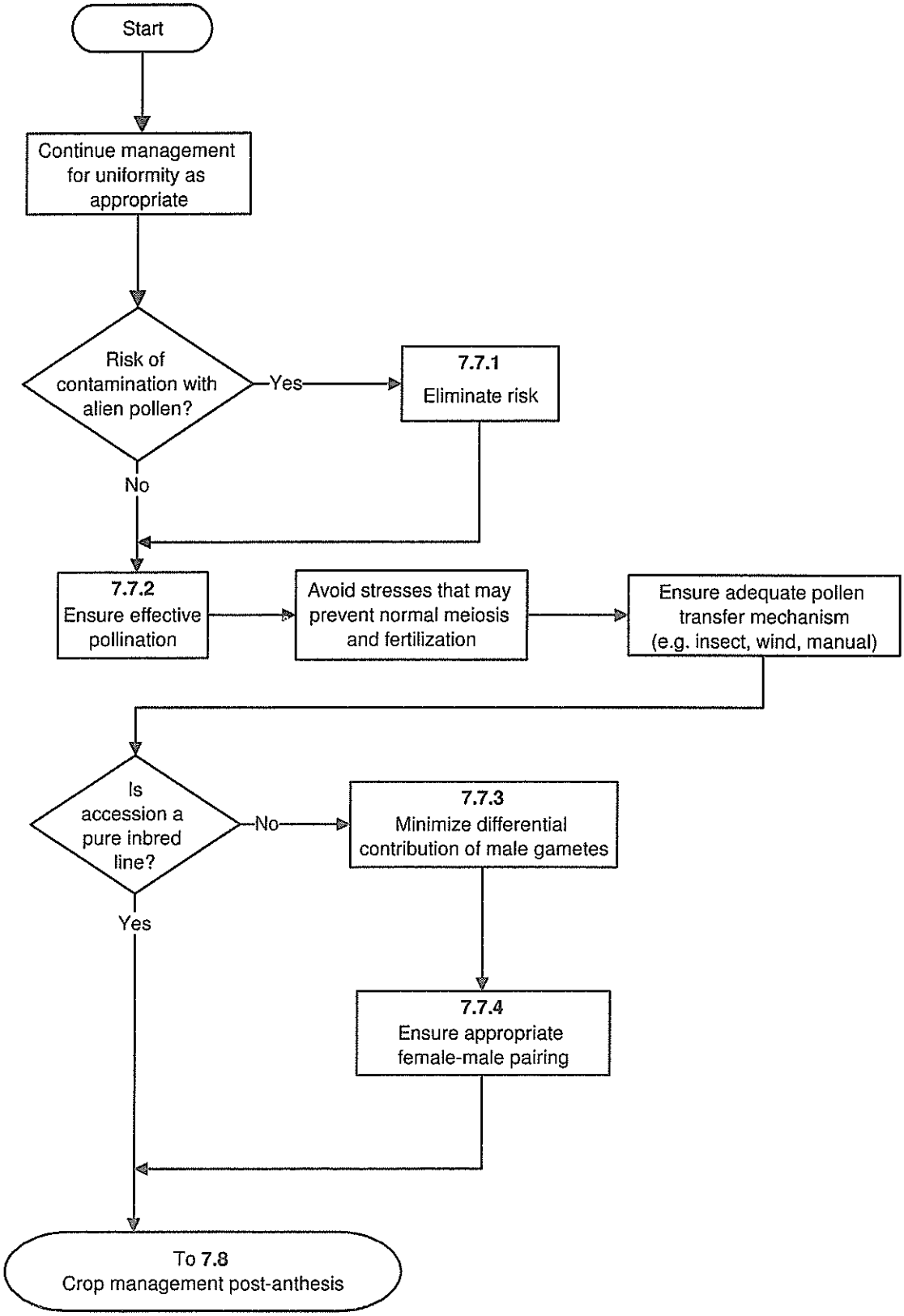
- eliminate pollination by alien pollen
- ensure effective pollination within the accession
- minimize differential contribution of male gametes to the offspring seed population
- ensure that pollination involves appropriate female-male combinations.

7.7.1 Elimination of alien pollen

Different options for eliminating alien pollen require implementation at different times during regeneration. The options available depend partly on the nature of the site selected for regeneration (section 7.1.3.2). The selected method of elimination may also require appropriate management before flowering (sections 7.4 and 7.6), possibly continuing throughout anthesis. For example, elimination of alien sources of pollen by regular clipping of nearby populations must be continued until the end of anthesis. Among the range of options available, some require action immediately prior to anthesis. These include:

- moving pots into a pollen-proof or pollinator-proof chamber for the duration of flowering
- erecting temporary pollen-proof or pollinator-proof nets or tents around the regeneration plot
- bagging selected flowers with pollen-proof or pollinator-proof bags.

In the case of manual pollination, extra care should be taken as the pollinator may serve as the carrier of alien pollen, in hand, clothing, pollinating implements, bags, etc.



Flow chart 7.7. Crop management during anthesis.

7.7.2 Ensuring effective pollination

Ensuring effective pollination within an accession requires good knowledge of the pollination biology of the species. The plants of an accession must produce a supply of viable pollen, and there must be an effective mechanism for pollen transfer from anther to stigma. In most species, ensuring a supply of viable pollen entails ensuring abundant flowering (section 7.1.2.1) and an absence of stresses that may prevent normal pollen development.

Additional care is required in species where some flowers or plants produce no pollen, as in the following cases.

- In monoecious species, there must be an appropriate proportion of male to female flowers. The proportion should be the same for all plants, but need not be 1:1; it should ideally equal the proportion required for full pollination of female flowers. Controlling this proportion requires a good understanding of the physiological basis of flower development.
- In dioecious species, it is necessary to ensure an appropriate proportion and distribution of male and female plants. In general, there should be equal numbers of males and females to avoid genetic changes at loci with different allele frequencies in males and females.
- Similarly, in populations that are polymorphic for male sterility there is a need to control the proportion and distribution of male sterile and fertile plants. To achieve adequate control it is usually necessary to understand the genetic control of male sterility.

In wind-pollinated species, effective pollen transfer requires sufficient wind to ensure random dispersal of pollen throughout the regeneration plot. In isolation chambers this will usually require a fan-driven air-circulation system.

In insect-pollinated species, effective transfer requires sufficiently large numbers of an appropriate pollinator during anthesis. The insect must be a species that preferentially visits flowers of the species, that effectively picks up pollen from anthers and deposits it on stigmata, and whose foraging behaviour promotes panmictic pollination within the accession. Controlling the species and number of pollinators is easiest when regeneration plots are contained within pollinator-proof cages.

In autogamous species, pollen transfer is often fully effective within flowers. In some species, pollination occurs only after a pollen-release mechanism is triggered, usually by an insect or other animal visiting the flower. This trigger may be operated manually in some species; otherwise there must be a supply of appropriate insects to operate the trigger. The proportion of self- and cross-pollination varies with species and with the availability of pollinators to effect cross-pollination. It is usually preferable to maintain the same proportion of outcrossing in regeneration plots as occurs naturally; to do this it may be necessary to ensure a supply of appropriate pollinators.

Where manual pollination is undertaken, the curator must devise an effective method for collecting sufficient quantities of uncontaminated pollen, which will depend on the floral morphology of the species. Depending on the chosen strategy for pairing plants (section 7.7.4), pollen from each plant may have to be kept separate or combined. The method for applying pollen to stigmata will also depend on the species.

7.7.3 Minimizing differential contribution of male gametes

For genetically variable accessions, the curator should attempt to minimize genetic changes that arise from differential contribution of male gametes to the offspring seed population. Exactly equal contributions from each parental plant

can be ensured by manual pollination, albeit at the expense of considerable skilled labour. If manual pollination is considered impractical, the curator should ensure that, as far as possible, all plants:

- produce an equal amount of pollen
- flower at the same time, or at least overlap sufficiently to ensure that appropriate pollen is released when ovules of other plants are receptive
- are positioned optimally for cross-pollination as appropriate.

Minimizing variation between plants in the time and total amount of pollen produced is achieved primarily through appropriate management before anthesis. As discussed in section 7.6, the objective is to reduce, if possible to zero, the environmental but not the genetic variance between plants.

If management prior to anthesis fails to achieve adequate uniformity of flower and pollen production, consider pruning flowers or inflorescences from the plants with most flowers, so that all plants are left with, as far as possible, the same number. This may be a continuation of a pruning programme started earlier (section 7.6), or may be the only pruning. It is most likely to be desirable for species with long flowering periods, which is most common in species with indeterminate growth.[5]

7.7.4 Ensuring appropriate female-male pairing

For accessions that are genetically variable and at least partially outbreeders, inappropriate female-male combinations lead to a loss of genetic integrity through the generation of recombinant genotypes in frequencies not representative of the original population. They arise in two situations as follows.

1. **Non-random mating where random mating is required.** This will change the relative frequencies of different recombinants. Examples include:
   - variation in flowering times within accession – each plant will be pollinated only by those plants that release pollen when its ovules are receptive, which could be only a small proportion of the total population.
   - low pollen dispersal distances – this can be a particular problem with insect-pollinated species. Although foraging patterns vary widely between insects, for many species insect flight-paths typically involve travelling to the nearest available flower. Cross-pollinations then occur primarily between adjacent plants, while plants at opposite corners of the regeneration plot may be rarely if ever crossed.

2. **Random mating where the original population was substructured with non-random mating.** This will change not only the relative frequencies of different recombinants but may even generate novel recombinants that were not present in the original. This is likely to be a problem only for wild species. It can be dealt with only if combined with an appropriate collecting strategy that takes population structure into account (section 7.3.3). **If regeneration is undertaken once without due regard for population structure, population structure is lost and no further measures can be taken.**

[5] Indeterminate growth: type of growth in which inflorescences develop only from lateral buds, while the shoot apex can continue indefinitely producing new lateral buds and therefore flowers. The result is an indefinitely long period of flowering and widely different maturation dates for seeds on different parts of a single plant.

Options providing different degrees of pollination control include the following.

1. Open pollination within accessions provides minimum control. It is usually applicable when random mating is required. There is a risk that there will be a preponderance of pollinations between plants that bear flowers adjacent in space or time. It may be necessary to take precautions to minimize this risk and maximize the randomness of cross-pollination.
   - Wind pollination – in enclosed isolation chambers, an active air-circulation system will usually be needed to achieve sufficient pollen exchange between plants. The spatial arrangement of plants in a regeneration plot may need to be adjusted in accordance with pollen dispersal characteristics of the species given the imposed air circulation characteristics over the regeneration plots.
   - Insect pollination – care must be taken to select insect species that are effective pollinators for the species and that have flight characteristics and pollination behaviour that maximizes the randomness of pollination. Such flight characteristics tend also to increase cross-pollination between accessions if regeneration plots are not totally isolated from each other. Therefore open-pollination by insects must usually be undertaken within enclosed isolation chambers. This necessitates additional pollinator management procedures. It must be possible to produce and maintain pollinator populations free of pollen outside the isolation chambers, introduce them in controlled numbers at appropriate density into the isolation chamber at anthesis, and maintain them as small viable effective populations within the isolation chamber for the duration of anthesis.

2. Manual pollination provides additional control, but at the cost of additional trained labour. It is important to ensure sufficient labour input, because if too few pollinations are undertaken there is a risk that the benefits of additional control may be outweighed by the disadvantage of obtaining too few seeds to maintain genetic integrity. Options include:
   - mass sibbing, gathering and mixing pollen from all plants in the accession and brushing the mixture onto stigmata of each plant
   - fully paired pollinations, i.e. cross-pollinating plants in all possible pairs
   - chain pollination, i.e. pollinating each plant with one other in a chain.

   The first option is the cheapest and fastest form of manual pollination, but provides the least control.

   The second option provides the most uniform possible contribution of pollen from each plant with the most uniform distribution of pollinations across receptor plants. A disadvantage is very large labour input. For example, with a population of 100 parents, there would be 4950 combinations of pairs for crossing excluding selfs and reciprocal crosses. In addition it may not be possible to undertake all paired crosses if there is too much variation in flowering time.

   The third option also provides uniform contribution of pollen from each plant but with much less effort than paired pollinations because of the smaller number of plant combinations used. Potential problems arising from wide variation in flowering date can be avoided by arranging the chain in order of the date of anthesis. The disadvantage is a less uniform distribution of pollinations across receptor plants than for paired pollinations.

3. A combination of open and manual pollination may be useful. For example, with insect-pollinated species, depending on insect behaviour in relation to plot structure, cross-pollination may be largely restricted to adjacent pairs of plants, while a plant at one end of the regeneration plot may rarely or never receive pollen from a plant at the other end. The same may apply to wind-pollinated species, depending on the pollen dispersal characteristics and air circulation patterns over the plot. In these cases it may be appropriate to augment natural pollination between adjacent plants, with specific additional hand-pollinations between plants at opposite ends of the plot.

4. For both open and manual pollination, sowing and planting at two or more different times within a regeneration plot can help promote uniform contribution of gametes by early and late-flowering genotypes, and enable hybridization between genotypes differing in flowering time.

Which of these options is selected depends partly on the species biology and partly on the choice of other options for regeneration and the success of previous management options (section 7.6) in securing uniform, healthy and vigorous populations.

7.8 Crop management post-anthesis

See flow chart 7.8. Objectives for crop management post anthesis are to:

- provide optimal conditions for uniform seed set and seed ripening in all plants
- maximize viability, vigour and 'storability' of seed
- maximize seed health.

Achievement of these objectives requires application of specific prior knowledge on the biological requirements of the species or accession.

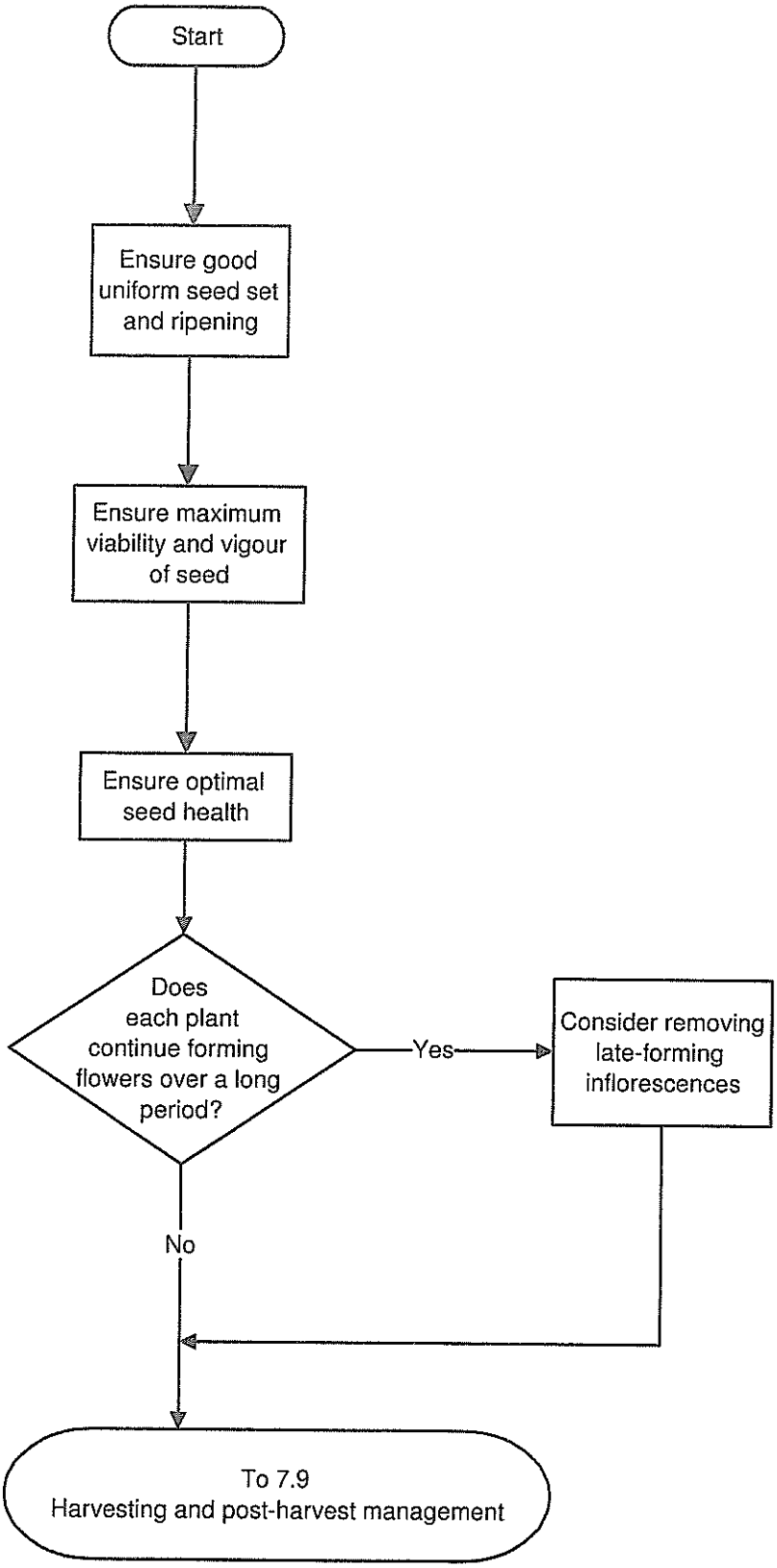
Particular attention must be paid to controlling pathogens and pests that reduce the quantity and quality of seed, and especially to preventing infection of progeny seed with seed-transmitted pathogens and pests.

For species with a long flowering period, especially those with indeterminate flowering, it may be desirable to prevent the formation or maturation of late inflorescences, for example by removing indeterminate shoot apices. The advantages are:

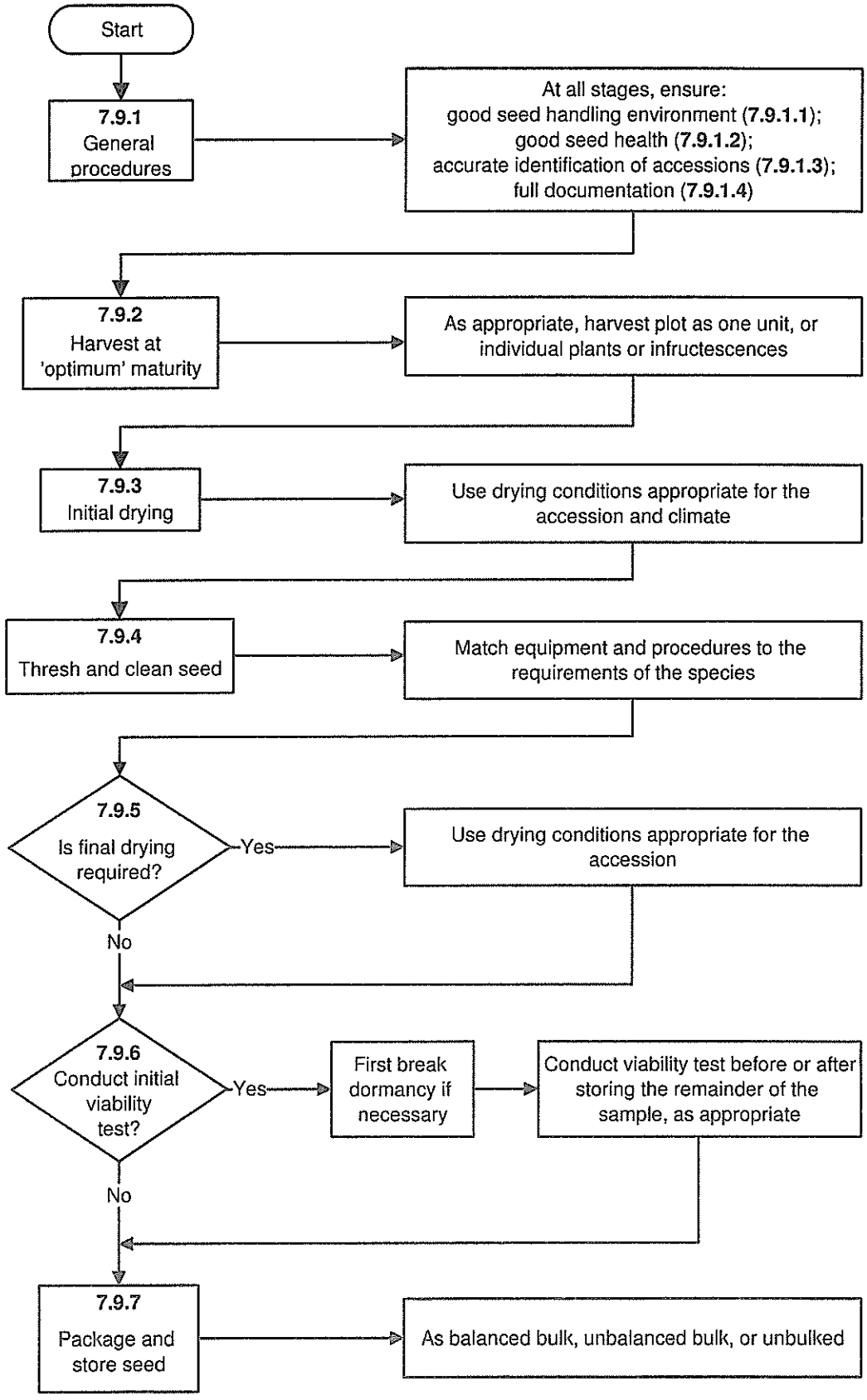
- reduced duration of pollen-control measures
- increased uniformity of ripening.

7.9 Harvesting and post-harvest management

- See flow chart 7.9. Procedures for harvesting and post-harvest management are again highly dependent on the biology and agronomy of the species concerned. The intention here is not to provide an exhaustive list of management options that may be found by reference to standard agronomic texts. Rather, it is to present the areas that need to be considered, and particularly to highlight those aspects for which regeneration requires management that differs from conventional crop management.



Flow chart 7.8. Crop management post-anthesis.



Flow chart 7.9. Harvesting and post-harvest management.

7.9.1 General procedures

7.9.1.1 Seed handling

A good seed-handling environment is desirable, preferably in a room dedicated to seed handling. At all stages strict attention must be paid to cleanliness, to:

- ensure clean, high-quality seed
- avoid admixing seed from different accessions, different plants, or other sources
- protect the health and safety of people handling the seed.

The seed-handling room should have the following characteristics:

- good lighting for close and detailed observations of samples
- smooth flat work area, easily cleanable and with no crevices where seed could become lodged
- draught-proof with limited access
- access to all necessary equipment such as sieves, forceps, lens
- controlled temperature and humidity where possible
- earthed to prevent build-up of static electricity
- controlled dust and particulates.

Equipment, whether for manual or mechanical seed handling, must be suitable for producing a sample that contains seed only, with no chaff, pieces of rachis, dead greenfly, dust, etc. The aim should be to produce 'standard seed' quality by setting equipment (e.g. column blower, sieves) to a predetermined standard suitable for the accession.

Machinery, tools such as screens and sieves, and work surfaces must be cleaned between each seed lot to avoid contamination. Particular attention must be paid to difficult areas, such as inside machinery.

Packets or other containers for seed should be secure, and of appropriate construction. They must at all times be labelled with the ID for the accession, and date and origin details, such as location and ID of the regeneration plot and an inventory lot code. Records should be maintained of the progress of seed through the regeneration system. Seed-handling operations must include procedures to eliminate errors in identifying accessions.

Some seed-handling procedures depend on the final method of packaging seed for storage (see section 7.9.7 for a discussion of preferred packaging methodology in relation to species biology and genebank policy).

- If seed of an accession is to be bulked for storage in a single container, it may be bulked at any stage from harvesting onwards. The curator should ensure that the stage chosen for bulking is appropriate and efficient. For the purposes of handling, packaging and labelling, the seed of one accession is treated as a single seed lot from the moment of bulking.
- If a balanced bulk is to be taken for storage in a single container, seed produced by each plant of an accession must be kept separate throughout harvesting and post-harvest procedures until the seed is ready for the balanced bulk to be made. That is, the seed of different plants should be treated as distinct seed lots, even if they belong to the same accession:
  - each plant must be harvested individually
  - separate labelled containers should be used for the seed produced by each plant
  - the seed produced by each plant must be handled separately
  - the documentation system must be extended to provide for individually labelling and tracking progress with each seed lot

* procedures for cleanliness should be extended to include avoiding contamination with seed produced by other plants of the same accession.

Separate handling of the seed of each plant stops only at the point of having clean dry seed that can be weighed or counted for creating the balanced bulk.

- If the seeds produced by each plant of an accession are to kept in separate containers in storage, then procedures should be largely the same as for balanced bulks. The main exception is that the final stage of forming a balanced bulk and preparing a single storage container is replaced with preparing a separate storage container for the seed of each plant.

7.9.1.2 Seed health

At all stages, good seed health must be ensured. Particular attention must be paid to storage pests and seedborne pathogens including viruses, because of the consequences of infection on the longevity of the seeds in storage and the genetic implications of possible genetic variation in resistance within the population. Specific diagnostic tests may need to be applied or developed where there are no readily visible symptoms of infection. For internally seedborne pathogens, specific procedures may need to be applied or developed to derive healthy seed from the affected seed lots.

Appropriate preventative techniques should be applied. For example, individual seed heads should be scrutinized for infected seed before threshing to avoid contaminating equipment. All equipment should be cleaned between seed lots. Known diseased seed lots should be isolated from non-diseased lots. Insects should be excluded. Bags should be kept off the ground or floor of the drying area if it presents a health risk. If possible, air should be filtered to keep out pests and pathogens.

In most species, humidity has a major impact on several pathogens such as mildews. Seed should be harvested in dry weather and stored in a clean dry atmosphere with some form of natural or forced air circulation. Heads must be dried in porous containers such as paper bags or muslin bags, not in waterproof containers such as plastic bags. Conditions should be maintained uniformly dry. Bags should be kept spaced well apart to allow dry air to circulate within and between them.

Fumigants and pesticides should be used if necessary, but with caution and only as a last resort. In general, they should not be used unless they are known to have no adverse effect on seed quality and longevity in storage, which requires good prior knowledge of the fumigant or pesticide. If they are used, appropriate health and safety practices must be followed to protect all persons required to handle, ship or dispose of the seeds.

7.9.1.3 Accession identity

Post-harvest management involves a considerable amount of seed handling and transport, with a correspondingly high risk of misidentifying seed samples at some stage in the absence of appropriate countermeasures. To avoid misidentification, the curator should establish seed-handling procedures that include:

- a protocol for double-checking identity at every stage

- wherever possible, cross-checking readily visible and distinctive characteristics (e.g. size, characteristic markings on seed coat or infructescence, etc.) against:
    - its recorded phenotype in the genebank documentation system
    - reference material retained for this purpose, e.g. seeds or infructescences from the first regeneration, and/or
    - photographs of reference material.

7.9.1.4 Information management

The entire regeneration history of all accessions should be recorded in the genebank documentation system. This history starts when the accession enters the genebank and is a record of seed movement as well as a biological record. All aspects of the regeneration history should be noted and the record updated at each event. Relevant data include:

- upon arrival of an accession into genebank
    - * date, donor, species, number of seed or plants or seed weight
    - * packet number, location in genebank
- regeneration required
    - * reason for regenerating
    - * how many seed germinated for regeneration
    - * how many plants used for regeneration
- regeneration details
    - * location, date, pot size
    - * management procedures
    - * pollination control procedures
    - * dates of peak anthesis, harvest, threshing, germination test and results etc.
    - * identity verification
    - * numbers of plants harvested
    - * quantity and quality of seed produced.

Computerized preparation of labels, bags, etc. is recommended as part of quality assurance and the minimization of misidentification.

7.9.2 Harvesting

Harvesting should be done at 'optimum' maturity. Optimum here means:

- with as many ripe seed per head as possible
- after seed cease to be sensitive to desiccation
- at or just prior to seed reaching physiological maturity to ensure it has not passed the threshold and entered the phase of declining quality, and
- before natural seed dispersal by fruit shattering, etc.

Unless the regeneration plots are in well-controlled environments, it may also be necessary to take into account other possible causes of loss, e.g. harvest during appropriately dry weather, before excessive losses to bird and other pests, and before excessive damage by bad weather.

Application of these principles requires knowledge of agronomy of the species and factors such as the number of days from peak anthesis to seed ripening and its dependence on weather conditions, genotype, etc.

Bags to hold harvested seed heads should be suitable for further ripening/drying, i.e. porous material such as brown paper or cotton enabling

good air circulation. They must also be appropriately labelled and secure for transport to drying/threshing area without loss or mixing of seed.

The harvested unit must be suitable for the subsequent threshing method, e.g. for hand-threshing, harvest the peduncle as well as the infructescence to provide a handle.

Options for harvesting depend partly on intended storage options (section 7.9.7). They include the following.

- Bulk harvest the whole plot by machine
  It is necessary to use purpose-built machinery for adequate cleanliness. The insides of commercial agricultural machinery cannot be cleaned adequately between regeneration plots. This option is feasible only if the intention is to form an unbalanced bulk of all seed for storage.
- Harvest plants individually, by machine or by hand
- Harvest infructescences individually, by hand
  For controlled pollinations with bagged inflorescences, there is an option to leave the bag in place until harvest, although this requires caution in relation to infestations of pathogens or pests inside the bag.

To prevent rapid seed deterioration, it is important to avoid delays in seed processing after harvesting. Appropriate seed drying should be initiated as soon as possible. If seed cannot be processed quickly, they should at least be placed in temporary holding areas where the drying process can begin.

7.9.3 Initial drying

Drying seeds under good drying conditions is a critical step in the process of obtaining seed of high quality (FAO/IPGRI 1994). Seed should generally be dried as soon as possible after harvest.

Drying should be a two-stage process. The initial drying stage aims to dry material to a moisture content low enough for effective threshing, but not so low that threshing damages the seed. Optimal seed moisture content for threshing is generally higher than that for final storage, so that for many species a second stage of drying after threshing is needed. For most species, slow drying is preferable to fast. Bags should be packed as loosely as possible to maximize air circulation.

Appropriate measures must be taken to ensure protection against storage pests including rodents and insects. These can include keeping seed off the ground and regular inspections; apply insecticides and other pesticides only with caution and only when other control measures fail (see section 7.9.1.2).

Options for drying include:

- outside in shade if the climate is suitable
- in dry glasshouse or shadehouse
- inside
  - passive drying in a seed drying room with a rack system to store seed widely spaced and good ventilation and air circulation;
  - active drying in a dehumidified chamber with temperature and humidity control.

The method chosen will depend on the local climatic conditions, available equipment, the seed characteristics of the species, the number and size of samples to be dried, and cost considerations. Drying outside may require additional control measures, e.g. against birds, insects and dew. In many cases the climate is too humid for drying outside or in a glasshouse or shadehouse. In the moist tropics seeds deteriorate rapidly in the high temperatures and high

relative humidities that occur outside and in glasshouses and shadehouses. In such cases it is essential to control both temperature and humidity. The preferred standard is to dry at 10-25°C and 10-15% relative humidity (FAO/IPGRI 1994).

7.9.4 Threshing and cleaning

The aim is to obtain clean, high-quality seed from each plant. Avoid threshing overdried seed and causing consequent seed damage.

Seed may be threshed manually or mechanically. Mechanical threshing is suitable only for 'robust' species. Only special-purpose equipment that can be fully cleaned between accessions should be used. Manual threshing is necessary for 'delicate' species.

Matching appropriate threshing and cleaning techniques to the seed and fruit characteristics of the species is a critical step in obtaining a high percentage of high-quality seed, although numerous other factors also can lead to low germination rates (section 5.1). Many species abort a large proportion of their seed, especially when growing in suboptimal conditions. This is the case in most non-agricultural situations. Inappropriate machinery or incorrectly set machinery may fail to eliminate aborted seed from the sample. The resulting seed sample will have a low percentage germination rate. This is commonly attributed as a characteristic of wild species, but may more often reflect inadequate knowledge of optimal cleaning procedures for the species.

Some species, e.g. *Calendula*, are polymorphic for seed morphology and size. Specialized cleaning procedures may be required to extract the full range of seed types of such species, including several passes through seed-cleaning equipment adjusted to a different setting at each pass.

7.9.5 Final drying

The preferred option for final drying depends on factors such as seed characteristics and intended storage temperature, which determine the optimal drying rate and target moisture content (Vertucci-Walters and Roos 1990), and climate, which determines the relative efficacy of the options in achieving the desired target. Drying to very low moisture contents is recommended for some species to improve their longevity in storage; however, it damages other species, and with these species overdrying must be avoided. Even for species whose seed longevity is increased at very low moisture content, the dried seed can be brittle and easily damaged. Increased care with seed handling is therefore necessary after the drying process is complete.

Options for drying include:

- drying in ambient conditions
  - outside in shade
  - in dry glasshouse or shadehouse
  - inside a seed-drying room
- drying in artificially dehumidified conditions
  - with self-indicating silica gel in small boxes
  - in humidity-controlled room
  - active, fan-assisted drying (to be avoided when there is a risk of drying too fast for maintenance of high viability).

Drying in ambient conditions is not feasible in most climates. Cost and reliability of artificial dehumidification may be important criteria. For example, silica gel is considerably cheaper than active dehumidifiers powered by

electricity, petrol, etc. It is also highly effective in achieving very low moisture contents, and so is suitable for species where this is appropriate. In addition, it is not dependent on a possibly erratic electricity supply nor is it subject to equipment failure, and is therefore reliable under all conditions.

Procedures for determining when the drying process is complete depend on knowledge of the species and experience with the drying system. To gain the required knowledge it may be necessary to test seed moisture content frequently during drying until final moisture content is attained. An experienced curator will be able to judge drying speed sufficiently to reduce frequency of testing to a minimum. Self-indicating silica gel has the advantage that its colour change on absorbing moisture can itself be sufficient to indicate when seed has dried sufficiently.

7.9.6 Initial viability testing

Ideally, germination rate should be tested after drying. Provided the test can be completed quickly, it should usually be conducted before storing seed. Depending on seed characteristics, seed may need careful rehydration before the germination test to avoid damage. For species with dormant seed, treatments to break dormancy should be applied only to the seed to be used for testing, not to the seed to be stored. If dormancy cannot be broken quickly (e.g. if several weeks of vernalization are required or a long period of after-ripening), seed should be stored immediately after drying, without waiting for completion of the test.

Genebank policy may provide for strategic testing of a representative sample of accessions. This should be in accordance with overall genebank policy for monitoring viability (section 7.2.1.1). The need for initial testing is greatest for accessions for which:

- prior knowledge on viability is poor
- initial viability is likely to be low
- there is a possibility of dormancy that needs to be checked.

7.9.7 Seed packaging and storage

After seed has been fully cleaned, dried, treated and tested, it is ready for storage. The location for storage (base, active or safety duplicate) will already have been determined (section 7.2). This section is concerned only with how seed is to be held in the predetermined location.

Options are as follows:

1. bulk all seed for storage in one container
2. form a balanced bulk by taking an aliquot of seed from each mother plant, and store in one container
3. use a separate container for the progeny seed of each plant.

Option 1 is preferred for all collection types if genetic variation within populations is low or zero, e.g. inbred lines and obligate inbreeders. It may also be used for variable accessions if variation between plants has been successfully masked by pruning (section 7.6).

Option 2 is preferred, at least for the active collection, if genetic variation within populations is high and has not been masked by pruning or other techniques to achieve balanced samples. It may also be used for variable populations in the base and safety duplicate collections if it is considered unnecessary to exercise the greater control over genetic change enabled by option 3. If possible, the size of the aliquot should equal the amount of seed produced by the plant yielding fewest seed. However, this is not possible if

some plants produce no seed or so few seed that using it as the aliquot would result in an unacceptably small seed sample. Option 2 reduces to zero both random and selective genetic changes arising from unequal contribution of female gametes, except where some plants produce fewer seed than the predefined aliquot. However, it has no effect on variation in pollen production and so in general is less effective than masking variation by pruning. Any effect it has on genetic changes arising from unequal contribution of male gametes will depend on options selected for control of pollination.

Option 3 should be considered, for the base and safety duplicate collections only, as a means of improving the maintenance of genetic integrity by improving the choice of seed for the next cycle of regeneration and possibly also by improving the control of pollination. If this option is selected for the base and safety duplicates, it will usually be combined with option 2 for the active collection. This option is a necessary part of a larger strategy for control of genetic integrity; thus the choice between options 2 and 3 will already have been made (section 7.3.3).

Seed to be stored in a single container should be thoroughly mixed. This will ensure that seed subsequently taken out for testing, distribution or regeneration will be random subsamples. They may then be placed in the containers in which they are to be held in the selected storage conditions. Full descriptions of the options for containers and storage conditions are given in Hanson (1985), Cromarty *et al.* (1990) and Genebank Standards (FAO/IPGRI 1994). Upon storage and completion of final documentation (section 7.9.1.3), the regeneration procedure is complete.

8 Concluding remarks

The decision guide presented here is only one step towards improving the effectiveness of germplasm regeneration programmes. In the longer term, two particular areas of development are envisaged to achieve further improvements. First, based on this decision guide, it should be possible to develop more prescriptive guidelines for individual crops or groups of crops, probably through the activities of the international crop PGR networks. Second, a large amount of research is required to gain the crop-specific knowledge necessary to optimize regeneration protocols, and to quantify the consequences (particularly population genetic and economic consequences) of the various options presented.

Nevertheless, despite the limited knowledge of many species, it is hoped that curators will immediately be able to use this decision guide to help establish or improve regeneration programmes specifically tailored to their own requirements and priorities.

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10 Glossary

Apomixis. An asexual breeding system involving seed production without meiosis and without fertilization. Obligate apomicts necessarily reproduce by apomixis. Facultative apomicts may reproduce by apomixis or sexually.

Balanced / unbalanced bulk. An unbalanced bulk is formed by bulking all the seed produced by a population of plants, thereby creating a single seed sample. Each plant in the parental generation is then represented in the offspring generation in proportion to the number of seed it produces. A balanced bulk is formed by taking an aliquot of seed from each mother plant and combining them into a single seed lot. Each plant in the parental generation is then equally represented in the offspring generation, at least in terms of seed production (not necessarily in terms of pollen contribution).

Biological duplicates. See Duplicate accessions.

Convergent selection pressures. Selection pressures acting to decrease differences between two or more populations that are initially distinct. Occurs when there is a single optimal (i.e. with highest evolutionary fitness) genotype, so that all populations evolve towards that genotype as a common end-point regardless of their initial genotypic composition. Usually used in the context of natural selection, the concept is equally applicable to artificial selection.

Determinate / indeterminate flowering.

Determinate flowering: an inflorescence arises at the apex of a shoot through differentiation of the apical meristem into a floral structure. The apical meristem thereby loses its functionality as a meristem, and further development of the shoot ceases with maturation of the inflorescence. The result tends to be relatively short flowering periods and uniform seed ripening.

Indeterminate flowering: inflorescences develop only from lateral buds, whereas the apical meristem retains its meristematic activity indefinitely. Thus new lateral buds, and therefore new inflorescences, can be produced repeatedly and indefinitely as the shoot grows. The result is generally a protracted period of flowering and widely different maturation dates for different seeds on a single plant.

Drift. an evolutionary phenomenon describing random changes in the genetic composition of a population caused by chance factors, such as sampling error and the effects of uncontrolled microenvironmental variation on growth, survival and reproduction. (compare Selection)

Duplicate accessions. Accessions held by one or more genebanks that are derived from the same original seed sample without deliberate selection and so are in some sense duplicates of each other. As a minimum, distinguish between **historical duplicates** and **biological duplicates**. Use of passport data to demonstrate that two accessions are derived from the

same original seed without deliberate selection is sufficient to demonstrate that they are historically duplicate, but in many cases such historical duplicates are biologically distinct from each other because of differential loss of genetic integrity during storage, seed exchange, and regeneration in different environments. Genetic characterization is necessary to determine whether they are also biologically duplicate. Van Hintum and Knüpffer (1995) introduce a more comprehensive terminology distinguishing different degrees of similarity.

Historical duplicates. See Duplicate accessions.

Inbreeder / Outbreeder. Obligate inbreeders are always self-pollinated. Obligate outbreeders cannot naturally self-pollinate and can be fertilized only by pollen from other plants. These are two extremes of a continuum: most species are intermediate, showing a greater or lesser tendency to self- or cross-pollinate.

Indeterminate flowering. See Determinate flowering.

Introgression. The repeated pollination of plants in one population with pollen from another population. Usually applied when the two populations are genetically distinct.

Joint frequency distribution (of alleles at multiple loci). The frequency distribution of all **combinations** of alleles at all loci. It is possible for the joint frequency distribution to change even if the frequency distribution of alleles remains constant at all loci.

Outbreeder. See Inbreeder.

Pleiotropy. Refers to multiple phenotypic effects of a single gene.

Rogueing. Manually removing atypical plants from a stand.

Selection. The process that results in non-random changes in the genetic composition of a population (compare Drift). These occur whenever genetic variation within a population is expressed as phenotypic variation among plants for any component of evolutionary fitness; that is, the offspring generation systematically contains more of certain selected gene combinations and less of others.

Artificial selection is selection deliberately imposed by man.

Natural selection is the process resulting in directed evolutionary change. It occurs not only in natural populations but also in artificial situations such as regeneration plots, for example by differential reactions of plants to the regeneration environment. It may include unconscious selection.

Quality threshold. See Thresholds for regeneration.

Quantity threshold. See Thresholds for regeneration.

Thresholds for regeneration. Minimum standards of a sample, below which it must be regenerated to produce a new seed sample of higher standard. Two thresholds must be defined: quality threshold defining the minimum standard for seed quality, and quantity threshold defining the minimum standard for seed quantity.

Transgressive segregation. A form of inheritance of continuous variation under polygenic control where, by recombination and segregation of genes at different loci affecting the same continuously variable character, the offspring generation may contain genotypes that are more extreme than the most extreme parental genotype.

Unbalanced bulk. See Balanced bulk.