Appendix III. Guidelines for the regeneration of accessions in seed collections of the main perennial forage grasses and legumes of temperate grasslands

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1. Introduction

"Timely regeneration must be a priority activity of all genebanks" (FAO 1996). The optimal protocol for regeneration depends on numerous factors, including 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, and operational constraints on genebank activities, such as funds, labour and equipment. It is therefore not possible to lay out a single uniquely optimal protocol. Rather, genebank-specific and even accession-specific decisions have to be made to establish the optimal protocol. In many cases there is not sufficient knowledge on which to determine the optimal solution; it is then necessary to make some pragmatic choices in the short term while undertaking research to enable further improvements in the long term.

A generalized decision guide (Sackville Hamilton and Chorlton 1997) provides help in the decision-making process. However, the choices are complex and multifaceted. It is necessary to progress beyond a general decision guide by providing more specific, prescriptive regeneration guidelines for particular species. This will improve conformity among genebanks by eliminating some of the need for decision-making by individual curators.

This document provides such prescriptive guidelines for the main perennial forage grasses and legumes of temperate grasslands. It is based on the principles presented in Sackville Hamilton and Chorlton (1997), which should be referred to for detailed discussion of the issues underlying the decisions presented here.

2. Background and assumptions

2.1 Taxonomic scope and characteristics

No attempt is made in these guidelines to cover all forage species, because they encompass too wide a range of life cycle characteristics. Taxa covered include those with the following characteristics:

1. Seed are small and sown at high density (typically about $400/m^2$ for dominant grasses, to $40/m^2$ for legumes and other minority components of seed mixtures). The resulting need for large numbers of seed generally rules out manual pollination as a tool for improving maintenance of genetic integrity.
2. Seed are long-lived, with good long-term survival in storage and with relatively well-known storage, dormancy and germination requirements. Seed survival characteristics have not been quantitatively determined as they have for some other species; nevertheless it is clear qualitatively that they are "easy" species for storage. IPGRI-preferred standards for storage and viability are therefore appropriate, there is a high degree of certainty over decisions, and relatively low priority attaches to additional research to improve knowledge of seed characteristics.
3. The species are self-incompatible outbreeders, so that
    a) each accession must be maintained as an interbreeding population
    b) there is a high risk of contamination with alien pollen if appropriate control measures are not taken
    c) genetic variation within populations is high.
4. The species are perennial, able to propagate vegetatively and with an indeterminate growth habit. Therefore there is potentially extremely high variation in fecundity between plants – some plants may produce zero seed, while the majority of the seed produced by a population may be produced by a small proportion of the plants in the population. The combination of this high variance in fecundity with high genetic variance within populations results in an exceptionally high potential for genetic

change during regeneration, even where contamination with alien genes is totally excluded.

5. Many of the species are native and naturally common in the areas where they are most used agriculturally. Sown populations readily become feral, persisting as naturalized populations, spreading out from their original location and introgressing with native populations. Native and naturalized populations may be abundant in paths, verges, fallow land, in the weed flora around experimental plots, and the seed bank in the soil. As such, wherever the species are used commercially or experimentally, there is a high risk of contamination with alien plants, seed or pollen from natural and naturalized populations.

In summary, the species covered by these guidelines present no particular problem in terms of seed storage, but in terms of the maintenance of genetic integrity they are probably the most difficult of all crop groups. The guidelines reflect this by attaching exceptionally high priority to limiting the loss of genetic integrity. Pending further research on alternative methodologies for the improved maintenance of genetic integrity, the guidelines are subject to future revision.

Both wind-pollinated (grasses) and insect-pollinated (legumes) species are covered. These require different protocols for pollination and the prevention of contamination with alien pollen, but otherwise are similar.

Categories of grassland species not covered by these guidelines include:

- inbreeders (mainly the annual species)
- apomicts (such as some *Poa* spp. and many tropical grasses)
- medium- to large-seeded species (including many tropical legumes)
- those with poorly known seed characteristics (including many nonagricultural species).

2.2 Types of collection

The following is assumed in relation to storage conditions:

1. Accessions are maintained in an **active collection** optimized for utilization rather than conservation, and maintained at 0 to 4ºC with 3-7% seed moisture content.
2. A sample of every accession is also held in a **base collection** maintained for conservation, under optimal conditions for long-term storage ("-18ºC or cooler with 3-7% seed moisture content": FAO/IPGRI Genebank Standards 1994) and with genetic integrity as far as possible intact. Seed in the base collection is not used for distribution. The preferred standard for regeneration purposes is to maintain the base collection at the same site as the active. It is acceptable to maintain the base collection at a distant site, although this makes it more difficult to achieve the preferred standard that all samples should usually be regenerated from the base collection (FAO/IPGRI Genebank standards 1994; see also section 3.3).
3. A duplicate sample of every accession is maintained in a **safety-duplicate collection**, also held under optimal conditions but at a distant site from the base collection. Seed in the safety-duplicate collection is not used for any purpose other than replacing accessions that have been accidentally lost from the base collection.

2.3 Units of seed usage

Definition of the fundamental units of seed usage is prerequisite to efficient genebank operation. The three fundamental units are as follows:

1. The **distribution unit** is the mean number of seed distributed with each request. This mean number may be varied in accordance with users' requirements and seed availability. Preferred standard: **mean 250 seeds; range 10-5000 seeds**.

2. The **test unit** is the number of seed required to test seed quality and viability. Preferred standard: **100 seeds**.
3. The **base unit** for regeneration is the number of seed needed to ensure the successful regeneration of a representative sample of the original accession, with genetic integrity maintained as far as possible intact and of sufficient size to meet future demands. The size of the base unit must make full allowance for all possible seed losses during regeneration and storage. Table 1 presents calculations for the preferred and acceptable base unit size.

2.4 Targets for seed production during regeneration

i. Seed quality

New seed produced for storage should as far as possible be free of any pathogen or pest, especially of storage pests and seedborne pathogens, and have ≥ 95% germination rate.

ii. Seed quantity

The target number of seed to be produced depends on whether the regeneration is for the active, base and/or safety-duplicate collections. Targets for number of seed to be stored in the active and base collection are given in Table 2. The target for storage in the safety-duplicate collection is one base unit, i.e. **800** seed preferred, **240** acceptable (Table 1).

iii. Genetic integrity

Genetic integrity deteriorates through two principal routes: (a) contamination with alien genes, and (b) other changes in genotypic composition that occur by random drift and by nonrandom selection even in the absence of contamination by alien genes. Standards for the former are given in Table 3.

Zero change in genotypic composition by drift or selection is not an achievable target. However, it is considered inappropriate to set quantitative targets. We merely set the qualitative target of minimizing changes as far as feasible within the constraints of available funding and infrastructure.

As outbreeders, each accession typically contains high levels of genetic variation among its component plants for many characteristics. Moreover, as perennials with the ability to propagate vegetatively and with an indeterminate growth habit, there is potentially extremely high variation in fecundity between plants. At one extreme, some plants may allocate all resources to vegetative propagation and so produce zero seed. At the other extreme, because of the indeterminate growth habit, some plants may attain a large size and then produce a large number of inflorescences. Typically, most of the seed produced by a population is therefore derived from a small proportion of the plants in the population, while most plants contribute little or nothing. As a result, the potential for degradation of genetic integrity through both drift and selection is exceptionally high in these species. Exceptionally high priority is therefore attached to measures that reduce such changes.

3. Regeneration protocol

The regeneration protocol outlined here highlights 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. It is assumed that the genebank has background knowledge of general agronomic requirements of the species.

3.1 Selection of location for regeneration

The location selected for regeneration should have the characteristics outlined in Table 4.

Quarantine regulations may also influence the choice of location for regenerating seed from newly imported seed or plants. It may be necessary or preferable to regenerate within quarantine facilities.

Table 1. Preferred and acceptable sizes of a base unit

	Preferred standard	Acceptable standard
Number of parent plants to be used for regeneration	100 plants	30 plants †
Safety factors, guarding against:		
Germination rate < 100%	2	2
Probability of crop failure > 0%	2	2
Other seed losses > 0%	2	2
Total base unit size	**800 seeds**	**240 seeds**

† The figure of 30 should be used with caution. It is lower than usually regarded as acceptable. In part it reflects the higher priority attached here to minimizing selection and contamination than to minimizing drift, and the resulting need for increased effort per parent plant. It is most acceptable for small original samples (e.g. of material collected vegetatively from pasture). It is not acceptable unless the protocol adopts preferred standards in relation to other measures for minimizing selection and contamination, such as regenerating inside isolation chambers. If these other preferred standards are not met, the acceptable standard should be increased to 50 plants.

Table 2. Preferred and acceptable targets for the number of seeds to be stored

(a) in the active collection

Use	Basis of calculation	Preferred standard	Acceptable standard
Viability monitoring	Expected number of tests	5	3
	• test unit size	100	100
	= number of seed required	**500**	**300**
Regeneration	0 if regenerating from base	0	**240**
	1 base unit if regenerating from active		
Seed distribution	Expected number of requests †	10	5
	• uncertainty factor ‡	5	3
	• distribution unit size	250	100
	= number of seed required	**12,500**	**1,500**
Target number of seeds for storage in active collection after regeneration		13,000	2,040

(b) in the base collection

Use	Basis of calculation	Preferred standard	Minimum standard
Viability monitoring	Expected number of tests	20	5
	• test unit size	100	100
	= number of seed required	**2,000**	**500**
Regeneration			
Replenishment of stocks in base collection	1 base unit	800	240
Replenishment of stocks in safety-duplicate collection	1 base unit	800	240
Replenishment of stocks in active collection	Expected number of times	5 §	1 ¶
	• uncertainty factor ‡	4	4
	• base unit size	800	240
	= number of seed required	**16,000**	**960**
Target number of seeds for storage in base collection after regeneration		19,600	1,940

† Standards cannot be set for expected number of requests: determining appropriate values for any genebank is the sole responsibility of the curator. However, it is necessary to enter values here in order

to establish appropriate values for target seed quantities. The values entered are intended to represent approximate figures in the range likely to be adopted by most genebanks.

‡ The uncertainty factor is a factor allowing for uncertainty of usage of seed in relation to the relative costs of producing more or fewer seed than are actually used. See Sackville Hamilton and Chorlton (1997).

§ Assuming adherence to the preferred standard (section 3.3), that samples in the active collection are always regenerated from the base collection.

¶ Assuming adherence to the acceptable standard (section 3.3), that samples in the active collection are regenerated from remnant seed in the active collection for up to three cycles before reverting to the base collection.

Table 3. Preferred and acceptable targets for contamination of accessions with alien genes

Cause of contamination	Preferred standard	Acceptable standard †‡
Misidentification of accessions caused by incorrect juxtaposition of plants and labels at any step during regeneration	0%	0.001%
Contamination with alien plants or seed from any source (other accessions, previous crops, wild or feral populations, seed bank) at any stage (seed preparation, seed-bed preparation, sowing, crop growth, harvesting, all post-harvest seed handling through to seed storage).	0%	0.01%
Contamination with pollen from any alien source (other accessions being regenerated nearby, or crops, wild or naturalized populations in the vicinity) at any stage.	0%	0.1%

† Although values are given for acceptable standards, high priority should be attached to achieving the preferred target instead, because of the detrimental consequences of lower standards in terms of loss of diversity in the collection (Sackville Hamilton and Chorlton 1997).

‡ The differences in values set as acceptable for different causes of contamination reflect the different costs and difficulty of prevention.

3.2 Selection of accessions

An accession needs to be regenerated when it falls below predefined threshold levels for quantity or quality. Thresholds are given in Table 5 for accessions already in storage, and in Table 6 for new material not yet entered into the collection.

Every effort should be made to ensure that enough seed is kept in the base collection to cover all usage, so that they should need to be regenerated only when they deteriorate in quality and never for inadequate quantity. Although Table 5 includes threshold quantity for seed in the base collection, falling below this threshold is regarded as a failure of the regeneration protocol.

Selection of accessions for regeneration involves the following steps:

i. Construct preliminary list of samples that may fall below threshold
ii. Determine which of these are actually in need of regeneration
iii. If necessary, prioritize accessions for regeneration
iv. Select regeneration protocol appropriate to accession status
v. In the event of problems, consider refining future regeneration protocol.

Table 4. Preferred and acceptable standards for the characteristics of the location used for regeneration

Location characteristic	Preferred standard	Acceptable standard
Latitude	Within 5° of site of origin	Within 10° of site of origin
Altitude	Within 300 m of site of origin	Within 500 m of site of origin
Soil	High fertility, permanently moist but well-drained, pH 5-7.5 depending on species	High fertility, permanently moist but well-drained, pH 5-8 depending on species
Method for elimination of alien pollination (grasses)	Plants contained within 100% pollen-proof isolation chambers, at least for the duration of anthesis	Outside, in sheltered site, surrounded by tall crop of densely packed plants ≥2 m high, ≥20 m thick, and with its edge ≤1 m from edge of regeneration plot, ≥50 m from nearest alien pollen source (other regeneration plot, crop, feral population, etc.) (increase distance from alien pollen if quality of barrier crop is reduced)
Method for elimination of alien pollination (legumes)	Plants contained within 100% pollinator-proof isolation chambers, at least for the duration of anthesis	Outside, in sheltered site, surrounded by ≥50 m thick crop with dense canopy of flowers of similar colour, morphology and scent to accessions, preferably conspecific male-sterile ≥50 m from nearest alien pollen source (other regeneration plot, crop, feral population, etc.), near to source of preferred pollinator
Accessibility	Sufficient to enable daily patrols and monitoring	Sufficient to enable biweekly patrols and monitoring

Table 5. Preferred and acceptable threshold levels for the quality and quantity of seed stored in base and active collections, below which seed should be regenerated

Criterion	Basis of calculation	Preferred standard	Acceptable standard
Germination rate		≤ 85%	≤ 70%
Quantity in base collection[†]	1 test unit	100	100
	+ 1 base unit	800	240
	+ 2nd base unit **if** there is an imminent need to regenerate the active collection from the base collection[‡]	0-800	0-240
	= total threshold	**900-1,700**	**340-580**
Quantity in active collection	1 test unit	100	100
	+ 1 base unit **if** the next regeneration cycle is to use residual seed from the active collection [§]	0	0-240
	+ 1 distribution unit	250	250
	* expected number of seed requests before the next possible regeneration cycle	2	1
	= total threshold	**600**	**350-590**

[†] Genebank procedures should aim to ensure that accessions do not fall below this threshold.

[‡] This will be the case if the sample in the active collection is at or below threshold and the genebank adheres to the preferred standard of regenerating active from base.

[§] This will never be the case if the genebank adheres to the preferred standard of regenerating active from base. It will be the case at least one in four cycles if the genebank adheres to the alternative standard of regenerating samples in the active collection from remnant seed in the active collection for up to three cycles before reverting to the base collection.

Table 6. Preferred and acceptable threshold levels for the quality and quantity of newly received seed samples, below which new seed samples should be regenerated before being added to the collection

Criterion	Basis of calculation	Preferred standard	Acceptable standard
Germination rate		≤ 85%	Regenerate regardless of germination rate ≤ 95% ≤ 70%
Health		As far as possible, free of any pathogen or pest	
Quantity	Threshold quantity for regeneration of seed stored in base	900-1700	340-580
	+ Threshold quantity for regeneration of seed stored in active	600	350-590
	+ 1 base unit for safety-duplicate	800	240
	= Total threshold	**2300-3100**	**930-1410**

i. Constructing the preliminary list

Samples to be considered include all seed samples held in the base or active collection, and all newly received samples not yet in any collection. Samples held in the safety-duplicate collection should not normally require separate consideration. Preferred standard is that accessions in the safety-duplicate are held under conditions at least as good as the base collection, and that enough seed are held in the base collection to ensure that they require regeneration only when quality deteriorates. Where this is achieved, samples held in the safety-duplicate collection will need regeneration at the same time as those in the base collection, and regeneration protocol should make this assumption. Where standards fail and seed in the base collection require regeneration because they fall below threshold quantity, regeneration of base and safety-duplicate will fall out of synchrony and a separate regeneration cycle will be needed at some stage for the safety-duplicate collection.

The genebank documentation system should be used to construct the preliminary list, and should indicate the location of the selected samples. The list should include samples that:

- are below threshold for seed quantity (which for newly introduced material will include material received as plants rather than seed), or
- might fall below threshold for seed quality. All seed whose quality has not already been tested fall into this category. This will include all newly introduced materials. It may also include stored seed, if the genebank has failed to meet acceptable standards for testing new seed samples before entering them in the collection.

For seed that has been stored following at least acceptable standards, it can be assumed that quality will not fall below threshold for several years. In the absence of quantitative data on the rate of loss of viability in storage, Table 7 provides an approximate guide based on previous informal experience: it is supposed that seed samples might fall below threshold quality if they have remained in storage longer than the critical number of years given.

Table 7. Critical number of years of storage in base and active collections, after which accessions are considered to be at risk of falling below threshold germination rate and therefore in need of a repeat germination test. It is assumed that the base collection is stored -18°C, and the active collection at +2°C, both at 3-7% seed moisture content.

Germination rate at	Last regenerated in-house using optimal	Collection type

last test	protocol for regeneration and storage?	Base	Active
>95%	Yes	100	20
>95%	No	50	10
>90%	Yes or No	15	5
>85%	Yes or No	5	3

ii. Determining which samples in the preliminary list need regeneration

Germination tests and seed health tests are required to determine which seed samples are actually below threshold for quality. The preferred standard is to assess all accessions identified to be at risk of falling below threshold. If this is not possible, for example if genebank capacity is not sufficient, acceptable standard is to:

1. Identify groups of accessions in the preliminary list that have been previously regenerated in-house at the same time and are likely to show similar germination rates.
2. Test one or two accessions from each such group.
3. Treat all accessions in the group as if they have that germination rate.
4. Raise the quality threshold slightly, to allow for untested accessions having lower quality.

No attempt should be made to group new samples that have not previously been regenerated in-house, as there are likely to be wide variations in germination rate between accessions from the same collecting expedition or in the same batch of seed donated from another genebank: all such materials should have germination rate measured.

With one possible exception, the list of samples in need of regeneration constitutes all those below threshold quantity, plus all those that tests have shown to be below threshold quality.

The possible exception is for new seed samples donated by another genebank. If these fall significantly below acceptable threshold, it may be preferable to reject the accession altogether rather than attempt to regenerate. A decision on whether to reject or regenerate must be taken in conjunction with the donor: if the donor retains a superior sample of the same accession and is therefore able to regenerate to a superior standard, the sample should be rejected and a repeat donation requested. Otherwise, high-priority regeneration should be undertaken and a duplicate sample returned to the donor if requested.

iii. Prioritizing accessions for regeneration

If the number of samples in need of regeneration exceeds genebank capacity, there will be a need to identify which ones are in most urgent need of regeneration. Regeneration may be delayed where it is less urgent. Priorities include:

1. If the list was drawn up on the basis of preferred standards for thresholds, these standards may be relaxed to acceptable standards (Tables 5 and 6), and priority attached to those accessions that fall below acceptable threshold.
2. Regeneration of newly introduced samples and accessions in the base collection takes priority over accessions in the active collection.
3. Regeneration of samples below threshold quality takes priority over those below threshold quantity, with one exception: if a germination test has been conducted on an accession with so few seed that satisfactory regeneration cannot be accomplished using the residual seed, then (a) the sample must be regenerated, and (b) the seed germinating during the germination test must be used as parental plants for regeneration.
4. Rank samples by quality, and regenerate as many as possible of those with lowest quality.
5. Rank samples by perceived value for conservation or utilization, e.g. attach high value to accessions that have been shown to be unique and highly distinctive, or to have particular alleles of research interest, or whose original collecting site has been destroyed.

Regeneration must never be delayed for newly received samples and accessions in the base collection that are below minimum acceptable threshold for quality.

Any accessions in need of regeneration but not selected for regeneration must be immediately put on hold, placed in optimal storage conditions if not already there, and not used for any other purpose until they can be regenerated.

iv. Selecting regeneration protocol appropriate to accession status

The above procedures should identify accessions in need of regeneration before normal regeneration becomes impossible. However, in some cases the process will fail. Where seed quality or quantity is so far below minimum that the normal number and condition of parental plants cannot be established, there will then be a need for some form of 'rescue regeneration'.

At the minimum, this will involve simply recording in the documentation system that a bottleneck has occurred where insufficient plants can be grown from the remaining seed to enable regeneration of a representative sample.

It may be possible also to "rescue" the accession from plants already in use for other purposes, e.g. germination tests, characterization, etc.

If the above fail, the next resort is to retrieve seed from the safety-duplicate collection.

Finally, where quality is so low that normal procedures would result in zero germination even for seed in the safety-duplicate collection, consider using technologies such as embryo rescue.

v. Refining the protocol

There may be a need to consider refining the above procedures if experience shows they are inadequate.

Preferred standard is that accessions in the base collection should need to be regenerated only when they fall below threshold quality. If it is found that more than 5% are being regenerated because they are below threshold quantity, then target quantities for storage in the base collection should be increased (section 2.4).

If germination rates for stored seed are above threshold in most cases (>90%), the number of years between tests may be increased (Table 7). Conversely, if too many (>5%) are too far below acceptable threshold, the number of years between tests should be reduced.

3.3 Selection of parental material

There are three components to the selection of material for use as parental plants: selecting the appropriate source, determining how many plants to grow from that source, and determining how those plants should be sampled from the selected source.

i. Source of parental plants

Samples to be entered into a collection for the first time are received either as living plants or as seed, which provide the only possible source of parental material for regeneration. In contrast, an accession already in a collection is preferably represented by seed samples in the base, active and safety-duplicate collections. Regeneration protocol must define which of these to use as parental material for the next generation of seed (summarized in Table 8).

Preferred standard is normally to use seed in the base collection as parental material for all regeneration, whether for replenishment of stocks in base, active or safety-duplicate collections (FAO/IPGRI Genebank Standards 1994). This preferred standard changes in two situations, both of which represent failures in the system:

1. Replenishing stocks in the active collection from seed in the base collection would cause the latter to fall below threshold quantity (which is against preferred standards). In this case, seed in the active collection must be regenerated from remnant seed in the active collection, for all regeneration cycles until seed in the base collection falls below

threshold quality. The curator should then also consider increasing the number of seed stored in the base collection the next time it is regenerated.

2. The accession either has been lost from the base collection, or has suffered or would suffer an unacceptable loss of genetic integrity. In this case, seed in the safety-duplicate collection should be used to regenerate base, active and safety-duplicate collections simultaneously.

Table 8. Preferred and acceptable sources of parental material for replenishing stocks in base, active and safety-duplicate collections

Source of parental material	Stocks to be replenished		
	Active	**Base**	**Safety-duplicate**
Active	Acceptable for ≤ 3 in 4 regeneration cycles according to Genebank Standards 1994; for perennial forages, regarded as not acceptable unless unavoidable. Preferred if too few seed in base collection	Not acceptable	Not acceptable
Base	Preferred; except: not acceptable if too few seed in base collection	Always, unless exceptional conditions necessitate regeneration from safety-duplicate	Same as for replenishing stocks in base: usually at same time and in same regeneration plot as base
Safety-duplicate	Only in exceptional conditions, where the accession is either completely lost from base or otherwise suffers unacceptable loss of genetic integrity.		

Acceptable alternative standard for replenishment of stocks in the active collection (FAO/IPGRI Genebank Standards 1994) is to alternate between active and base as source of parental material. This can include regenerating from remnant seed in the active collection for up to three successive regeneration cycles before reverting to seed in the base collection for one regeneration cycle. However, this is relatively unacceptable for species with high genetic variance within accessions and high potential rates of loss of genetic integrity. These guidelines are for such species, and therefore it is recommended to adopt the preferred standard wherever possible. This departure from FAO/IPGRI Genebank Standards 1994 is reflected in Table 8.

Preferred standard for replenishment of stocks in the safety-duplicate collection is to regenerate at the same time and in the same regeneration plot as the base collection, using the same set of parental seed from the base collection; regenerated samples for storage in base and safety-duplicate collections should be appropriate random samples of the seed produced in the regeneration plot, which should therefore produce sufficient seed to satisfy requirements of both.

Where seed in the base collection need regeneration because they are below threshold quantity (which is against preferred standard), the seed produced should be used to replace only the base collection, not the safety-duplicate collection as would normally be the case. Consequences of this are that seed in the safety-duplicate collection will then be superior in terms of genetic integrity, but have lower seed quality. The subsequent cycle of replenishment of stocks in the base collection should if possible be undertaken using seed from the safety-duplicate. This will not only resynchronize quality in base and safety-duplicate collections, but also maintain superior genetic integrity.

ii. Number of parental plants

The preferred standard is at least 100 plants established in the regeneration plot (i.e. 100 plants surviving after losses due to <100% germination and establishment). Acceptable standard is 30.

If the number of plants that can be established in the regeneration plots is less than 30, a bottleneck should be noted in the documentation system.

iii. Identity of parent plants

Preferred standard for wild populations is to adopt an integrated strategy for collecting, regenerating and storage that maximizes retention of original population structure and genetic integrity. For regeneration it should be possible to select particular parental plants that best represent the genetic structure of the original population sample. Achieving preferred standard requires use of multiple storage containers for each accession in the base collection. Each container should hold the progeny of one plant (vegetative cutting or seed heads) collected from the original population. For regeneration, an equal number of seed is then sampled at random from each container, to make up the required total number of parent plants.

Acceptable standard is to ignore population structure, thoroughly mix seed of each accession and use a random subsample as parental plants for regeneration.

3.4 Preparation of regeneration plots

Preferred plot size: 100 plants by 20-cm spacing = 4 m^2.

Preferred standard is to use pots, as these provide superior control over soil, weeds, soilborne pests and pathogens, plant growth rate and contamination with alien plants; and the resultant mobility provides superior control of contamination with alien pollen and a means to improve throughput capacity.

Acceptable standard is to use field plots, but this necessitates very considerable care in areas such as follows:

- **Soil.** The regeneration plot must be as uniform as possible in terms of nutrients, soil structure, physical and chemical composition. Consider a physical and chemical analysis of the soil. If necessary, apply soil ameliorative treatments (e.g. fertilizers, lime, drainage, irrigation, ploughing, soil structuring, preheating).
- **Weeds, pests and pathogens.** Determine whether such problems can be reduced during preparation of regeneration plots by the application of appropriate pregermination treatments for elimination of weeds, pests and pathogens. Ensure that any pregermination treatment selected does not adversely affect seed production.
- **Contamination with alien seed and plants.** Preventing contamination involves either:
    - using a novel site with no prior history of the species being present, whether naturally or as part of previous trials or regeneration plots, or
    - rigorous elimination of plants and seed in the soil, e.g. by sterilizing soil, digging out the soil and replacing it with the sterile compost. A single cycle of ploughing to encourage germination followed by spraying or deep ploughing to kill emerging seedlings is not usually sufficient to eliminate all seed from the seed bank.
- **Contamination with alien pollen.** Preferred standard is to erect pollen-proof or pollinator-proof cages over the regeneration plots. Acceptable is to isolate from other regeneration plots and other sources of pollen using a combination of distance and partial barriers (Table 4), eliminating all near sources of pollen. Preparation of the regeneration plot needs to take into account the intended method of control of contamination.

3.5 Preparation of seed

If appropriate or necessary, use seedlings already germinated from previous germination test (section 3.2). Otherwise, start with a new seed sample. The former may be preferred if the germination test produced enough seedlings and used seed from the desired source, or may be necessary when too few other seed remain.

Ensure 100% accuracy in the identification of accessions throughout bagging, labelling and transporting seed. Use built-in cross-checking mechanisms, including labels that stay with the seed wherever possible, dual labelling inside and outside bags, preprinted and pre-ordered sets of labels and labelled bags, and two personnel to cross-check each other.

Ensure zero contamination of seed samples with seed of other accessions. Use only purpose-built seed-preparation facilities (work surfaces, machinery, etc.) containing no crevices or internal lacunae where seed may become lodged. Completely clean all surfaces and implements after preparing each accession.

If necessary, break seed dormancy. Scarification (physically with sandpaper, or chemically with sulphuric acid) is a common requirement for forage legumes.

Avoid use of *Rhizobium* inoculants for legumes, as host-strain specificity is likely to increase variance between individuals. Use mineral nitrogen instead.

Apply proprietary seed dressings to reduce disease incidence or delay the onset of disease.

Sow in seed trays. Transplant seedlings to pots (preferred) or as spaced plants in field plots (acceptable). Preferred pot volume approximately 1-2 L. Preferred spacing in the field approximately 20 cm.

3.6 Crop management

3.6.1 Before anthesis

Inspect plots and plants regularly. As far as possible ensure complete control of weeds, pathogens and pests. Do not thin plants.

As far as possible promote uniform induction of flowering in all plants. Vernalization over winter is a common requirement for flower induction in many temperate forage species.

If using field plots, ensure continued absence of all potential sources of alien pollen both within and near the regeneration plots.

If necessary, prune large plants to reduce variation in size between plants. Prune plants to prevent competition between them. If necessary, restrict growth uniformly by using small pots or low fertilizer application.

Where possible, verify accession identity while the plants are growing, by comparing their phenotype against the documented phenotype of the accession. This will be possible only for accessions with visually distinctive characteristics of high heritability that have been recorded in the genebank documentation system. For some visually variable species such as *Trifolium repens* this may be feasible for a large proportion of accessions. For others such as *Lolium perenne*, it will not be feasible for most accessions.

3.6.2 During anthesis

Ensure no stresses, such as excessive heat or drought, that might interfere with normal meiosis and pollination.

Prune plants at the beginning of anthesis so that all plants have a similar number of inflorescences at a similar stage of development, i.e. remove early inflorescences from plants with many.

If required for the chosen method of elimination of alien pollen, move pots into a pollen-proof or pollinator-proof chamber for the duration of flowering, or erect temporary pollen-proof or pollinator-proof nets around the regeneration plot.

In the absence of sufficient research on pollination patterns within regeneration plots, and given the expense of manual pollination for the large number of seeds required, the preferred standard is currently to permit open-pollination, using the smallest possible size of regeneration plot. For wind-pollinated species in isolation chambers, use an active