ENSCONET Curation Protocols & Recommendations

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ENSCONET CURATION RECOMMENDATIONS

SEED CLEANING

For more detail please see Topic 1.

- Ideally, all seed lots should be cleaned thoroughly until all accompanying plant parts have been removed. However, in some cases, it may be necessary to bank the collections as received and to record this on the computer record.
- Seeds of wild species should be cleaned manually but using certain mechanical cleaning equipment. Where equipment is used care needs to be taken to clean it thoroughly between using it for different collections.
- Staff need some knowledge of seed and fruit morphology and how to interpret structures under the microscope.
- It is advised that seed cleaning operations are performed under an extraction hood with a dust filter or in a contained area by staff wearing dust masks of a suitable standard.
- During cleaning, seeds must be inspected for signs of damage using a stereo-microscope.
- Debris generated during seed cleaning should be destroyed preferably by controlled incineration.
- Cut tests or X-ray analysis should be performed to evaluate the number of “empty” seeds in the cleaned seed-lot.

DRYING & MOISTURE MONITORING

For more detail please see Topics 2 & 3.

- Until new data are available, it is recommended that seeds are dried to equilibrium with about 15 % RH (approximately 3.5 – 6.5 % moisture content depending on seed oil content) at 10-20 °C if they are then to be placed at sub-zero temperatures. Ultra-drying to below these moisture levels is recommended if above-zero storage is to be used. Warm or oven based drying is not recommended.
- Where possible, seeds or fruits that are at obviously different stages of ripening should be separated into sub-samples and each sub-sample dried once mature.
- For maximising drying rate, seeds should be spread out in a thin layer and air directed over them.
- Mature unripe seeds and fruits for 2-3 weeks under conditions similar to those where they were collected.
- Gradually dry seeds extracted from mature fleshy fruits at ambient conditions (60-70 % RH, 20-25 °C, for 1-2 weeks) before transferring them to dry room conditions.
- Use equilibrium relative humidity (eRH) to monitor drying but beware of potential sources of error. This can be measured using hygrometers, dataloggers or indicating silica gel.
- A drying room that can be adjusted to a given value of RH is recommended for a medium to large seed bank (say, a few 100 – few 1000 accessions per year). For small-scale operations involving small quantities of accessions (say, less than 100 per year), silica gel drying or use of an incubator dryer are recommended.
- Seeds with a high moisture content should be first dried under ambient conditions for 2-3 days to remove free water prior to desiccant drying. If using silica gel a ratio of 1:1 by volume of desiccant to seeds should enable seeds to be dried to a safe level (<30 % eRH).

PACKAGING

For more detail please see Topic 4.

- For long-term storage use re-sealable transparent glass containers if regular access is required; use flame-sealed glass containers or double packed re-sealable glass containers if access is not required. High quality foil bags should perform well if well sealed and preferable double packed.
- Preferably package seeds in a humidity controlled environment.
- Include a humidity indicator silica gel sachet in the container for monitoring seal performance and protection against catabolites. These must be equilibrated to the seed bank drying conditions (usually 15 % RH).
- Periodically check and replace seals (if applicable).
- Leak test batches of containers employing a standard leak test procedure.

STORAGE

For more detail please see Topic 5.

- A storage temperature below 0 ºC (usually between -18 and -20 ºC) is recommended for the long-term storage of most orthodox seeds.
- For conventional storage at non-cryogenic temperatures, lowering temperature below -35 ºC would not appear to benefit seed longevity sufficiently to warrant the extra costs.
- For very short-lived orthodox-seeded species, cryo-storage in or above liquid nitrogen may be advisable.
- All samples must be duplicated at a second bank. This duplicate bank should be sufficiently far away that it would not be subject to the same catastrophic loss that could destroy the main collection.

GERMINATION

For more detail please see Topic 6.

- It is recommended that germination tests are carried out on as many accessions as possible after seeds have been dried and processed and if feasible after banking. Ideally, germination tests should be carried out before and after drying to check if there are indications that germinability is being affected by desiccation. If resources are limited and only one initial test can be carrried out it is recommended that this should be within the first month after banking.
- Germination percentage should be expressed in terms of the number of seeds which could be physically capable of germination (total number of seeds minus damaged or empty seeds).
- While a sample size of 200 seeds is ideal, two replicates of 50 or 25 seeds are acceptable in most cases.
- In order to avoid imbibition damage, seeds of susceptible species should be humidified before testing.
- Seed germination is an extremely plastic trait and requirements can vary considerably between species, populations, collection years and even between intervals of storage. Use published literature or databases such as RBGK's Seed Information Database (Liu *et al.*, 2008: http://data.kew.org/sid/sidsearch.html) and LEDA traitbase (http://www.leda-traitbase.org/tomcat/LEDAportal/index.jsp) as a guide. When there is no information (even about re-

lated species) be guided by the climate, ecology and seed structure of the species.

- Seeds of many wild species show dormancy. Appropriate dormancy breaking techniques should be used.
- The substrate for sowing could be agar, filter paper or sand. Agar (usually 1 %) has the advantages that it: requires low maintenance; has low risk of imbibition damage; maintains applied chemicals at constant concentration; allows white radicles to be visible on a dark background; allows seedling plugs to be removed for transplanting.
- In all cases deionised or distilled water should be used.
- The seeds should not be touching one another on the germination medium.
- If possible, constant temperatures should be avoided except during most stratification treatments. Continuous light should be avoided due to the risk of inhibiting germination.
- For germination tests, a germinated seed might be defined as one with a protruded radicle of 1-2 mm long.

VOUCHERS AND VERIFICATION

For more detail please see Topic 7.

- Except in the few cases where populations are well known, a pressed specimen should be collected to represent the population from which seeds have been sampled. Obviously care should be taken if rare or endangered populations are involved.

DATA

For more detail please see Topic 8.

- When recording data about each seed collection it is vital to remember that the data will be meaningful to users of that data both now and during the lifetime of the collection (perhaps 200 years hence). The data therefore must be objective and checked against agreed standards.

REGENERATION

For more detail please see Topic 9.

- Regeneration is an expensive operation that is difficult to do well and is best avoided if
- possible by the collection of large, high quality seed collections in the field. In some cases, it may be better to recollect directly from the wild (should that still be possible) rather than carry out regeneration.
- Seed bank staff should do their best to reduce the risks of selection, genetic drift or hybridisation with closely related material though it has to be accepted that perfection will rarely be attained. They should use whatever knowledge there is about the species' breeding systems to inform pollination method (e.g. with out-breeders do not force them to inbreed and ensure genetic isolation from similar collections). Additionally, wek or off type plants should not be discarded as they may contain different genetic material.
- High-quality horticulture is essential to minimise losses of individuals (by growing in similar to conditions to the wild) and maximise seed output (by reducing competition and optimising flowering and seed set).
- Ideally plants should be crossed as paired reciprocal crosses, accepting that in most cases

this will be too time consuming and crossing in as many combinations as possible will be necessary; contribution of pollen from different parents should be equalised.

- Hand pollination on several dates will cover early and late flowering.
- Where possible, seed-lots from different mother plants should be kept separate and equal quantities from each used to create the next generation. Where this is not possible, equal seed quantities should be harvested from each maternal parent and bulked; the next generation should be selected at random and should consist of no less individuals than the previous generation to avoid bottlenecking.
- Growing conditions and regeneration results should be fully documented.

SEED DISTRIBUTION

For more detail please see Topic 10.

- Seed lists should be made up of collections with adequate quantities of seed, acceptable germination levels and verified identity; they should not contain collections with collecting agreement restrictions or be of species that are known to be strongly invasive.
- It is important that seed banks maintain a base stock of seed that is not distributed. This is either achieved by physically separating a conservation (base) sample from the used (active) sample or by having a failsafe stock control system on the seed bank database.
- Because of national sovereignty and ownership issues related to genetic resources, seed samples are usually sent out under the terms of a legally-binding material transfer agreement (MTA) that controls what the seeds may be used for, whether they can be passed to a third party and how benefits arising from the use will be shared.
- Plant health and CITES and the Habitats Directive must be considered when moving material across national borders.
- Samples should be sent in a foil packet and accompanied by relevant passport and germination data.
- The seed bank should keep records of recipients and uses of the distributed samples. This information is essential in demonstrating the immediate value of seed bank collections.
- Where possible, seed bank managers should discuss the types of collection required by potential users; this would help ensure maximum use of the collections.

INTRODUCTION

Aim of this document

The aim of this document is to provide the best available advice to those involved in, or embarking upon, the long-term conservation of wild-collected seed samples particularly in Europe but also more widely. It is intended as a guide rather than as a prescriptive manual. For more detailed manuals, curators are referred to (Crop seed - Rao *et al.*, 2006; tree seed - Schmidt, 2000; wild species – CPC, 1986; Bacchetta *et al.*, 2008; Offord & Meagher, 2009); for detailed background text see Smith *et al.*, (2003) and especially the chapter by Terry *et al.* (2003). For each topic, recommendations are given for seed banks holding wild species. As such, they build on the Genebank Standards produced by FAO / IPGRI (1994). The opportunity is also taken here to highlight areas for further research.

Topics covered

Seed drying, seed moisture monitoring, packaging, seed storage, seed germination, seed cleaning, verification of identity, curation data handling, seed regeneration and seed sample distribution (see Figure 2 for the sequence of activities in a fairly typical wild species seed bank while Figure 3 shows these activities in relation to the layout of seed bank facilities).

Figure 1 Seeds in long-term storage. (© RBGK)

Figure 2	Key elements of a generalised seed banking procedure (From Smith *et al.*, 2003)
Planning & Collecting	Planning and permission seeking ↓ Field collection of labelled seed and voucher (pressed) specimens plus field data recording ↓ Rapid shipment of the seed to the bank
Processing & Testing	↓ Unpacking of consignment, check for problems, drying/mounting of vouchers ↓ Data record creation about each accession ↓ Assessment of likely seed storage characteristics (if unknown) ↓ Seed cleaning[1] ↓ X-ray analysis or cut-test ↓ Seed quantity determination ↓ Drying ↓ Determination of seed moisture status ↓ Initial germination test[2]
Storage & Utilisation	↓ Packaging and security duplication[3] ↓ Storage under cold conditions ↓ Characterisation and evaluation[4] ↓ Distribution of characterised sub-samples to users (through time) ↓ Germination re-tests (through time) ↓ Regeneration / multiplication of collections (when required)[5]

1 Cleaning is sometimes preceded by a period of drying. Small samples may be multiplied at this stage with procedure after harvest as for field collected material.

2 This step is sometimes left until after banking if confirmation is desired that seeds survive both desiccation and freezing. It requires an easily re-sealable container.

3 Duplication should be well away from bank.

4 As appropriate.

5 Procedure after harvest as for field collected material.

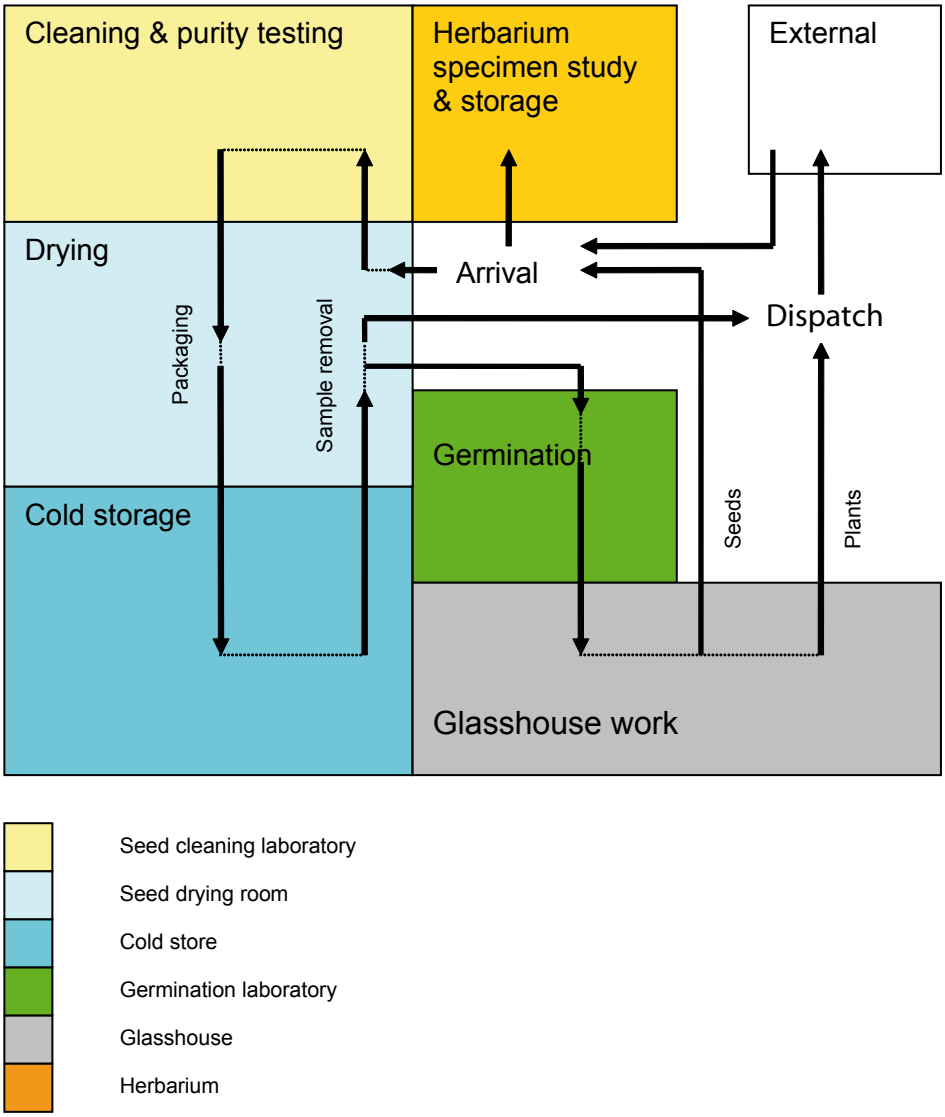

Figure 3 Chart of main seed bank processes and facilities

TOPIC 1. SEED CLEANING

Summary written by Gianni Bedini (Pisa Botanic Garden) based on the experiences of UVEG, MNHN, RBGK and Pisa Botanic Garden, and subsequent discussions.

General comments

Seed cleaning is the act of removing all parts and tissues surrounding seeds, as well as any non-plant material (like e.g. associated insects or soil particles) and seeds of other species (see MSBP Technical Information Sheet 14 http://www.kew.org/msbp/scitech/publications/14-Seed%20cleaning.pdf). Although it is time consuming and requires expertise, significant laboratory space and specific equipment for it to be carried out conveniently and thoroughly, the process is beneficial in several ways:

- It saves space in cold storage. If cleaning is carried out before drying, it also saves space and reduces the drying required in the dry room / cabinet.
- It aids the visual inspection of the seed lot for empty, immature, damaged or infested seeds (that are useless for conservation purposes) and their subsequent removal.
- It removes pathogens (fungi, bacteria and viruses) which are more likely to be carried in the plant debris than the seed. This makes compliance with plant health regulations easier when seeds are despatched to users.
- Seed quantity can be more accurately determined with cleaned rather than un-cleaned seed-lots.

Ideally, all seed lots should be cleaned thoroughly until all accompanying plant parts have been removed. This is not practically possible for all species especially with certain fruit types and small seeds. Dehiscent fruits and large seeds are relatively easy to clean. Separating tiny seeds from pericarp fragments of comparable size, form and density can prove too difficult a task to be carried out in a practical time. Similarly, extracting seeds from indehiscent fruits (samaras, achenes and nuts) can take too long or can lead to seed damage. In these cases, it may be necessary to bank the collections as received and to record this on the computer record.

Seeds of wild species should mostly be cleaned manually but the use of certain mechanical cleaning equipment is acceptable. Automated processes, common in the bulk processing of agricultural, horticultural and forestry seeds, are not suitable for cleaning large numbers of small and variable (e.g. with respect to size and morphology) wild seed collections. The time taken to clean the automated equipment between samples to avoid cross-contamination is not cost effective with large numbers of small samples. Some collections, especially those deriving from populations of threatened species, may number only a few seeds. Furthermore, the equipment may need to be calibrated for each different species and cannot cope with the large amount of variability in fruit / seed shape, size and mass, within samples. Based on studies with crop seeds some types of equipment may cause sufficient mechanical damage to the seed such that longevity is reduced.

Seed cleaning of wild seed collections requires greater skill than many might expect. Staff need some knowledge of seed and fruit morphology at the family level and how to interpret structures under the microscope such that techniques applied to seed cleaning remove structures that are unnecessary for storage and yet does not cause damage. Adapting techniques for different types of fruit/seed is therefore the key to good seed cleaning.

Most seed cleaning is facilitated by drying. The exception is fleshy fruits where seed extraction prior to drying is advisable.

While simple cleaning procedures can take place in the field directly after collecting, most operations will be better performed in a laboratory with adequate bench space, dust protection and the following equipment:

Sieves, bungs & brush

Dust extraction system and / or masks

Rubber gloves

Seed aspirator (blower)

Stereoscope & dissection equipment

Washing facility

Debris disposal

Figure 4 General seed cleaning laboratory. (© RBGK)

TOPIC 1A. EQUIPMENT AND COMMON OPERATIONS

General comments

Sieves, bungs & brush

Stainless steel sieves of various wire mesh sizes are generally used in seed banks. They allow the separation of seed from its associated impurities in two or more fractions. Objects larger than the mesh size will remain on the sieve, while others will go through. If the debris is larger than seeds, then a mesh size slightly larger than seeds can be used to collect debris on the mesh and the cleaned seeds in a tray placed under it. If, vice versa, the debris are smaller than seeds, then a mesh size slightly smaller than seeds can be used to collect cleaned seeds on the mesh and the debris under it. A combination of different mesh sizes can be used to separate seeds from smaller and larger debris. This method does not work if the debris is the same size as the seeds. In this case, seeds must be stored with the debris or separated by another method e.g. by rolling the collection down an inclined surface, or by using a blower (see below).

The un-cleaned seed lot can be gently rubbed against the mesh with a rubber bung. Rubbing helps in breaking apart brittle debris, causing them to sift through the mesh, but vigorous rubbing may damage the seeds by squeezing or rupturing them against the mesh. If seeds are brittle, they can be rubbed by hand: in this case, the operator should wear latex or PVC gloves to protect the fingers while retaining sufficient sensibility to feel the seeds under the fingertips.

Figure 5 Cleaning seeds using a sieve and bung. (© RGBK)

Seeds must be inspected for damage with a stereo-microscope: if damage is noted such as undue abrasion to the seed coat or snapping of elongated Poaceae caryopses or Asteraceae achenes, then gentler rubbing should be

applied. It is also important to check the different fractions: if there is a mixture of debris and intact seeds, then they must go through the sieve again.

Sieves, trays and benches must be cleaned thoroughly between different seed lots to avoid cross-contamination. A steel brush is the best option to clean sieves.

Dust extraction system or masks

Especially when sifting / winnowing seeds or dry fruits, a lot of dust is produced, composed of fine particles that might be inhaled by operators and deposited on laboratory equipment. Some of this dust can cause lung irritation or allergy. Therefore, seed cleaning operations should be performed under an extraction hood with a dust filter or in a contained area by staff wearing dust masks (of a suitable standard e.g. EN 149: 2001 FFP3). Obviously, protection is only as good as the filtration, so filters in hoods and dust masks should have a regular cycle of replacement.

Figure 6 Clean air hood for seed cleaning. (© RBGK)

Seed aspirator (blower)

This equipment allows the separation of seeds from debris based on differences in density. It generates an adjustable airflow which passes through a seedlot placed on a fine mesh fitted in a hollow cylinder. The air flow captures “light” materials that are blown along the cylinder and collected in a trash catcher on top of the cylinder, while “heavy” materials will remain in the cylinder. It is based on the same principle as winnowing, but it is more reliable because the airflow can be adjusted to the desired value.

It is important to check that separation is as effective as practically possible, leaving all properly-formed seeds on the mesh and all debris (i.e. devoid of potentially viable seeds) in the trash catcher. This is obtained via a precise adjustment of the airflow, which must be set through a trial-and-error basis. With a small sample in the basket, the airflow is initially set at low speed, just enough to lift some debris to the trash catcher, as can be judged by looking at the cylinder, normally built from transparent material. The debris in the catcher is then checked under a stereoscope for viable seeds. If no seeds are carried in the debris, then the airflow intensity is increased by small increments and the procedure repeated. When properly-formed seeds are found in the trash catcher, the airflow must be set back to the previous adjustment. Blowing the seeds can be very effective when applied after sieving.

As with other techniques, the equipment (basket, cylinder, trash catcher) needs to be thoroughly cleaned between different seed-lots to avoid cross contamination of one sample by another. Static electricity can build up, especially in the cylinder, because of the friction generated by the airflow., for this reason, anti-static cloths / sprays are recommended.

Stereo-microscope & dissection equipment

A stereoscope is useful in many tasks associated with seed cleaning, including checking the purity of a seed sample, checking the debris fraction for the presence of intact, viable seeds, and performing cut tests.

Figure 7 Stereo-microscope. (© RBGK)

Cut tests are performed to evaluate the number of "empty" seeds (i.e. with endosperm and/or embryo lacking or disrupted). Because it is a destructive test, it is performed on sub-samples (e.g. 50 seeds) of the seed lot (see also x-ray analysis). Seeds in the subsamples are cut with a scalpel or other appropriate dissecting tool and checked for the presence of intact endosperm and fully-formed embryo, resulting in the evaluation of the ratio filled/empty seeds. If the proportion of empty seeds is very high e.g. >50%, then the sample might be recleaned, though only when it is practical to do so, e.g., if staff time was available.

Washing facility

Seeds extracted from fleshy fruit may need a quick wash to remove residues of pericarp still attached after extraction (see below). Water can also be used to separate seeds by flotation: empty seeds will float while filled seeds will sink upon immersion. This method is generally not recommended for use in seed banks as dried seeds might be affectd by rapid imbibition.

Debris disposal

Debris can contain pathogens and viable seeds of invasive species. If the seed bank processes exotic materials, both items can be a serious threat to native ecosystems if they have a chance to escape the seed bank. Therefore, all debris should be accurately confined while in the seed bank: a good option is to place them in plastic bags fitted in clearly labelled "debris" bins. When filled, the bags can be closed tightly and sent to an authorized waste disposal area. Medical-standard incineration is the safest treatment to prevent diffusion of live material, and it should be applied whenever possible.

Figure 8 Plant waste bin for incineration. (© RBGK)

Cleaning fleshy fruits

Immature fruits should be matured under high RH conditions before seed is extracted and dried. Seed can be extracted by cutting, splitting, squeezing the fruit, or squashing it through sieves. A tepid water wash may follow if needed to remove sticking residues of pericarp. The seeds are then spread in a thin layer and placed in the drying room. Where a mucilage layer covers the seeds, drying enables it to be removed easily by gentle hand rubbing.

Special case 1: sticky fruit flesh

Daphne alpina subsp. *alpina* produces small drupes with very little, sticky flesh. When the drupes are squashed through sieves, the stones are squeezed out surrounded by residues of pericarp which make them stick together in big lumps. The sticky flesh does not dissolve and is not removed by water. A more practical solution, in this case, consists in rolling the stones in ash until they are completely coated. Thus, the flesh becomes less sticky and can be removed by gently rolling the fruits on a sieve whose mesh size is smaller than the stones.

Special case 2: over-ripe fruits in the field
Fleshy fruits (e.g., Solanaceae berries) may require partial or full cleaning if they are over-ripe, or have been damaged or crushed during collecting, in order to avoid fermentation prior to cleaning. Using a sieve and cool running water, as much flesh as possible should be removed from the fruits. The seeds should then be placed in the drying room or, if in the field, left to air-dry on a fine wire mesh or thick filter paper until surface-dry before being packed and transported in cloth bags.

Special case 3: fruits unripe at dispersal
Woodland herbs (e.g., *Anemone nemorosa*) and aquatic species (e.g. *Caltha palustris*) may disperse fruits with immature seeds. In these cases, the seeds should not be extracted immediately after collecting; rather, the fruit should be matured in high RH conditions (after-ripening) before the seeds are extracted.

Cleaning dry seed heads
Dry seed heads are generally cleaned by the sieve and bung method. Seed heads are placed in a sieve of appropriate mesh size, such as not to allow the seeds to pass through, and then rolled about with the bung until the seeds are released and inert, dry materials are crushed into small parts that pass through the mesh.

If used too vigorously, the bung may damage the seed. It is therefore important to check the seed while cleaning; if any damage is observed, then a more delicate pressure must be applied. This is true especially for brittle seeds, which might break apart when pressed against the mesh. In this case, seed can be rolled about by hand. PVC or latex gloves can be worn to protect the fingers.

Sieving is pursued to a reasonable end point. If further cleaning will result in damage to the seed, or is too time consuming, then another technique may be applied to the seed lot, such as winnowing, or the collection can be banked in an imperfect state and this fact recorded on the database. If appropriate, sub-samples can be cleaned at the time when withdrawn for germination or distribution.

Special case 1: dehiscent dry fruits
Seeds that have been collected fully mature within dry, bulky fruits or capsules are rapidly and straight-forwardly cleaned by carefully opening the fruits and separating the seed by hand. Capsules, siliques and legumes can be split open along their natural dehiscence lines and gently shaken or “milked” on a tray to yield their seed-load free of contaminants: with some precaution, this task can be performed effectively in the field. In the case of abruptly dehiscent fruits, they must be kept in a tray or box covered with paper or cardboard to prevent loss.

Special case 2: indehiscent dry fruits
Fruits such as nuts, achenes and samaras are dispersed as diaspores and should be stored as such. As a general rule, the removal of any structure from the natural dispersal unit (such as wings, hairs, paleas and lemmas, etc.), especially when it is integral with it, or tightly attached to it, should be treated with caution and preferably avoided, as it is likely to damage the seed.

X-ray analysis
This technique allows a check on empty or damaged/infested seeds or number of seeds in non-cleanable fruit. It is a high-tech alternative to the cut-test, requiring specific equipment (X-ray machine and in cases of non-digital machines, photographic consumables, dark room and a light box) and trained staff. In comparison with simple cut-tests, it allows faster, more accurate and better documented evaluations. Although non-destructive, as a precaution against genetic damage, only a sub-sample of the collection is tested and these seeds are not returned to the main collection. The

X-rayed seeds might be used for germination testing (test results should be equal or worse than those for untreated seeds but note that potential genetic change of material).

Possible components of a seed-lot and their appearance in an x-ray test:

	Component of viability test	**X-ray test appearance**
Non-dormant* seeds	Yes	Filled
Dormant* seeds	Yes – use Tetrazolium test or general appearance to distinguish from dead	Filled
Dead seeds	Yes	Filled
Empty seeds	No – exclude	Empty
Insect-damaged seeds	No - exclude unless damage is superficial	Damaged
Debris	No - exclude	Debris

* See Topic 6 for definitions

% viability = (Non-dormant seeds + dormant seeds) /
(Total seeds - empty seeds - insect-damaged seeds) x 100

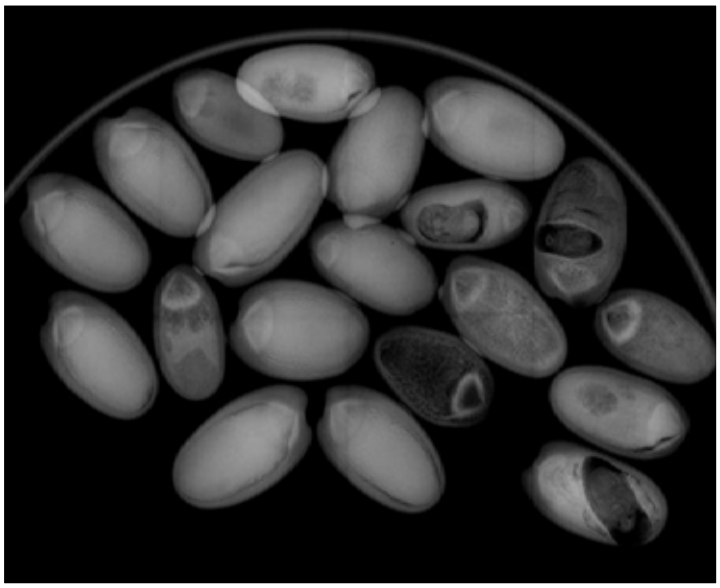

Figure 9 X-ray of *Albizia bernieri* showing empty, maggot infested and healthy seeds. (© RBGK)

Seed quantity estimation

Seed quantity estimation is important for planning the use of the collections (germination tests, duplication, conservation, propagation protocols, regeneration, etc.). The suggested procedure is as follows:

- Weigh overall seed-lot
- Weigh five samples of 50 seeds
- Calculate the mean weight of 50 seeds
- Calculate the 95 % upper confidence limit (UCL)
- Estimate the seed number: (overall seed-lot weight / UCL) x 50

Note that the suggested procedure leads to underestimating the seed number, which is preferable to overestimating as would occur with a simpler calculation involving only the weight (wt) of a single sample of 50 seeds, i.e. (overall seed-lot wt / 50 seed wt) x 50. In this case, if the single sample weighed was significantly lighter than average, the result would be overestimated.

An alternative method of seed quantity estimation would be to monitor collection weight.

TOPIC 2. SEED DRYING

Summary written by Costantino Bonomi (MTSN) based on the key presentation by Robin Probert (RBGK), additional presentations by UPM and NBGB, and subsequent discussions.

General comments

Appropriate drying is the key to maximising seed longevity in most species[1]. There is the temptation to assume that cooling is the key to seed banking. However, empirical models demonstrate the importance of drying to long-term seed storage.

Figure 10 Maturation series for *Fritillaria tubaeformis* showing natural point of dispersal. (© MTSN)

TOPIC 2A. HETEROGENEITY OF SAMPLES AND SPEED OF DRYING

General comments

Seeds and fruits should be collected when they are fully ripe and usually at the point of natural dispersal (Figure 10). Of course, in the case of highly serotinous (requiring fire to release seed) species, fruits will be collected many years before the point of natural dispersal. At the other extreme, some species possess fruits that undergo rapid, abrupt dispersal or, as is the case in orchids, that disperse seeds before the capsule has fully dehisced. In these cases it may be necessary to collect fruits before dispersal has started. Fruits are then allowed to ripen and undergo dispersal in a cloth bag in order to capture the seeds.

Seeds and fruits of most wild species are characterised by heterogeneity in their ripening time. This variation poses problems when applying post-harvest treatments including drying. Rapid drying may severely reduce the longevity of unripe (immature) seeds and those extracted from ripe fleshy fruits. A number of recent studies (Probert *et al.*, 2007 and reviews by Hay & Probert, 1995 and Probert & Hay, 2000) consistently show that seed quality will continue to increase if immature seeds and fruits are subjected to delayed or slow drying treatments. Moreover, a recent study at RBGK (Butler, *et al.*, 2009) has shown that immature seeds are remarkably resilient to fluctuations in RH and can not only recover from periods at intermediate RH levels but continue to mature when they become more or less fully hydrated again.

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1 Most species produce seeds that are desiccation tolerant when ripe. These are termed ‘Orthodox’ species. For further information on seed storage classification - see MSBP Technical Information Sheet 10 http://www.kew.org/msbp/scitech/publications/10-Desiccation%20tolerance.pdf; for further information on the effect of drying on seed longevity – see Pritchard & Dickie, 2003; to check whether anything is known about the status of a given species – see the Seed Information Database (*Liu et al.*, 2008: http://kew.org/data/sid/).

Recommendations

Maturation of unripe fruits (and hence seeds) for 2-3 weeks under conditions similar to those where they were collected is recommended. Usually this maturation process will manifest itself in a change in fruit colour, often from green to red or straw-coloured. Seed colour within fruits will also change during this process, often from white to a darker colour.

For seeds extracted from mature fleshy fruits, gradual initial drying at ambient conditions (60-70 % RH, 20-25 °C, for 1-2 weeks) is recommended; all other fully mature seeds can be dried under the conditions recommended below.

Where possible, seeds or fruits that are at obviously different stages of ripening should be separated into sub-samples and the unripe seeds or fruits allowed to mature and dried as detailed above; ripe seeds and seeds extracted from ripe non-fleshy fruits should be dried immediately (under the conditions recommended below) to minimise viability loss. There is concern about the rate at which different seeds reach equilibrium under dry room conditions. Seed bank staff should take special care to check that larger seeds with thick seed coats are properly dried before banking.

Research priority

More assessments of drying rate on seeds of different species would be useful.

TOPIC 2B. AIR CIRCULATION

General comments

With the exception of seeds extracted from fleshy fruits and unripe seeds (see above), drying rate should be reasonably rapid in order to minimise the time that seeds spend at high moisture contents and to maximise the throughputs of the drying method. Drying rate is affected by air-speed over the surface of the seed. Faster air removes moisture from the surface of the seed and maximises the moisture (water potential) gradient between the outside and inside of the seed. Seeds kept close together in a large or sealed bag, with poor air circulation, may create a locally damp environment that is detrimental to longevity even if placed under suitable drying conditions.

Recommendations

For maximising drying rate, seeds should be spread out in a thin layer and air directed over them. Forced air circulation might be effectively employed in this respect.

TOPIC 2C. MONITORING DRYING

General comments

The equilibrium relative humidity (eRH) created by the seeds within a closed space is best used as a measurement of the extent of drying rather than seed moisture content (mc). The eRH gives a measure that is independent of seed oil content.

If the oil content of the seeds is known, moisture content can be deduced from the eRH value (see: http://data.kew.org/sid/viability/mc1.jsp). This avoids the need for destructive moisture content determinations though these may be required if oil content is unknown.

See Probert *et al.* (2003) for general measurement of eRH. See Cromarty *et al. (*1990) for the conversion of eRH to mc and vice versa.

Recommendation

Use eRH to monitor drying (see below).

TOPIC 2D. TEMPERATURE AND HUMIDITY CONDITIONS USED FOR DRYING

General comments

Drying at hot or warm (>40 °C) temperatures is not recommended, particularly over prolonged periods. There is some evidence that carefully-monitored drying at high temperatures, such as sun drying, is not detrimental. However, there is the danger of over-exposure of the seeds to the high temperatures once they have reached the desired moisture level. Furthermore, large seeds can suffer cracking injury by high temperature drying. The drying temperatures that are widely used by seed banks lie in the range 10-20 °C. There is divergence of opinion on the final moisture level that should be achieved and consequently on the relative humidity conditions that are suitable for drying. Many seed banks store seed that has been dried to equilibrium with about 15 % relative humidity (approximately 3.5 – 6.5 % moisture content depending on seed oil content). Other seed banks use much drier conditions (Pérez-García *et al.*, 2007 & 2008). However, others use slightly higher RH values (20-25 %) because the water potential (equivalent to eRH) of packaged seed, dried at 15 % RH, could theoretically drop to sub-optimal levels for long-term storage when placed at sub-zero temperatures (Vertucci *et al.*, 1994). However, there is evidence that the latter phenomenon is not universal or always so marked. Some of the conflict of evidence is due to the different experimental methods and species used. However, this remains as one of the great unanswered questions in seed conservation.

Recommendations

Seed bank managers cannot wait for the issue of optimal moisture content to be resolved. It is not possible at this time to recommend a single target moisture value for use under all circumstances. Therefore, until new data are available, it is recommended that seeds are dried to equilibrium with about 15 % RH (approximately 3.5 – 6.5 % moisture content depending on seed oil content) at 10 - 20 °C if they are then to be placed at sub-zero temperatures. Ultra-drying to below these moisture levels is recommended if above-zero storage is to be used.

Research priorities

Further investigation is highly desirable to get greater clarity with respect to target moisture level. Of course, it may well be that different species have different optimal eRH values for storage.

A collaborative experiment should be set up to provide comparative data for seed storage at various moisture contents (at the drier end of the scale) to assesses if a general default target value of drying can be recommended across different taxonomic group of species or different bio-geographical regions showing different ecological requirements. The main focus of this experiment should be species that are suspected from existing data to be short-lived in seed bank conditions. These species should be more sensitive to the impact of different drying conditions on potential longevity.

Seed banks should publish their seed storage data (including the storage conditions used) in either peer-reviewed journals, technical reports or on-line, e.g. the Seed Information Database (Liu *et al.*, 2008: http://kew.org/data/sid/).

Suggested experiment

Each partner to the experiment should select at least one (possibly more) species with short-lived orthodox seeds, known germination requirements and large seed-lots. The seed-lots should be subjected to different drying regimes followed by a controlled aging and germination experiment. For instance, seed-lots could be dried to three different moisture levels (25, 15 and 5 % RH) at a common temperature (15 °C). These moisture levels, more or less correspond to three levels recommended by different banks namely National Center for Genetic Resources Preservation at Fort Collins, USA, the widely used FAO / IPGRI (1994) gene-bank standards and ultra drying (used by banks such as UPM) respectively. Seeds could then be stored at 3 different temperatures: 30, 5 and -20 °C. Viability would be monitored at 6 monthly intervals over 10 years using 4 replicates of 25 seeds per test. The total seeds required per partner per species would be: 3 x 3 x 21 x 4 x 25 = 18,900 seeds. P50 values could then be identified and compared across the different species investigated.

TOPIC 2E. DRYING EQUIPMENT

General comments
Methods include use of:

(a) Sealed containers with drying agents. These agents are hygroscopic substances, such as silica gel, used to remove water vapour from the air. This method is cheap but final moisture content can be controlled only within broad limits (other than with labour-intensive methods) which are dependent upon seed:gel ratio, the moisture status of the gel and time. It can be used for relatively small numbers of collections.

(b) Incubator dryers. An effective way to dry seeds is to employ certain types of cooled incubators where humidity can be controlled to low values. These values are monitored using a data logger probe. In effect, a small drying room (see below) is created. Air is circulated within the incubator by means of a fan. This method allows final moisture content to be controlled within fairly fine limits and is suitable for larger seed quantities than (a).

(c) Drying room. This is the most expensive option but is advisable for banks with high annual intake of collections. The room can be adjusted to given RH and temperature conditions using sorption dryers and cooling equipment. The dryers normally use Silica Gel or Lithium Chloride (LiCl) as the drying agent. The dryers are connected to cooling equipment and hence to a well-insulated room via duct work (the process air flow). A separate set of duct work allows the moisture removed during the automatic regeneration phase in the dryer to be exhausted outside the building. The amount of regeneration of the drying agent is determined by controls linked to an hygrometer inside the drying room. Collections can be left in the dry room and processed when staff time allows. Readers are also referred to Linington (2003) regarding drying room design (http://www.kew.org/msbp/scitech/publications/SCTSIP_digital_book/pdfs/Chapter_33.pdf).

(d) Sealed containers with a saturated salt solution. Saturated solutions of salts such as Lithium Chloride generate a specific RH in a closed environment and actively maintain it, absorbing and desorbing water vapour. Lithium Chloride is useful because it is relatively temperature insensitive for this purpose. Seeds placed in the container but above the solution (on a platform) will dry to equilibrium with the RH at the temperature of the room. This approach could be used for very small quantities of seeds though care needs to be taken with spillage of the solution. Some chemicals can be hazardous e.g., ammonium chloride and sodium nitrite.

Figure 11 Drying incubator. (© UPM)

Figure 12 Dry room. (© MAICh)

Recommendations

Warm or oven based drying is not recommended.

A dedicated drying room that can be adjusted to a given value of RH is the recommended choice. This is a key facility in a modern seed bank with a medium to large (say, a few 100 – few 1000 accessions per year) annual intake of seeds. This solution is flexible and can be adapted to most applications.

Freeze-drying techniques were briefly discussed in the workshop, concluding that sufficient data on the effect of this technique is not currently available and that there is the risk of freezing in wet samples that would jeopardise the long-term conservation of seeds. As a precaution, this method is not currently recommended.

For small-scale operations involving small quantities of accessions (say, less than 100 per year), a variety of desiccants could be used such as silica gel or even roasted seeds and charcoal. In all cases desiccants will need to be dried in an oven prior to use, monitored and actively managed during the drying process to ensure that they are still active. Purpose built, cabinet style, seed dryers and incubator dryers (contact RBGK for details) are available as well as seed drying kits such as RBGK's mini seed bank (see: http://shop.kew.org/kew-mini-seed-bank.html). See also cabinets used by UPM at http://www.etsia.upm.es/banco_germoplasma/inicio_bgv_archivos/Page497.htm.

The ratio of desiccant to seeds for drying to safe levels (to avoid over-drying) very much depends on the moisture status of the seeds at the start of drying. Freshly collected seeds with a high moisture content will require large amounts of desiccant and this may need to be renewed before the seeds are dry. It is therefore recommended that seeds are allowed to dry under ambient conditions for 2-3 days to remove free water (ie. that which is easily lost) prior to desiccant drying. The seeds should then be placed in a sealable container above a layer of the chosen desiccant.

The water holding capacity of desiccants varies. If using silica gel a ratio of 1:1 by volume of desiccant to seeds should enable seeds to be dried to <30 % eRH (Probert, 2003) which will provide safe medium term storage for most species. For charcoal a ratio of 3:1 will be needed.

Figure 13 Silica gel drying bin. (© RBGK)

TOPIC 3. MOISTURE MONITORING

Summary written by Robin Probert (RBGK) based on the key presentation by Costantino Bonomi (MTSN), an additional presentation by PAV-UNI and subsequent discussions.

TOPIC 3A. MEASUREMENT OF EQUILIBRIUM RELATIVE HUMIDITY

General comments

The advent of reliable relative humidity sensors over the last decade or so has transformed our ability to effectively monitor moisture at various stages of the seed conservation process. Relative humidity sensors are at the heart of the control systems used in dry rooms and they function to warn if equipment fails or if drying conditions move out of range. But arguably the most important advance in seed conservation technology in recent years has been the routine use of relative humidity sensors to provide a means for monitoring seed moisture (see MSBP Technical Information Sheet 5. http://www.kew.org/msbp/scitech/publications/05-eRH%20moisture%20measurement.pdf). These sensors measure the relative humidity of the air in equilibrium with a sample of seeds. If required, the measured equilibrium relative humidity (eRH) can be related to seed moisture content by reference to a moisture sorption isotherm.

The major advantage of such methods, compared to conventional moisture content determination, is that the test is usually non-destructive and therefore valuable seeds are not wasted. Another advantage is that the measured value is not dependent on seed oil content because seeds with different oil contents equilibrated under the same RH and temperature conditions have different moisture contents but the same hydration status (water potential).

However, instruments for measuring seed moisture non-destructively have not meant that conventional seed moisture content determination is now redundant. Gravimetric moisture content determination (for general information see ISTA, 2008) still plays an important role in most seed banks and when combined with eRH values, determined over a range of moisture levels, at a given temperature, is the basis for constructing moisture sorption isotherms.

A range of relatively inexpensive desk-based and hand-held devices are now routinely used in seed banks to monitor seed moisture status during and after seed drying and in seed research. Only dew-point hygrometers provide a direct measure of a psychrometric parameter of the air at equilibrium, the dew point temperature (for further discussion of the psychrometric properties of air, see Probert, 2003).

Other hygrometers tend to rely on the physical or chemical change in a substance which is directly related to a change in humidity against a calibrated standard. Such instruments include hygrolytic, capacitance and resistive sensors. Modern digital hygrometers operate with software that calculates the required output units from psychrometric principles. Thus displays in relative humidity (% RH); water activity (aw); water potential (MPa); and dew-point temperature (°C) are common.

Instruments with sample chambers designed for seeds or other hygroscopic materials tend to measure eRH, or water activity (identical to eRH but expressed on a scale from 0 to 1).

Figure 14 Silica gel hydration seris. Full hydration to partial hydration (left to right) (© MTSN)

Recommendations

Probe type hygrometers

Meteorological (probe-type) hygrometers can be used to measure seed eRH provided that the sensor has an air-tight seal at its probe end and that the sensor itself can be located into a suitable 'sample chamber' also through an air-tight seal.

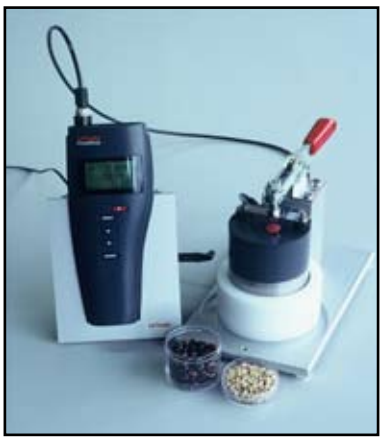

Figure 15 Rotronic hygrometer for measuring the eRH of seed samples. (© Rotronic Instruments (UK) Ltd)

Self contained dataloggers

Miniature RH / temperature dataloggers can be used to record the eRH of seed samples inside sealed containers. Such instruments can be used for example, in monitoring the behaviour of seed collections as seed containers are moved from ambient to sub-zero temperatures and back again.

Dataloggers also can be used for monitoring the moisture status of seed collections in transit. Located amongst the seeds inside a single cloth bag or amongst a number of collections boxed up for shipment, dataloggers can provide important clues to explain the final quality of collections arriving at a seed bank.

Figure 16 Datalogger recording eRH in a sealed container of seeds. (© RBGK)

Low cost mechanical and electronic hygrometers

Mechanical and small electronic hygrometers can be purchased for under 50 Euros. Although generally much less accurate, these instruments could be used to roughly determine the moisture status of seeds. Such instruments placed amongst seed collections during a collecting mission could be used to indicate whether certain collections needed to be dried further. The instruments could also be used to check that ambient conditions were suitable for drying and subsequently to confirm that drying had occurred.

Indicating silica gel

An ingenious and very cheap way of monitoring the progress of seed drying is to use moist indicating silica gel as a surrogate for seeds. Silica gel beads held inside porous packets can be placed amongst seeds during drying. As the seeds dry, the silica gel loses water and changes colour.

Figure 17 Range of mechanical and electronic hygrometers. (© RBGK)

Research priorities

Two issues emerged during and subsequent to the workshop which should be considered for further work.

Seed moisture content database

Since most seed banks routinely record seed moisture content and an increasing number of banks also routinely record eRH values, it would be useful to compile this information into a shared database (e.g. the ENSCONET database). Because moisture content at a given eRH is a good indicator of seed oil content, such a database would add significantly to our understanding of the taxonomic distribution of oily seeds.

Drying problems in certain species

Evidence gathered by RBGK indicates that species in certain families such as the Fabaceae, especially species with relatively large seeds, can be slow to dry. The potential risk is that seed collections could be banked before they are completely dry. This problem is compounded by the difficulties described below in getting accurate eRH measurements for such seeds unless they are crushed.

Further data on the moisture content and eRH of seeds during seed drying and at the point of seed banking needs to be gathered for targeted families.

TOPIC 3B. USING HYGROMETERS FOR MONITORING SEED MOISTURE

General comments

A number of possible sources of error need to be considered when using hygrometers for monitoring seed moisture.

Seeds with impermeable seed coats

As a precaution, it is recommended that seeds with physical dormancy (i.e. seed coats impermeable to water, see Table 1) are cut open or crushed immediately prior to testing. To avoid the possible rapid gain or loss of water from the seeds as the internal tissue becomes exposed, it is extremely important that crushed seeds are transferred to the sample chamber of the hygrometer as swiftly as possible. The obvious disadvantage of such treatments is that the seeds are destroyed.

Table 1. Families containing genera reported to possess physical dormancy. For further details refer to Baskin (2003)
Anacardiaceae
Bixaceae (including Cochlospermaceae)
Cannaceae
Cistaceae
Convolvulaceae (including Cuscutaceae)
Cucurbitaceae
Dipterocarpaceae (subfamilies: Montoideae and Pakaraimoideae but not Dipterocarpoideae)
Fabaceae (subfamilies: Caesalpinioideae, Mimosoideae and Papilionoideae)
Geraniaceae
Malvaceae (including Bombacaceae, Sterculiaceae and Tiliaceae)
Nelumbonaceae
Rhamnaceae
Sapindaceae
Sarcolaenaceae

Sample chamber selection and sample size

Ideally, the largest sample chamber that can be completely filled with the seeds to be tested should be chosen. When sample size is limiting, the following rule of thumb is recommended. A sample chamber should be selected such that the weight of seeds to be measured in grams is at least 10 % of the total air space in cm^3. For example, for a 70 cm^3 sample chamber the weight of seeds should be at least 7 g (see Probert *et al.*, 2003 for further details).

Measuring non-equilibrated samples

When measuring the eRH or water activity of non-equilibrated seeds (for example seeds actively drying), it is extremely important that ample time is given for the seeds to attain equilibrium inside

the hygrometer sample chamber. In order to give ample time, it is strongly recommended that measurements are datalogged, if possible, or at least that values are periodically checked and noted so that a time to equilibrium graph can be plotted.

Importance of temperature

We know from moisture sorption isotherms that equilibrium values are temperature dependent (ISTA, 2008). Consequently, if there is a discrepancy between the temperature of the seeds to be measured and the temperature of the hygrometer sample chamber, an incorrect value could be recorded. Such a situation could arise for example, if a water activity hygrometer was being used to check the moisture status of collections held in a seed bank. Usually such measurements would be performed at room (or dry room) temperature. If the seed collection to be tested had not fully equilibrated to the temperature of the room, and was significantly colder, then an inaccurate value could be recorded. This inaccuracy could be higher or lower than the correct value depending on how quickly the seeds were transferred to the sample chamber and on the ambient RH. For example, if very cold seeds were exposed to the open air, even for a couple of minutes, there is a significant risk that water molecules would be absorbed onto the surface of the seeds by condensation. This (additional) water would be quickly lost to the sample chamber atmosphere during measurement giving an inaccurate high reading.

Thus it is strongly recommended that if seed samples are to be measured at a temperature different from that where they have been held, then ample time is allowed for the sample to equilibrate to the measuring temperature before the container is opened and the sample is transferred to the hygrometer sample chamber.

Recommendations

The following guidelines for routine, laboratory monitoring of seed moisture status (taken from Probert *et al.*, 2003) are recommended:

- Choose a suitable sample chamber size to minimise the total headspace above the seed sample.
- The weight of sample in g must not be less than 10 % of the volume of the total headspace in cm^3.
- If the seeds are known to possess impermeable seed coats (presence of physical dormancy) the seeds should be cut, coarsely ground or crushed immediately before placing into sample chamber. Note that the pestle and mortar need to be cleaned and dried between samples.
- The temperature of the seed sample must be fully equilibrated to the sample chamber temperature before starting the measurement.
- Remember that the human body is constantly releasing water vapour through the skin and in the breath. Minimise direct handling of seeds, avoid touching the inside surfaces of the sample chamber and breathing on an exposed sensor.
- Manufacturer's claims for equilibration time should be treated with caution. We recommend allowing at least 30 min for accurate measurements of seeds that are at a stable moisture status and substantially longer if the internal and external tissues of the seeds are thought not to be in equilibrium.
- Wherever possible, measurements should be datalogged and final equilibration time should be interpreted from graphical data.
- Sensors should be regularly calibrated in line with manufacturer's recommendations.

Many hygrometers are designed to operate by battery as well as mains power and can therefore be used in the field. The sensor head can be exposed to the air (not attached to the sample chamber) to give measurements of ambient RH and temperature and samples of seeds at the point of collection can be loaded into an appropriately sized sample chamber for measurement of seed eRH.

TOPIC 4. PACKAGING

Summary written by Costantino Bonomi (MTSN) based on the key presentation by Cesar Gomez Campo (UPM), additional presentations by RBGK and MAICh, and subsequent discussions.





Figure 18 Selection of packaging types. (© NBGB, UPM, MAICh & RBGK).

General comments

Appropriate packaging is essential to keep seeds dry and thus maintain their longevity during storage (see Gomez-Campo, 2002). The low temperatures used in seed storage are often characterised by a high relative humidity which increases the risk of moisture ingress into seed containers. Even if this process is very slow due to the low temperatures, given enough time, it will eventually be detrimental to the seeds. Providing an efficient barrier to water vapour transfer is the key for long-term conservation. Should it be overlooked, it might jeopardise the overall safety of seed bank collections. It is therefore essential to carefully select validated moisture-proof containers and implement systems to monitor their performance.

The first issue to address is therefore the selection of appropriate moisture-proof containers (see particularly, Gomez-Campo, 2002 and Manger *et al.*, 2003 for a description of different containers). Very few materials are completely moisture proof. Soft plastic (PET-like) is not recommended, hard plastic (PVC-like) might be better performing but requires further investigation and is currently not recommended. At present, glass is the best performing material according to the information available. Some concerns (see Gomez-Campo, 2006) have been expressed about the effective barrier provided by tri-laminate (polyester, aluminium and polythene layered) foil bags over long periods but these are still widely used in seed conservation institutions (see also Walters, 2007 and Gomez-Campo, 2009). One disadvantage is that these cannot be monitored by visual inspection because they are not transparent.

In order to monitor the effectiveness of container seals during long-term storage, it is recommended that small silica gel indicator sachets are placed inside the containers. Silica gel has the additional benefit of being capable to absorb ethylene and other potentially harmful gases produced by the seeds themselves as catabolites during the slow ageing process. It is important that the silica gel sachets are equilibrated to the drying conditions adopted by the seed bank to avoid the risk of over drying.

Other critical issues to address are the seal of the container and the accessibility of the content if re-sealable containers are used. The seal might need to be periodically checked and substituted. It is recommended that natural rubber seals are used; these have performed well in current testing and use (RBGK data).

Containers have been regularly leak-tested (see below for method) by different seed banks and this is a recommended procedure that should be carried out by all seed banks to counter batch to batch variation in container performance.

There is some evidence that oily seeds might benefit from vacuum packaging as oxygen in the air could promote lipid peroxidation (see also Ellis & Hong, 2007). However vacuum packaging also increases the risk of moisture ingress because of the negative pressure and, in the case of irregularly-shaped fruits held in foil bags, the risk of pin-holing. Additionally, the extra creasing might potentially increase the stresses on the bag. Note that vacuum sealing of other containers such as tins cans is possible.

Leak test procedure

Place 1 g of indicating silica gel beads per 1 l volume of empty container (0.1 %, weight to volume ratio). The silica gel should be oven dried and the container equilibrated to dry room conditions. Place containers under test in a moist (sealed box containing water) environment at room temperature for a minimum of 4 weeks and then transfer to -20 °C for at least one year and preferably for as long as possible. At least 10 containers per batch should be tested and it is recommended that the pass level should be set at 100 %. A single failure indicates that the entire batch should be rejected.

Recommendations

- Choose container using decision tree illustrated below.
- Preferably package seeds in the dry room or in another humidity controlled environment.
- Use transparent glass containers (screw top vials and bottles, clamped vials and clamped jars performed best in the current practice across existing seed banks); avoid plastic bags.
- Include a humidity indicator silica gel sachet in the container for monitoring seal performance and protection against catabolites. These must be equilibrated to the seed bank drying conditions (usually 15 % RH). Employ an annual inspection routine to check for leakage.
- Periodically check and replace seals (if applicable).
- Double or triple package (as in the Russian doll way of packaging) to increase protection against water vapour intake particularly for 'base' collections that will remain untouched for long periods.
- Leak test batches of containers employing a standard leak test procedure.

Decision Tree for container selection

1 You need to move accessions between different institutions or otherwise transport them over long distances	use foil bags (low weight and low risk of breakage)
1 You need to store accessions without moving them for long distances	
2 you aim at short term storage	either use foil bags or glass containers
2 you aim at long term storage	
3 you need to access your collection regularly	use re-sealable transparent glass containers with a leak control system or split the collection into several flame-sealed glass containers
3 you do not need to access your collection	use flame-sealed glass containers (for small collections), double packed re-sealable glass containers with indicating silica gel sachets and a regular inspection routine or well sealed high quality double packed foil bags.

Research priorities

- Use material science university departments to further investigate the physical properties of different materials (including hard plastics) and container closure systems to design a container for long-term seed storage that combined moisture-vapour proof properties, accessibility and potential affordability.
- Obtain more data on the effects of vacuum packing oily seeds.

TOPIC 5. LONG-TERM SEED STORAGE: TEMPERATURE

Summary written by M. Elena González-Benito (UPM) following the key presentation by Hugh Pritchard (RBGK), an additional presentation by MAICh and subsequent discussions.

Overall comment

It is well known that the reduction in seed moisture content (see topic 2D) and / or storage temperature prolongs longevity in orthodox seeds (see Roberts & Ellis, 1989; Pritchard & Dickie, 2003). Therefore, the use of a storage temperature below 0 ºC (usually between -18 and -20 ºC) is recommended for the long-term storage of most orthodox seeds (FAO Gene Bank Standards, 1994; Rao *et al.*, 2006).

With this general recommendation in mind, the following topics will be discussed, which should be helpful when designing a seed bank for wild species especially where there is no published information on seed storability: (1) the storage temperature to use; (2) the identification of seeds that store poorly at -20 ºC and steps that may be taken to ameliorate this problem; and (3) the opportunities and risks of cryo-storing seeds.

Topic 5A. STORAGE TEMPERATURE

General comments

The benefit of using low temperature for seed storage has been quantified through the seed viability equations (Ellis & Roberts, 1980). As storage temperature is decreased, seed longevity increases. However, the benefit diminishes as the temperature falls (Tompsett, 1986; Dickie *et al.*, 1990; Walters *et al.*, 2004). Deciding which storage temperature to use is a matter of balancing cost and technical effort against longevity. Most conventional freezers run at -18 to -20 ºC, so this range of temperature is often used. Within this range of storage temperatures, seed moisture contents of 3.5 – 6.5% are recommended (see Topic 2D). However, it should be noted that empirical data validating the use of such conventional storage temperatures as an optimum balance between cost and longevity is essentially lacking. This said, lowering temperature below -35 ºC would seem unlikely to benefit seed longevity sufficiently to warrant the extra costs.

Temperatures as low as -196 ºC, obtained in cryo-storage using liquid nitrogen as refrigerant, can be recommended for short-lived desiccation tolerant seeds (Stanwood, 1985). Hypothetically, there is a 175-fold increase of longevity when compared to conventional storage at -20 ºC (Dickie *et al.*, 1990; Pritchard & Dickie, 2003). More recently, work on dry lettuce seeds has suggested half-lives of 500 and 3400 years in vapour-phase and liquid-phase nitrogen, respectively. This is at least 50-times longer than that expected at conventional seed bank storage temperature (Walters *et al.*, 2004). Cryo-storage could be also recommended for seed storage of endangered or endemic species for which only small amounts of seeds are available, and of course for tissues (embryos, embryonic axes, shoot-tips) of recalcitrant seeds.

There is evidence that some orthodox seeds fully tolerate seed bank-type drying but lose germination capability or have shorter longevity than expected after storage at about -20 °C or at other sub-zero temperatures (Ellis *et al.*, 1990; Pritchard *et al.*, 1999). For example, dry seeds of *Cattleya aurantiaca* (Orchidaceae; Pritchard & Seaton, 1993) and *Cuphea carthagenensis* (Lythraceae; Crane *et al.*, 2003) showed low germination after storage at -18ºC, possibly as a result of lipid transformation / crystallisation. In *C. carthagenensis*, germination was partially recovered after heating prior to imbibition (Crane *et al.*, 2003). The treatments needed to recover germinability (if no damage to the seed has occurred) could be species-specific and may include: slower imbibition; imbibition at a higher temperature; and pre-heating before imbibition (see Pritchard & Nadarajan, 2008).

Recommendations

A storage temperature below 0 ºC (usually between -18 and -20 ºC) is recommended for the long-term storage of most orthodox seeds.

For conventional storage at non-cryogenic temperatures, lowering temperature below -35 ºC would not appear to benefit seed longevity sufficiently to warrant the extra costs.

Research priorities

In view of what has been reported, if a species shows low germination after storage at a sub-zero temperature, it does not necessarily mean that the seeds are dead or that sub-zero storage is inappropriate; further studies are important to clarify the situation with respect to the species. Specific dormancy breaking and/or imbibition treatments may need to be studied. Additionally, storage at different temperatures could be attempted.

A study that examined the cost effectiveness of using different conventional sub-zero storage temperatures would be useful.

Topic 5B. CHOICE OF LOW-TEMPERATURE FACILITY

General comment

Walk-in cold room and freezers

The choice between a walk-in cold room and an upright or chest freezer depends on the number of accessions and the volume of the accessions (seed size). When numbers are small, use of a freezer may be desirable. Upright freezers have the advantage compared to chest (top-lidded) ones that accessions can be reached more easily. They are more efficient in that the number of accessions per unit laboratory floor area is greater than for chest freezers. If freezers are selected, it is advisable to have a back-up freezer in case of failure equipment failure.

If capacity exceeds 10-15 m3 then a walk-in room is more efficient (Cromarty *et al.*, 1990). In such cases, two small rooms with independent freezing systems may be better than one big room especially if there is no intention of filling the storage space completely in the near future (see also Linington, 2003 for discussion about seed bank design).

Whatever the choice of facility, it is important to bear in mind that an appropriate labelling / bar-coding system is essential for easy and rapid sample retrieval. Rapid sample retrieval is particularly important where freezer doors need to be kept open. The accession number, location number (e.g., in UVEG this is Shelf / Rack / Container such as AB/04/10B – see Figure 19) and bar code shown on the label need to be recorded on the seed bank's data base.

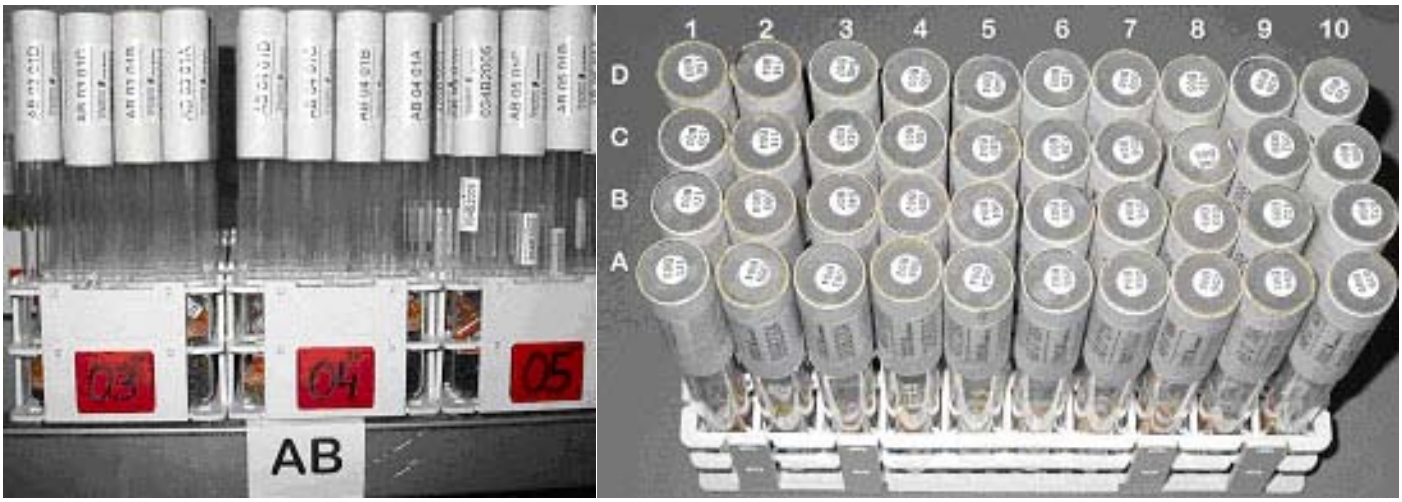

Figure 19 Organisation of collections in the UVEG seed bank. (© UVEG)

Base and active stores and duplicates

Traditionally, seed banks have split each collection between a base sample that is kept untouched for long-term conservation and an active sample that is more readily accessible and for which samples are removed for use. The base samples have usually been kept in less accessible packaging (sealed glass or double packed) and stored under long-term storage conditions. In contrast, the active samples have been stored in readily opened containers (or even in open dry storage) at temperatures that are more conducive to staff access. Potentially, there are problems with genetic divergence of the two samples (see Topic 9, Regeneration). Furthermore, it can be difficult to decide how much of the collection should be required for distribution and how much for long-term conservation. The RBGK (Millennium Seed Bank) operates an active and a base system but keeps both samples under long-term storage conditions. The majority of the sample is held untouched in the base sample.

A major benefit of seed banking is that a large amount of genetic diversity can be curated at one location. This concentration of diversity is also a fundamental weakness in that one accident could destroy extremely valuable (and in some cases irreplaceable) material. Therefore it is essential that in addition to doing everything practical to safeguard the bank's collections, a sample is duplicated (in effect as a separate base sample) at another bank geographically well away.

Cryo-storage

Although storage in liquid nitrogen (-196 ºC) or in its vapour phase (approximately -150 ºC) can prolong the life span of seeds, it is not usually the first choice for seed storage due to technical / practical reasons (see below). A cost-benefit analysis of cryo-storage compared with conventional storage appears not to have been published for over thirty years. Such an analysis would be helpful.

Cryo-preservation is most obviously appropriate when storing orthodox seeds with short longevity or when only very small quantities of seeds are available from a population or species (e.g., endangered or narrow endemics).

Figure 20 Cryo-preservation in liquid nitrogen. (© RBGK)

Although the seeds of hundreds of species have been shown to tolerate liquid nitrogen treatment and / or storage, there are practical challenges when using cryo-storage:

- Liquid nitrogen is a hazard due to burning and asphyxiation. Specialised staff training, appropriate clothing and safety procedures are essential. Because spilt liquid nitrogen evaporates and rapidly displaces oxygen, it is essential that oxygen alarms are installed where liquid nitrogen is stored and used.

- There must be a supplier of liquid nitrogen nearby.

- Big liquid nitrogen containers (e.g. 600 litre capacity for 12,000 cryovials) are expensive. Liquid nitrogen containers must be regularly filled to the required level to keep the samples at the appropriate temperature (approx. -150 ºC). Small cryogenic tanks (e.g. 30 litres with a maximum capacity of 855 vials of 2 ml) can be manually topped up; however, the big ones require a supply container. Although the initial cost of the big containers is high, the liquid nitrogen supply price is lower when compared to the maintenance of small containers as the transport of liquid nitrogen increases the price.

- The method is only appropriate for relatively small quantities of seeds per accession.

- For the optimal handling of the seeds, three main aspects should be considered (Pritchard & Nadarajan, 2008): 1) seed moisture content should be lower than the high moisture freezing limit (Stanwood, 1985) ; 2) fast cooling or re-warming can mechanically damage very dry seeds, but this can be avoided; 3) slow imbibition, particularly avoiding immersion in water at inappropriate temperatures, is recommended either before or as part of the germination test.

Apart from the advantage of extra longevity in cryo-storage it should be noted that cryo-tanks have a lifespan about five times that of freezers (J. Puchalski, pers. comm.) and intermittent electricity supplies are eliminated.

Recommendations

For very short-lived orthodox-seeded species, cryo-storage in or above liquid nitrogen may be advisable.

All samples must be duplicated at a second bank. This duplicate bank should be sufficiently far away that it would not be subject to the same catastrophic loss that could destroy the main collection.

Research priorities

A cost-benefit analysis of cryo-storage compared with conventional storage would be helpful.

TOPIC 6. BASIC GERMINATION TESTING

Summary written by David Draper and M. Elena González-Benito (UPM) based on the key presentation by Costas Thanos (NKUA), additional presentations by PAV-UNI and RBGK, and subsequent discussion.

General comments

In a seed bank, it is important to know the viability of seeds that are being stored. Seed viability is defined as the number of seeds that are alive in a seed lot, and have the potential to give rise to a plantlet (Gosling, 2003; Rao *et al.*, 2006).

As the longevity of a seed lot depends on the seed quality, it is important that seeds have high viability when they are stored, or at least to have known viability. The most reliable method for testing viability is a germination test, i.e. to germinate seeds in optimum conditions after applying a dormancy breaking treatment, if necessary. The problem arises when, as happens with many wild species, optimum germination conditions and the method for breaking dormancy is unknown.

There are also biochemical tests that can be used (e.g. Tetrazolium chloride, TTC), which can be used to identify viable seeds without germinating them. Such tests can be useful as a means of distinguishing between dead and dormant seeds. However, they have the disadvantage that they are not as accurate as the germination tests and the results are sometimes difficult to interpret.

Obviously it is important that we know how to germinate seeds so that they can be used in the future. Therefore, the following section focuses on the germination test and procedures necessary for the production of normal seedlings.

Recommendations

When to test?

Ideally, germination tests should be carried out before and after drying and after banking to check if there are indications that germinability is being affected by desiccation and freezing. However, for the majority of seed banks this may not be feasible. If resources allow it is recommended that germination tests are carried out on as many accessions as possible after seeds have been dried and processed but before they are banked and shortly after banking. If resources are limited and only one initial test can be carried out it is recommended that this should be within the first month after banking. The result of this test will be the benchmark against which future retests will be measured.

How many seeds to test?

Before carrying out the germination tests, an estimation of empty / damaged seeds should be established. Germination percentage should be expressed in terms of the number of seeds which could be physically capable of germination (total number of seeds minus damaged or empty seeds).

The recommended sample size of 200 seeds (two replicates of 100) for agricultural species (FAO / IPGRI, 1994; ISTA 2008), may be too large for collections of wild species, where seed numbers may be limited. This is particularly true for endangered species with small populations or species with large seeds. In these cases, a germination test comprising 100 or even

Figure 21 Germinating test result. (© RBGK)

50 seeds is considered acceptable with the sample divided into two replicates of 50 or 25 seeds respectively (please note that there are no definitive rules on this, for instance, some institutes such as MAICh use three replicates of 30 seeds). The recommendation is also useful with seed banks storing large numbers of accessions and with restricted availability of resources and / or personnel.

Humidification of dry seeds

Seed containers should be warmed up before sample removal (see Topic 10). For species known to be susceptible to imbibition damage, for example large seeded Leguminosae (Fabaceae), seeds should be humidified after removal from storage. This helps avoid imbibition damage i.e. the damage to very dry or aged seeds due to rapid intake of water causing leakage of cell contents because cell membranes have not had chance to repair. Seeds can be placed inside a plastic container with a hermetic closure (similar to those for food storage) or in desiccators, that in turn have a container of water; this water will generate a humid environment. Seeds are left in the container for 24 hr prior to sowing but must not be in physical contact with liquid water.

Choosing the germination conditions

The seed germination response is extremely plastic and requirements can vary considerably between species, populations, collection years and even between intervals of storage. However, prior knowledge from the published literature or databases such as RBGK's Seed Information Database (Liu *et al.*, 2008: http://data.kew.org/sid/sidsearch.html) and LEDA traitbase (http://www.leda-traitbase.org/tomcat/LEDAportal/index.jsp) of germination requirements for a given or related species can be an important guide and in some cases there are trends at the genus and even family level (e.g. light requirement in Campanulaceae and hard seededness in Cistaceae).

When there is no such prior knowledge (as is often the case), germination requirements can be predicted from information about the ecology (habitat and climate preference) and seed structure of the species in question. For example, seeds with copious endosperm and small embryos (likely morphological dormancy) from regions with cold winters and / or hot dry summers (likely physiological dormancy) are more likely to possess morpho-physiological dormancy and therefore require prolonged incubation at conditions that mimic the season prior to germination in the wild.

Dormancy breaking treatments

Seeds set to germinate under optimum conditions may still not germinate, although they are viable, because they are in a dormant state. Dormancy could be due to characteristics of the embryo or of the seed covering (see a recent review on dormancy: Finch-Savage & Leubner-Metzger, 2006). Seeds of many wild species may show dormancy.

Some of the causes of dormancy are (see Baskin & Baskin, 1998):

- *An underdeveloped embryo.* Generally this is the case in seeds with small embryos and copious endosperm. In this type of seeds (with morphological dormancy) the embryo has to grow before the seed is able to germinate (Baskin *et al.*, 2006). Usually warm or cold stratification is required, according to the timing of natural dispersal (e.g., spring or autumn). However, if an undeveloped embryo does not begin to grow immediately after seed dispersal, it could also be physiologically dormant. If this is the case (morpho-physiological dormancy), seed must first experience a period of cold or warm stratification before the embryo begins to develop.

- *Hard (water-impermeable) seed / fruit coats.* These occur in many species of the following families: Anacardiaceae, Bixaceae, Cistaceae, Combretaceae, Convolvulaceae, Curcurbitaceae, Fabaceae, Geraniaceae, Malvaceae, Rhamnaceae, Rutaceae, Sapindaceae and Violaceae. Scarification of the seed coat can be done mechanically (e.g. by chipping, filing or rubbing with sand paper) or thermally (e.g. by immersion in hot water) prior to sowing is required in these cases

to enable water uptake. A third traditional methos is chemical (acid) scarification but it is not recommended due to obvious hazards and the need for careful calibration. Of the methods, careful hand chipping or filing is advisable when dealing with small quantities of seed.

Figure 22 Chipping a hard coated seed with a scapel. (© RBGK)

• *Chemically inhibiting* substances are present in the germination unit, either the embryo or the seed coat. Dormancy may be removed by (cold or warm) stratification, leaching in running water or application of GA_3.

Germination conditions: water/agar, temperature, light

The substrate for sowing could be agar, filter paper or sand. The first two are used inside Petri dishes, and the latter is generally used for large seeds (Rao *et al.*, 2006) in trays or larger plastic containers. The paper should be high quality. It needs to be uniform in order to obtain reproducible results.

Agar (usually 1 %) has some advantages over paper:

- Low maintenance (it does not require regular watering), and therefore there is less variability
- Lower risk of imbibition damage
- Constant concentration of applied chemicals (fresh agar after 3 weeks)
- White radicles are easily visible on a dark background
- It is possible to remove seedling plugs for transplanting

However, if the germination test lasts over 4 weeks, agar could start drying out and the seed may need to be transferred to fresh agar. To minimise this agar plates can be held inside polythene bags or wrapped with parafilm. It should also be considered that some seeds may need to be surface sterilized prior to sowing. Seeds can be surface sterilized by soaking in a 10 % solution of domestic bleach (sodium hypochlorite) followed by a thorough rinsing in deionised water.

In all cases deionised or distilled water should be used. For the filter paper, the volume of water depends on the thickness of the paper; the paper should not be so wet that a film of water forms around the finger when is pressed (Rao *et al.*, 2006).

The seeds should be evenly distributed on the surface; they should not be touching. The containers are then placed in the incubators. The moisture level of the substrate should be visually checked when scoring, especially when high temperatures are used (25-30 ºC). If filter paper is used water should be replaced regularly (every 2-3 days).

Figure 23 Incubator similating day/night conditions. (© RBGK)

Incubation conditions, wherever possible, should try to simulate conditions in the natural habitat at the time of seed germination. Therefore, it is generally better to germinate seeds using diurnal alternating temperatures with light provided during the daytime phase (Baskin *et al.*, 2006). If possible, constant temperatures should be

avoided except during moist stratification treatments. Continuous light should be avoided due to the risk of inhibiting germination (the high irradiance reaction – HIR). Mediterranean species usually have cool optimum germination temperatures (10-20 ºC), and in many cases alternating temperatures of 20/10 ºC or 20/15 ºC (light/dark) work well. Subtropical, tropical and alpine species usually germinate best at warmer temperatures (20-25 ºC). Photoperiods of 8, 12, or 16 hr are generally used. For several alpine and temperate species, a period of cold stratification (e.g. 0 ºC for 4-20 weeks) is known to increase seed germination.

Although a preference for germination in the dark is relatively uncommon this should be borne in mind. There is a greater chance of a dark requirement in relatively large seeds and has been reported in some Cucurbitaceae. *Galanthus nivialis* also germinates better in darkness.

Possible online sources of information on incubation conditions and dormancy breaking pre-treatments are Ellis *et al.* (1995) and the Seed Information Database (Liu *et al.*, 2008: http://kew.org/data/sid/).

Depending on the number of accessions to be germinated and the personnel availability, germinated seeds should be scored regularly and at least once a week. If germinated seeds can be scored every 1-2 days an accurate rate (speed) of germination can be determined. The duration of the test depends on the species and conditions used.

Germination scoring

For germination tests, a germinated seed might be defined as one with a protruded radicle of 1-2 mm long. It is important to have a clear definition for each species before starting scoring. Germinated seeds are removed and counted in each dish. It is also important to remove infected seeds to avoid the spread of infection (Rao *et al.*, 2006) but these must be recorded. Alternatively, a midly infected sample could be surface sterilised and be resown. The duration of germination scoring period varies among species, and could vary from one week to several months. Four to six weeks is typically required for many wild species. The number of germinated seeds at each scoring time is recorded on a data sheet. Other observations should be also included such as the number of infected seeds and abnormal germination.

At the end of the germination test, the non-germinated seeds should be cut-tested to check the number of: full, firm and fresh looking seeds (probably dormant), mouldy and soft seeds (dead) or empty seeds. The mean germination percentage is calculated from the results of all the replicates taking into account the empty / damaged seeds. If the germinated seeds have been scored frequently (every 1-2 days) the germination rate (speed) can also be calculated. For example, T50 is a germination rate index which is defined as the time required for the germination of half (50 %) of the seeds that eventually germinate under the particular set of conditions.

The germination data (final germination percentage and germination rate) should be included in the seed bank database, together with the pre-treatments applied and the incubation conditions used.

Research priorities

An assessment of the relationship between radicle emergence and normal seedling production in wild species would be useful. The link between the two ‘forms’ of growth gives cross-reference to the ISTA approach to seed germination testing. Photographs taken of seedlings would be useful for identification.

TOPIC 7. VOUCHERS AND VERIFICATION

Summary written by Simon Linington (RBGK) based on the key presentation of Gianni Bedini (Pisa Botanic Garden) and subsequent discussions

General comments

The correct scientific name for the species represented by the seed collection is essential. The correct name links the seed collection to everything that is known about the species. Seed users want to be confident of the identity of their material (Goldblatt *et al.*, 1992) to prevent erroneous results. Vouchers are essential to clarify unexpected results (Funk *et al.*, 2005).

Sometimes plant populations are so well known or the collector is so familiar with the species that the field identification can be accepted. More usually, one or more pressed specimens (see Bridson & Forman, (1998) for technique and associated information) are collected in the field. These should represent the plants in a population from which seeds are collected and are used to confirm identification by reference to known specimens in a herbarium. Usually, the specimen (known as a 'voucher') is collected at the same time as the seeds. However, the plants at this time may not be suitable for verification. Consequently, it is sometimes necessary to anticipate this and collect the vouchers before the seeds.

The RBGK (Millennium Seed Bank Project) currently advises that all duplicated specimens of woody species are taken from the same individual that represents the average characteristics of the population and from which some of the seed was harvested; this ensures all herbaria receiving the duplicates have identical material. However, it could be argued that each duplicate specimen should be harvested from a separate individual and labelled as such to give greater information about morphological variation.

Figure 24 Herbarium specimen. (© RBGK)

Verification can be made by an experienced botanist in the field or later in the herbarium. Botanical keys are one of the most common tools used for indentification. Verifiers should be recorded as should any change of name designation.

Please see Bridson & Forman (1998) for details about voucher curation.

It is important that the seed bank receives name changes applied to the herbarium specimen as a result of taxonomic revision. Therefore, the herbarium label should have some note that it is linked to a seed banked sample.

Recommendations

Except in the few cases where populations are well known, a pressed specimen should be collected to represent the population from which seeds have been sampled. Obviously care should be taken if rare or endangered populations are involved. Photographic images are also useful.

TOPIC 8. DATA

Summary written by Simon Linington (RBGK) based on the key presentation of Gianni Bedini (Pisa Botanic Garden) and subsequent discussions

General comments

Collections without good data are nearly useless. When recording data about each seed collection it is vital to remember that the data will be meaningful to users of those data both now and during the lifetime of the collection (perhaps 200 years hence). The data therefore must be objective. It should be assembled in a uniform way. This is achieved by using data standards. ENSCONET has published data standards (see ENSCONET database schema, 2009). These standards should be kept with the data.

Collecting data (see ENSCONET Collecting Manual, 2009) are recorded in the field. The collection management data are linked to the collecting data and are added to as the seed collection and the accompanying herbarium voucher are processed. If well structured, the data can help the seed bank manager track the processing of the material. Recording collection supply and use is important.

Syntax, namely the rules governing data entry, is important, as it is with all data recording. Therefore, data checks need to be carried out on input e.g. no future dates; month <= 12 etc.

Curation data can be grouped (see for example, Bone *et al.*, 2003):

- Cleaning
- Drying
- Packaging
- Storage
- Verification
- Distribution

Detail of individual fields and their standards are shown in the ENSCONET database schema (2009). The notes fields can be used to record variations of procedure but beware that there is the danger that these might not get picked up in general data searches.

Where possible, use of the International Transfer Format developed by BGCI is suggested. These will facilitate data sharing and decrease the data preparation when submitting data to shared databases.

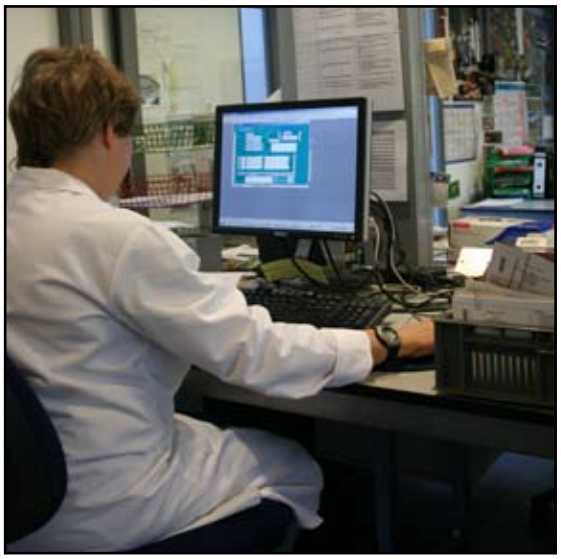

Figure 25 Good data management is essential. (© RBGK)

TOPIC 9. SEED REGENERATION

Summary written by Albert-Dieter Stevens (BGBM) based on the key presentation of Simon Linington (RBGK) and subsequent discussions

General comments

Definition & purpose

Seed regeneration is the generation of a new seed-lot either when viability falls to a given level in an old seed collection or when seed numbers in a collection fall to a given low level. The latter is often termed 'seed multiplication'.

An essential element of seed regeneration is the maintenance of genetic integrity of the original sample. The two concerns are maintaining the occurrence of different alleles and, if possible, maintaining the frequency of those alleles. It is an expensive operation that is difficult to do well and that is best avoided if possible by the collection of large, high quality seed collections in the field. In some cases, it may be better to recollect directly from the wild (should that still be possible) rather than carry out regeneration.

Figure 26 Regeneration of *Bromus bromoideus* (extinct in the wild). (© RBGK)

The viability level at which seed collections need to be regenerated is called the 'regeneration standard'. This is usually set at a high level (75-85 %) to avoid genetic damage that is linked to loss in viability and to avoid deteriorating field establishment. Because seed collection viability is normally monitored at 5 or 10 year intervals by means of a germination test, it is essential that seed dormancy is first removed. Due to statistical error, as the regeneration standard is approached, it can be difficult to be certain whether the collection viability is still above the standard. A procedure called 'sequential testing' whereby sample size is increased until the above decision is resolved has been put forward (see Ellis, *et al.*, 1985). However, because this is an involved process, most banks will either failsafe by regenerating early or will accept the risk that regeneration might be carried out late. Consideration should be made that field establishment will be significantly lower than laboratory germination. Therefore, numbers of plants raised should take this into account.

Seed multiplication needs to be carried out when seed numbers collected in the field are low (e.g. for small populations of endangered species) or when seeds in storage have fallen to a given level. This level should be set so that there are enough seeds for several (say three) attempts at regeneration (say, 500-1000 viable seeds). An ideal situation (rarely achieved) would be where a collection was used up such that the need to regenerate for low seed numbers coincided with reaching the regeneration standard. Seed numbers in storage fall for two reasons. Firstly, seeds are used for monitoring the collection. This fall is reasonably predictable based on the frequency of retest monitoring and size of sample used. Secondly, seeds are supplied to users for research or conservation work. This reduction is rather unpredictable; some collections may be very popular and others rarely used. A number of banks split each seed collection into a base (conservation) sample and an active (distribution) sample. In this way, the base collection is rarely touched and the

active is used for distribution. In most banks, the active collection is held under shorter-term storage conditions than the base collection. At others (e.g. RBGK), the two are kept under similar long-term conditions. In the case of very popular samples, the number of seeds in the active collection may rapidly decline. This active sample may then be multiplied to generate a fresh sample. There is the danger that if this is done too often (especially at a site away from that where it was collected), the active collection may diverge genetically from the base collection. Therefore, periodically, samples should be removed from the base collection to regenerate the active collection.

Accessions with low viability should be regenerated with priority compared to accessions with low number of seeds.

Risks

Seed regeneration involves risks to the genetic integrity of seed accessions due to selection, genetic drift (the random loss of rarer alleles in small samples) or hybridisation with closely related material (especially of the same species) growing nearby (see Breese, 1989 for a clear, detailed discussion). Seed bank staff should do their best to reduce these risks though it has to be accepted that perfection will rarely be attained.

Hybridisation is a major risk with out-breeding species. It should be noted that many inbreeding species exhibit a low level of out-crossing; this level can increase under certain environmental conditions. Even some apomictic species may be facultative out-breeders. Selection can take many forms. The obvious one is brought about by growing up the material under conditions very different to those in the wild. The selection is brought about by climatic and soil (including microbial) differences compared with the original site. There may be pest or disease selection pressures under cultivation not experienced by the material in the wild. There can also be competition effects if the plants are grown at significantly greater density than might occur in the wild. Collection managers need to be aware that standard horticultural practice is to weed out off-types or weak plants; clear instruction needs to be given not to do so.

Further selection can result from differences both in timing and amount of the production of flowers, pollen and seed. The effects of this can be limited by equalising the seed contributed by each female parent.

Unless part of the collection is held back for safety, there is also the risk of complete loss through adverse environmental conditions. Obviously, a balance must be achieved between splitting the collection for safety and creating a bottleneck by regenerating samples that are much smaller than the original field collection such that alleles are not carried forward to the next generation or that inbreeding depression (in the case of out-breeders) occurs.

A further risk is mixing the seed after harvest with that of morphologically similar collections. This risk should be no greater than under other seed collecting / processing circumstances provided commonsense is used.

Genetic aspects of regeneration

The aim of regeneration is to achieve complete random mating between a constant number of parents at each generation and to equalise the reproductive output of parent plants (see Breese, 1989). In the wild, not all plants in a population contribute equally to the next generation for a variety of reasons and this is why single samples collected in the wild rarely fully represent the genetic diversity of a species present at a site.

In regeneration, the aim is to minimise the loss of genetic variation such that genetically, wild sampled seed is the same as that from the first regeneration, the second regeneration etc. Ideally, regeneration should be carried out as few times as possible and using the maximum number of individuals. This said, one 'bottleneck' can cause significant loss of diversity. For instance, if 50 individuals are sampled in the wild then at least 50 should be used for regeneration. If 10 individuals are used for the next regeneration, significant diversity may be lost. Even if subsequent regenerations use 50 individuals, the bottleneck will have had a strong influence. There is the danger that random sampling to choose the seeds to raise the plants for regeneration may not be random enough. For instance, by chance, 10 of the 50 plants might have come from one plant sampled in the field. This problem is overcome by keeping seed harvests from individual plants separate (see below).

It is essential that the breeding system of the species is considered. A breeding system is defined as "all factors, apart from mutation, affecting the degree to which gametes (that) fuse at fertilisation are genetically alike" (Thain & Hickman, 2000 - see also Annex 1). However, it is important to note that breeding system can vary between populations of the same species (not all species are obligate in their breeding system) and incompatibility systems are known to break down at the end of flowering in many species.

Obligate in-breeding taxa (e.g., species with closed (cleistogamous) flowers) and apomictic taxa do not require controlled pollination. However, all out-breeders must be subject to a controlled pollination management (see below).

It is important to note that if out-breeders are forced to inbreed e.g., by being reduced to a few individuals then it is possible to get 'inbreeding depression' as the frequency of homozygosity increases. For instance, it is possible to overcome the self-incompatibility system in *Brassica oleracea* (by opening the flower bud and pollinating the stigma) and force it to inbreed. However, this eventually would lead to inbreeding depression. Similarly, when in-breeders are encouraged to outbreed then you can get 'out-breeding depression' and frequency of heterozygosity increases. In both cases, the fitness of the individuals is reduced.

Method of pollination

Under most circumstances, pollination must be carried out artificially by a variety of means that replicate the effect of pollen vectors but most often by brushing pollen from the anthers of one plant onto the stigma of another plant.

Timing of pollination is essential for successful fruit set. The receptivity of the stigmas is time sensitive as is the opening of anthers and release of pollen. Furthermore, the natural life of pollen varies from 30 minutes in some grasses to much longer than a day with Gymnosperms and some fruit trees (Richards, 1997) to many days, in orchids for example (Pritchard, H.W., pers. comm.). The point of pollen release might be taken as a guide to pollen ripeness (Pritchard, H.W., pers. comm.). Because stigma ripeness is more difficult to judge, hand pollination should be carried out on several dates.

The ideal method for ensuring that genetic variation is maintained during regeneration is by paired reciprocal crosses (see Breese, 1989). The individuals to be regenerated are randomly paired and then (in the case of monoecious plants) crossed both ways (reciprocally) with each plant acting as both a maternal and paternal parent. To avoid mix up, plants should be tagged. Ideally, the seed harvests from each parent should be kept separate. The next best method is to keep the seed from each pair separate. While maintaining genetic integrity, this may not be that practical because curation of the seed will be made more complicated. For instance, seed will need to be bulked

for germination monitoring and for providing samples for users. The alternative more practical approach which reduces genetic integrity is to bulk the harvests from each plant and in doing so, equalise the contribution of each parent to the bulk. Some institutes (see Chorlton *et al.*, 2003) keep the individual seed harvests as a conservation sample, a 'balanced bulk' and the remaining 'unbalanced bulk' (i.e. from plants producing excess seed) for supplying seeds for use where genetic representation is not important or less so.

In many cases, paired crossing will be too time-consuming. Under these circumstances, crossing between all parents selected for regeneration (a poly-cross) can be carried out. The number of individuals that contribute to the seed-lot can be maximised by carrying out hand pollination on several dates to cover early and late flowering. It is important to ensure that one or more parents do not dominate in pollen contribution. Again, the seed from individual parent plants should be kept separate if possible. Less good will be to bulk the seed, equalising the contribution from each parent.

Figure 27 Insect pollination should be avoided because it may results in hybridisation. (© RBGK)

The least satisfactory option is to allow totally random pollination using natural vectors such as breezes or any appropriate insects that might be present.

Genetic isolation

During regeneration it is necessary to avoid contamination by accidentally introducing pollen from one collection of a species onto plants of another collection of the same species or its close relative. This is achieved by cleaning pollination brushes and forceps carefully and by sensible isolation procedures.

Simple isolation can be achieved through keeping collections physically separated. The distance will depend on whether insect or wind pollination is involved and what physical barriers (e.g. glasshouse walls or insect-proof mesh) separate the collections. Obviously, within the confines of most institutes / botanical gardens, isolation distances will be limited. Therefore, the options are usually to prevent other related collections from flowering during pollination (possible with perennials) or to bag flowers up in breathable half film / half paper 'bakery' bags (or similar) prior to pollen release and carefully introduce the required pollen by briefly removing the bags.

Where bags are used, it is important that flower and fruit development is encouraged without

increasing damage due to excess of humidity and infections. Bagging should be applied at early bud stages and bags can be removed if fruit development has started.

Growing conditions

It is impossible to standardise regeneration processes for all species and recommendations and protocols must be developed on a species bases. Species specific ecological information, research, and comprehensive documentation of all steps in cultivation are of crucial importance for regeneration.

Sufficient seeds must be sown to yield the required number of plants for regeneration. If sowing is direct into compost or soil, germination will be less than that obtained in the laboratory. Additionally, there could be mortality of plants before they reach reproductive stage. A factor will need to be allowed for each in estimating the number of seeds required.

Regeneration should take place under similar ecological growing conditions to the original populations (light, water, soil type and pH, temperature etc.) but exclude predators, weeds, and pests. Plants will need to be spaced to minimise competition. A high number of species might not set seed in the first year(s), therefore long term maintenance and stability of the regenerating plots should be considered. Initiation of flowering and fruit ripening might require specific environmental triggers or conditions which would have to be applied or controlled artificially. This can be important for fruit ripening and seed maturation regarding water supply or temperature. Germination and subsequent growth phases should be planned according to seasons so that seed maturity can coincide with favourable weather conditions. The plants should be inspected at regular intervals. These inspections are an opportunity to confirm that the correct material is being grown.

Harvesting should take place at the stage of natural dispersal. In a number of cases such as dehiscent capsules, it may be necessary to bag the seed heads just prior to dispersal.

Documentation

There is the tendency for horticulturalists to keep information in their heads rather than to write it down. For many wild species, there will be little in the literature about cultivation let alone about regeneration. Therefore, staff should be encouraged to document every aspect of the regeneration process. This information if databased could prove unique and thus very valuable to other seed banks and those involved in conservation or restoration projects.

A suggested data form is shown below in Annex 2. When data are shared more widely, it is useful to have some characterisation of the regeneration site including soil type, temperature regime, light conditions, watering regime, preparation and planting scheme.

Although not on the form in Annex 2, data about the proportion of flowers pollinated that set seed and seed yield (number) per plant would be useful in guiding future seed multiplication efforts.

Research priorities

Research on genetic diversity would help increase our knowledge about the minimum size of samples and number of plants for efficient sizing of regeneration populations. Reliable identification (genetic fingerprinting) of accessions, which are *relatively* easy and cheap to run, would help to monitor integrity of samples regenerated.

Lack of information on breeding systems of most taxa renders the proper management of accessions for regeneration difficult. Research on reproductive biology (e.g. outcrossing rates, sexuality versus apomixes) would help proper adaptation of regeneration procedures.

ANNEX 1. SUMMARY OF KEY PLANT BREEDING SYSTEMS

See Richards (1997) for much more detail.

♂ - male (stamen = anther containing pollen grains + filament)
♀ - female (carpel = ovary containing 1 or more ovules + style + stigma)
♂♀ - hermaphrodite – can relate to either plants or flowers

Flower organisation	Plant	Flowers		Note
Dioecy[1]	♂ or ♀	♂ or ♀	Outbreeding (obligatory)	A
Monoecy[2]	♂♀	♂ or ♀	Outbreeding	B
Hermaphrodicity	♂♀	♂♀	See below	C
Self - incompatability				
- Gametophytic	♂♀	♂♀	Outbreeding	D
- Sporophytic	♂♀	♂♀	Outbreeding	E
Different types of flower in species [a]	♂♀	♂♀	Outbreeding	F
Anther and stigma separated – pollination requires insect visit [b]	♂♀	♂♀	Outbreeding	G
Pollen release and stigma receptivity separated in time [c]	♂♀	♂♀	Outbreeding	H
Inbreeding	♂♀	♂♀	Inbreeding	I
Asexually created seeds [d]	♂♀	♂♀ or ♀	Asexual	J

[a] Heteromorphy, [b] Herkogamy, [c] Dichogamy (Protandry - ♂ ripe first on plant; Protogyny - ♀ ripe first on plant), [d] Agamospermy.

[1]&[2] Many variations e.g.

Flower organisation	Plant	Flowers
[1] Gynodioecy	♂♀ or ♀	♂♀, ♂ or ♀
[1] Androdioecy	♂♀ or ♂	♂♀, ♂ or ♀
[2] Gynomonoecy	♂♀	♂♀ or ♀
[2] Andromonoecy	♂♀	♂♀ or ♂

See Richards (1997) for more detail.

Note		Comments	Examples
A	Dioecy	Scarce. Look at flowers	Salicaceae. Tropical forest trees
B	Monoecy	Scarce. Look at flowers. Often display dichogamy.	Large wind- or water-pollinated plants / those with capitula / umbels. Cyperaceae
C	Hermaph-rodicity	Very common. Look at flowers. Natural pollination can give important clues to breeding system	
D	GSI [3]	Commoner cf. SSI. Found in most orders of flowering plant. Experimentation or literature.	Some Poaceae, *Corylus avellana*
E	SSI [4]	Experimentation or literature.	Many Brassicaceae, Asteraceae
F	Hetero-morphy	Different types of flowers – often reciprocal herkogamy (see below). Look at flowers. Morphs are typically cross-compatible but within-morph incompatible.	Rubiaceae, Boraginaceae, Plumbaginaceae, Primulaceae (Pin / thrum in *Primula*)
G	Herkoga-my	Anther and stigma well separated. Look at flowers.	Orchidaceae, Primulaceae
H	Dichogamy	Experimentation or literature	Caryophyllaceae (protandry), some Brassicaceae, Rosaceae (protogyny)
I	Inbreeding	Correlated characteristics (see below) or closed flowers ('cleistogamy'), experimentation or literature	Many pioneer annuals, crop species
J	Agamos-permy	Some species are able to produce their seed sexually or asexually. Literature	*Taraxacum*, some *Ranunculus*

[3] GSI (Gametophytic Self incompatibility). See Richards (1997). Same alleles control recognition factors operating in both pollen and stigma. Factors operate independently in the diploid stigma and in the haploid pollen. When pollen and stigma carry same factor there is incompatibility and the pollen tube does not grow down to the ovule.

Pollen genotypes	S1 & S2	S1 & S2	S1 & S2
Stigma genotype	S1S2	S1S3	S3S4
	Incompatible	Partially compatible	Fully compatible

[4] SSI (Sporophytic Self incompatibility). See Richards (1997). Control of the pollen grain comes from the sporophytic anther that gave rise to it. There is dominance of the factors shown in the pollen grains but independent expression of the alleles in the stigma.

Pollen phenotype	S1 (assume S1 > S2)	S1	S1
Stigma genotype	S1S2	S1S3	S3S4
	Incompatible	Incompatible	Fully compatible

ANNEX 2. POSSIBLE DATA FORM FOR REGENERATED COLLECTIONS

Site	
Location at site	
Collection no.	
Genus	
Species	
Infra-specific taxon	
Anticipated breeding system	
Conservation status	
Date sown	
Number of seeds sown	
Date germinated	
Date seed collected	First Last
Collector(s) of seed	
Isolation (please tick)	No control: open pollination Spatial: nearest member of same genus at least ……metres Spatial: nearest member of same species at least ……..metres Physical barrier to pollen/insects (specify) Other (specify) Unknown
Controlled pollination (please tick as appropriate)	Date pollinated: Self pollinated Cross pollinated with same accession Cross pollinated with another accession (specify collection no.) Details:
No. plants in accession	
No. plants from which seed harvested	

Relation of seeds to original collection made from wild (please tick)	Seeds harvested from plants raised from vegetative material taken from wild (generation 0) Seeds harvested from plants grown directly from wild-collected seed (generation 1) Seeds harvested from plants grown from generation 1 seed (generation 2) Unknown generation = 9
Herbarium specimen (please tick)	Collected from plant from which seed harvested (see next 7 rows) Unable to collect suitable specimen Not collected as already verified (see 8 rows down)
Date of collection of herbarium specimen	
Collector of herbarium specimen	
Plant lifecycle (please tick)	Annual Biennial Perennial
Plant height (metres)	
Other description of plant	E.g. habit, flower colour, odour, latex and anything not apparent from the herbarium specimen:
Photograph taken (please tick)	No Yes Location of photograph:
Details of previously verified material	Verifier: Institute: Date: Material verified (wild living, wild cultivated, herbarium specimen etc):
Collection status (please tick)	Complete More seed to follow (specify details) Herbarium material to follow (specify details)
Notes (flowering date, fruiting date etc)	

TOPIC 10. SEED DISTRIBUTION

Summary written by Albert-Dieter Stevens (BGBM) based on the key presentation of Simon Linington (RBGK) and subsequent discussions.

General comments

Seed samples are distributed from wild species seed banks for a variety of purposes. This distribution might be internally within the parent institute or externally (nationally / internationally). These uses involve pure and applied research, education, breeding, species re-introduction and habitat restoration. The applied research and breeding may involve the private sector.

Many seed banks issue seed lists (some on-line) for users to select from. These lists should be made up of collections with adequate quantities of seed, acceptable germination levels and verified identity. Collections should be excluded if they cannot be distributed because of restrictions placed on them under the terms of any collecting agreement e.g., by a government, national park or land-owner. Species that are known to be strongly invasive or that are known to be harmful should not be included (or if they are included should have a clear warning). This said, nearly all species have the potential to be invasive given the right conditions, so a general warning to users about this risk is important. If possible, all relevant data (including germination conditions) should be linked to the seed list; if not, the data relating to the requested seed should be distributed either as hardcopy with the consignment or electronically. If any handling charges are payable, this should be made clear on the seed list; in most cases, realistic handling charges will barely cover costs of administrating the recovery of this money.

It is important that seed banks maintain a base stock of seed that is not distributed. This is either achieved by physically separating a conservation (base) sample from the used (active) sample or by having a stock control system on the seed bank database that signals when all the active portion of the sample is used up. In this latter case, a failsafe seed stock system will be necessary whereby the data management system shows that there are less seeds in the bank than are actually present. This can be achieved to a high level by determining the upper confidence limit of the mean weight of five samples of 50 seeds and dividing this value into the total seed weight multiplied by 50 to give total seed numbers (see Topic 1).

Because of national sovereignty and ownership issues related to genetic resources (arising from the Convention on Biological Diversity http://www.cbd.int and see also the International Treaty on Plant Genetic Resources for Food and Agriculture http://www.planttreaty.org/), seed samples are usually sent out under the terms of a legally-binding material transfer agreement (MTA) that controls what the seeds may be used for, whether they can be passed to a third party and how benefits arising from the use will be shared. In effect, the agreement ensures that the conditions under which the seed was collected are passed on to the user. In most cases, the seed is sent out on receipt of a signed MTA. The alternative approach is to use a 'shrink-wrap' agreement whereby the user accepts the terms of the agreement the minute they take receipt of the samples. An example MTA can be found on the Millennium Seed Bank website (http://data.kew.org/seedlist/msa.pdf). It is important that users are asked the uses to which the samples will be put; this can be included as part of the MTA.

Another issue that needs to be considered when distributing seed is plant health. Within the EU, seeds can move without hindrance. When seeds are moved into or out of the EU, then the relevant plant health legislation will need to be consulted. Furthermore, national authorities need to be contacted in all cases over the movement of CITES (http://www.cites.org) and Habitats Directive (http://ec.europa.eu/environment/nature/legislation/habitatsdirective/index_en.htm) species.

Containers should be warmed up before samples are withdrawn to prevent condensation forming on the seeds (warming time is dependent on container size but 24 hr should be sufficient for most commonly-used containers). These samples should be drawn randomly and then packaged in foil bags (to prevent moisture uptake prior to use) within crush-proof (padded) envelopes for shipment. Plant health and other import documentation should be in a plastic pouch on the outside of the envelope.

RBGK have found that a self-checking serial number (a six figure number with a check digit calculated from that number) is useful in reducing errors when preparing seed orders.

The seed bank should keep records of who has been sent seed samples (this also helps prevent repeat orders) and the uses to which the seed has been put. This information is essential in demonstrating the immediate value of seed bank collections. Where possible, seed bank managers should discuss the types of collection required by potential users; this would help ensure maximum use of the collections.

Figure 28 Active Collections in the Millennium Seed Bank. (© RBGK)

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