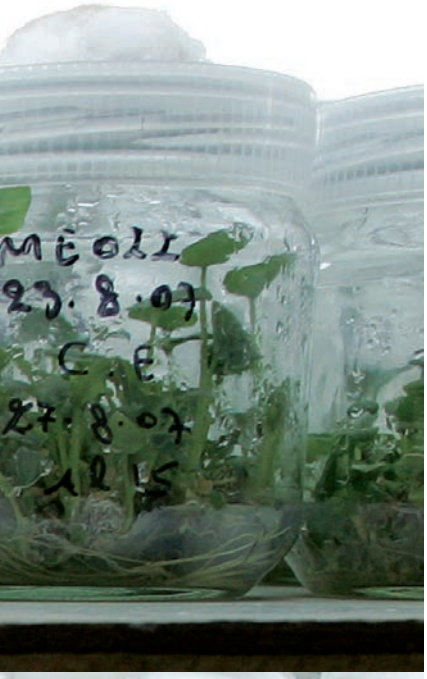

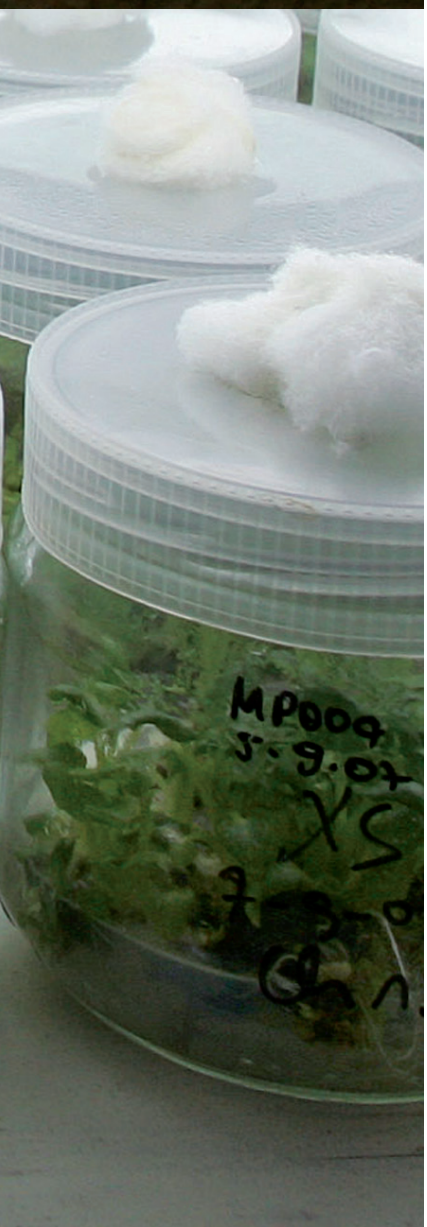

6

Genebank standards for *in vitro* culture and cryopreservation

The Standards for *in vitro* culture and cryopreservation are broad and generic in nature due to the marked variation among non-orthodox seeds and vegetatively propagated plants. This variability is a function of the inherent biology and metabolic status of the plants concerned, which influences their differing responses to various manipulations and often requires modifications of basic approaches to be made on a species-specific basis. These various features necessitate an introduction to the phenomenon of non-orthodoxy and storage behaviour of non-orthodox seeds to better understand the scientific basis of these standards.

Phenomenon of non-orthodoxy

Understanding the desiccation tolerance and sensitivity in orthodox compared to non–orthodox (intermediate and recalcitrant) seeds is of fundamental importance for cryopreservation. At maturity, orthodox seed water content would generally be in the range 0.05 – 0.16 g g^{-1} [1] (5 percent - 14 percent [wmb]), although some species are shed at much higher water content, undergoing substantial dehydration after this. Unlike recalcitrant seeds, all orthodox seeds acquire desiccation tolerance, which is genetically-programmed and entrained before, or at the start of maturation drying. Recalcitrant seeds do not dry during the later stages of development and

1 In this document, the term water content (wmb here is wet mass basis) is used in preference to moisture content, as recalcitrant seeds are hydrated (wet) rather than moist (barely wet). Also, the figures given are expressed on a dry mass basis (g H_2O g^{-1} dry matter [g g^{-1}]), which is considered to be more explicit than expression as a percentage of the wet mass.

are shed at water contents in the range of 0.3–0.4 – >4.0 g g^{-1}. Because they are desiccation-sensitive, the loss of water rapidly results in decreased vigour and viability, and seed death at relatively high water contents. This is due to their metabolic activity (Berjak and Pammenter, 2004) with little or no intracellular differentiation occurring, thus exposing membranes to the damaging consequences of dehydration stress (Walters *et al.*, 2001; Varghese *et al.*, 2011). A spectrum of differences in post-shedding physiology also occurs in intermediate seeds. Seeds showing intermediate behaviour can withstand water loss to ~ 0.11 to ~ 0.14 g g^{-1} (Berjak and Pammenter, 2004). They have the capacity to perform some of the important mechanisms and processes governing desiccation tolerance. However, they are not long-lived in the dehydrated condition, particularly at chilling temperatures for some species.

The variability in physiology of recalcitrant seeds is frequently also intraspecific. Seed or, embryo/embryonic axis water content can vary significantly in collections from the same locality from year-to-year, and also for material from the same locality, within any one season. This means that the parameters (water content, response to drying) must be assessed for each species. Additionally, seeds harvested late in a season are usually of considerably inferior quality compared with those harvested earlier (Berjak and Pammenter, 2004). The provenance of the population from which seeds are collected is also a major factor in the properties and responses of recalcitrant seeds. Thus, even if they are of the same species, seeds developing along a latitudinal gradient can show remarkably different characteristics. (Daws *et al.*, 2006; Daws *et al.*, 2004).

Seed developmental status has emerged as a critical consideration when recalcitrant germplasm is to be cryostored. Early during seed ontogeny, all seeds are highly desiccation-sensitive. Desiccation sensitivity in recalcitrant seeds increases as the processes of germinative metabolism are manifested (Berjak and Pammenter, 2004). The early events of germination in recalcitrant seeds are initiated soon after they are shed, without the 'punctuation' between the end of development and the start of germination imposed on orthodox seeds by maturation drying.

Depending on the species, recalcitrant seeds will initiate germinative metabolism after being shed. Those species with fully developed embryos on shedding, generally initiate germination virtually immediately, with a concomitant increase in desiccation sensitivity. In some other species, seeds are shed with under-developed embryos, necessitating the completion of development prior to the onset of germinative metabolism. These developmental differences dictate the duration for which the seeds can be wet-stored (i.e. hydrated storage

at the shedding water content). It is now known that recalcitrant seeds cannot be dehydrated to a water content precluding germination (so-called sub-imbibed storage), as this actually shortens the hydrated storage life span. Slight dehydration actually stimulates the onset/progression of germination, thus shortening the time before an extraneous water supply is required to support the process (Drew *et al.*, 2000; Eggers *et al.*, 2007).

In general, recalcitrant seeds from temperate provenances are chilling-tolerant, while those from the tropics and sub-tropics and of the same species, are more likely to be chilling-sensitive. Chilling sensitivity is also an issue for the storage of intermediate seeds, particularly those from the tropics and sub-tropics. When dried to water contents that are not injurious in themselves, the storage life span of such seeds is curtailed at temperatures ≤10 °C (Hong *et al.*, 1996).

Seed-associated microflora (fungi and bacteria), especially those associated with the interior surfaces, e.g. of the cotyledons or embryonic axis, is generally a major problem with recalcitrant seeds, particularly of tropical and sub-tropical origin (Sutherland *et al.*, 2002). The conditions of hydrated storage, being moist and often necessarily at benign temperatures, encourage fungal proliferation, with the probability of hyphae penetrating the embryo tissues. This has a major deleterious effect and curtails hydrated storage life span significantly.

Under field conditions, unless seedling establishment is rapid, recalcitrant seeds will gradually lose water, the rate depending on the species-specific nature and morphology. Under conditions of slow water loss (days to a week or more), desiccation damage accumulates and the seeds of most species will have lost viability when the embryos/embryonic axes are at a water content of around 0.8 g g^{-1} (Pammenter *et al.*, 1993). Thus when handling or storing recalcitrant seeds, great care is normally exercised to maintain water contents at the levels characteristic of shedding.

The response of explants to dehydration depends on the rate of drying and size of the explants. Often recalcitrant seeds are too large to dry rapidly, and too large to cool rapidly on exposure to cryogen (as is required to obtain successful cryopreservation). Thus, excised embryos or embryonic axes are explants of choice, since they can be dehydrated to water contents that will minimize ice crystallization, which are ≤0.4 g g^{-1}. Embryos/axes can be dried in a stream of air (flash-dried) (Pammenter *et al.*, 2002), which significantly curtails the time during which metabolism-linked desiccation damage can occur. It is not that the embryos/axes have become desiccation-tolerant, but simply that they dry before lethal damage has accumulated, providing the time needed to subject them to cryogenic temperatures. In cases where embryo/axes prove impossible to manipulate for successful cryostorage, alternative

explants, such as shoot apical meristems excised from seedlings developed from seeds germinated *in vitro*, can be used.

In addition to cryopreservation, other means of conservation for species producing recalcitrant or otherwise non-orthodox seeds include *in vitro* conservation that could involve slow growth of seedlings/young plants/plantlets. In some instances, slow-growth conditions may be imposed *ex vitro*. In the last instance, plantlets may be derived from embryogenic callus (which itself might be amenable to cryopreservation) and conserved *in vitro*, possibly under slow-growth conditions.

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6.1 Standards for acquisition of germplasm

Standards

6.1.1 All germplasm accessions added to the genebank should be legally acquired, with relevant technical documentation.

6.1.2 All material should be accompanied by at least a minimum of associated data as detailed in the FAO/Bioversity multi-crop passport descriptors.

6.1.3 Only material in good condition and of consistent maturity status should be collected, and the sample size should be large enough to make genebanking a viable proposition.

6.1.4 The material should be transported to the genebank in the shortest possible time and in the best possible conditions.

6.1.5 All incoming material should be treated by a surface disinfectant agent to remove all adherent microorganisms and handled so that its physiological status is not altered, in a designated area for reception.

Context

Acquisition is the process of collecting or requesting germplasm (seeds and other propagules[1]) for inclusion in the genebank, together with related information. Adherence to legal requirements is essential, and both national and international

1 In this context, a propagule refers to vegetative portions of a plant such as seeds, buds, corms, cuttings and other offshoots, used to propagate a plant.

requirements must be fulfilled as appropriate. During the acquisition phase, it is important to ensure that passport data for each accession is as complete as possible and fully documented (Alercia *et al.*, 2001).

There is a need to ensure maximum quality of the germplasm and avoid conservation of immature seeds and seeds that have been exposed for too long to the elements. The way that seeds and other propagules are handled after collection and before they are transferred to controlled conditions is critical for quality. Unfavourable extreme temperatures and humidity during the post-collecting period and during transport to the genebank could cause rapid loss in viability and reduce longevity during storage. The same applies to post-harvest handling within the genebank. The seed quality and longevity is affected by the conditions experienced prior to storage within the genebank. As recalcitrant seeds are metabolically active and have high water contents at maturity, the way they are handled after collection is critical for successful long-term conservation of the material. As field-grown material is frequently contaminated with fungi and/or bacteria, it is necessary to have a set of measures in place to reduce the risk of deterioration of the material in the post-harvest state.

Material must be as clean as possible. Therefore, transfer of field material into pots and short periods of glasshouse growth is recommended. In these cases, plants should be watered from the bottom and, in extremely infected material, pesticides may support later disinfection of the explants. Visibly infected material should be excluded from the beginning or eliminated when found.

Technical aspects

Plant genetic resources within the Multilateral System of the ITPGRFA are accompanied by a Standard Materials Transfer Agreement (SMTA). For material acquired or collected outside the country where the genebank is located, the acquirers should comply with the relevant national and international legislation. Phytosanitary regulations and any other import requirements must be sought from the relevant national authority of the receiving country.

Passport data is needed to identify and classify the accessions. Many accessions are wild species, making collection of accurate field data absolutely imperative. The multi-crop passport descriptors should therefore, include a herbarium voucher, as well as GPS coordinates and photographic images of habit, habitat, and the substratum as much as possible. If fallen material is collected, it should, be recorded as such and kept separate from that harvested from the parent plant. The sample size should include an

adequate number of individuals/accessions, large enough to establish an appropriate protocol for cryopreservation, and/or to place samples in long-term cryostorage.

There is a need to ensure maximum seed and propagule quality and avoid conservation of immature or over-ripe material (in the case of seeds) that has been exposed for too long to the elements. Collecting well-matured clean and high quality propagules, will ensure maximum longevity in storage. Fallen material and fruits (seeds) showing abrasions or signs of weathering should be avoided. Late-season seeds appear often to be of inferior quality to those produced earlier (Berjak and Pammenter, 2004). It is advised not to collect late-season recalcitrant seeds of any species. Seasonality needs also to be considered when using bulbs and tubers, which develop new shoots only in some seasons, in woody plants that have dormant buds only in winter, and young inflorescence explants or pollens which are available only in the flowering period.

Many of the fruits bearing recalcitrant seeds harbour fungal contaminants, even when not visible. This is a serious problem, and surface disinfection prior to transport is important for removal of any superficial contaminant. High temperatures and humidity during the post-collecting period and during transport to the genebank exacerbate this problem and could cause rapid loss in viability and reduce longevity during storage. However, seeds and other propagules may be chilling-sensitive and elevated temperatures may either hasten germination or damage the seeds. Thus, transport temperature must be neither too low nor too high, generally not below ~ 16 °C and not above ~ 25 °C.

The problem of fungal contamination also applies to post-harvest handling within the genebank and fruits should be thoroughly surface-disinfected prior to opening. Similarly, for any imported accessions, contamination can result from containers and wrappings, which need to be incinerated as is generally stipulated by national Plant and Seed Health regulations. Fruit pulp, fibres, etc. must be completely removed from seed outer surfaces, but water must not be used, as seeds could well become (further) hydrated and affect the water content of the seeds. It is also important to gather information about the fruit and seeds weight prior to water content determination (see Standard 6.2).

Wherever possible (as in the case of hard-coated fruits), seeds should be transported within the fruits, both for protection and to avoid dehydration. Water loss both stimulates germinative metabolism and shortens storage life span, thus it is important that water content are maintained upon collection and during transport, by maintaining high relative humidity (RH) in the storage containers. Special plastic bags should be preferred, which are not vulnerable to breaking as are glass tubes.

Insulating packaging will help in keeping the temperature stabile, and can be especially relevant during long transportation.

Recalcitrant seeds produced in hard-coated fruits generally remain in better condition for longer periods, than if the seeds are removed from the fruits. Soft fruits, or those which are damaged or have dehisced should immediately be surface-decontaminated, the seeds extracted and the fruits removed and destroyed. If long transport periods are involved, it is advisable to extract, manually clean and surface-disinfect the seeds prior to transport. Ideally, a disinfection kit comprising water purification tablets or sodium hypochlorite (NaOCl), water (sterile, if possible, or boiled on site) and sterile paper towelling should be carried on field expeditions.

Under tropical conditions, other measures such as the storage of plantlets under shade (Marzalina and Krishnapillay, 1999) or *in vitro* field collecting (Pence *et al.*, 2002; Pence and Engelmann, 2011) may be applied. Minimum transportation times are also necessary when *in vitro*-collected material is used.

For *in vitro*-cultured explants, surface decontamination starts often by 70 percent ethanol followed by NaOCl diluted from pure stock solution or as constituent of a commercial bleach with a concentration of active chlorine amounting to about 3 percent. Detergent droplets may support the effect. Other substances may be used as well (e.g. calcium hypochlorite) in appropriate concentrations. The explant needs to be trimmed to the final size after surface disinfection. Note that the disinfectant will enter cut surfaces resulting in dead zones that need to be removed upon trimming.

Contingencies

When a consignment is contaminated or deteriorated, all material and its packaging must be incinerated, despite the financial implications.

Delays of a consignment in national quarantine facilities are a known hazard. In such cases, steps must be taken to minimize such delays, including the use of couriers.

Under conditions of a 'poor' fruiting season, it is preferable to postpone collection to a subsequent fruiting season. If circumstances dictate that fallen fruits have to be collected, only those that are newly-abscised, should be considered.

Occasionally, seeds of particular species react badly to NaOCl and/or to the commonly used fungicides, in which case safe alternatives must be used (Sutherland *et al.*, 2002). Note that 70 percent (v/v) ethanol in sterile/boiled water could be used.

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6.2 Standards for testing for non-orthodox behaviour and assessment of water content, vigour and viability

Standards

6.2.1 The storage category of the seed should be determined immediately by assessing its response to dehydration.

6.2.2 The water content should be determined individually, on separate components of the propagule, and in a sufficient number of plants.

6.2.3 The vigour and viability should be assessed by means of germination tests and in a sufficient number of individuals.

6.2.4 During experimentation, cleaned seed samples should be stored under conditions that do not allow any dehydration or hydration.

Context

Maintaining seed viability is a critical genebank function that ensures germplasm is available to users and is genetically representative of the population from which it was acquired. As a first step to preservation, it is important to ascertain the seed storage category by assessing the response of the propagule to dehydration. The response to drying will in turn determine the treatment needed for cryostorage. A number of factors influence drying rate, including RH, seed size, the nature of seed coverings, the flow rate of air over the seeds, and the depth of the layer of seeds (Pammenter *et al.*, 2002).

The rate and uniformity of germination of a seed sample, or of seed-derived explants, is a reliable indicator of vigour, while the totality of germination (i.e. what

proportion/percentage of seeds or explants tested finally germinated) reveals the overall viability of the sample. Viability should not be not less than 80 percent in a sample.

Technical aspects

Water content determinations and assessment of vigour and viability should be carried out as a single operation, and are issues to determine before selecting the type of drying technique. The number of procedures that can be investigated is determined by the number of seeds available. Three methods for screening seeds can be used for seed categorization. These includes a method that can discriminate between intermediate and recalcitrant seeds (Hong and Ellis, 1996), one which is designed for cases when seeds are limited (Pritchard *et al*., 2004), and one that assesses axis water content, rather than that of whole seeds. Irrespective of the method chosen, dehydration imposed during the screening procedure must never be carried out at elevated temperatures, which are damaging. The recommended temperatures for tropical/sub-tropical species and those of temperate origin are 25 °C and 15 °C respectively (Pritchard *et al*., 2004). A drying time-course assessing loss of viability with declining water content should be determined for each new accession.

The water content present within different components of recalcitrant seeds is critical for their successful cryopreservation. Water content determined on a whole recalcitrant seed basis, gives no indication of the water content of the axis. Therefore, water content determinations must be carried out separately for axes, embryos, fleshy cotyledonary tissue or endosperm (Berjak and Pammenter, 2004) and measured individually (not on pooled samples). In many cases, the dry mass of axes of recalcitrant seeds may be as little as a few milligrams, necessitating a 6-place balance.

It is important to determine water content of each newly arrived accession immediately after the propagules have been cleaned, to avoid further drying. Even if other accessions of the same species have been collected, one cannot assume that water contents will be similar. Because the composition of the axes and storage tissues of recalcitrant or otherwise non-orthodox wild species is generally unknown, drying is recommended to be carried out at 80 °C until constant weight is attained. When tissues are dried at 80 °C, the time taken to attain constant weight is generally between 24 and 48 h. After the drying period,

it is imperative that samples reach room temperature, without absorbing water, before being re-weighed.

A minimum of 10 seeds is recommended to be tested for water content (determined on an individual seed/embryo/axis basis). Additional seeds will be required for any biochemical analyses undertaken.

Seeds and the embryos/axes excised from them should be at their most vigorous stage of development when newly harvested. Intact seeds are best germinated on 0.8 – 1 percent water agar in closed plastic containers or Petri dishes, which will provide common conditions for all such assessments. It is important that the surfaces of seeds are disinfected prior to being set to germinate, or prior to excision of embryos or embryonic axes. Dormancy is not a common feature of recalcitrant seeds, and seeds should normally commence germination in a relatively short interval after being set out. However, the time will vary among species depending on the extent of embryo development. It is essential that all germination/viability testing is done under the same controlled conditions per species. Production of morphologically abnormal seedlings/plantlets (Pammenter *et al.*, 2011) should be noted and quantified, as abnormality can occur as a result of imposed stress (e.g. dehydration of recalcitrant seeds, embryos or embryonic axes). A minimum of 20 seeds is recommended for viability testing.

When handling recalcitrant seeds, great care is normally exercised to maintain water contents at the levels characteristic of shedding. However, intact recalcitrant seeds are almost invariably too large to be cooled to cryogenic temperatures. Hence, the explants, embryos or the embryonic axes, need to be excised from the seeds and dehydrated. Further to this, it is essential that the bulk of the cleaned seed sample be stored under conditions that preclude changes in water status. If exposed to the atmosphere for any length of time, the water content of seeds will change and seeds shedding at relatively high water contents would become somewhat dehydrated.

Contingencies

If a genebank does not have a temperature- and humidity-controlled drying room then, for whole seeds, bench-top drying in bell jars or drying in the shade in monolayers could be used. Specimens in any Petri dish not closed before extraction from the drying oven, will have to be replaced in the oven, as dry tissues rapidly adsorb water vapour, especially in a humid environment.

Excised embryos/embryonic axes will generally not germinate as rapidly as will the intact seeds. When working with excised embryonic axes, often shoot development will not occur. In such cases, root production will be the criterion on which vigour and viability are assessed.

In cases where embryos/axes prove impossible to manipulate for successful cryostorage, alternative explants must be used. These can be of a variety of types, but the most suitable are shoot apical meristems excised from seedlings developed from seeds germinated *in vitro*.

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6.3 Standards for hydrated storage of recalcitrant seeds

Standards

6.3.1 Hydrated storage should be carried out under saturated RH conditions, and seeds should be maintained in airtight containers, at the lowest temperature that they will tolerate without damage.

6.3.2 All seeds should be disinfected prior to hydrated storage and infected material should be eliminated.

6.3.3 Stored seeds must be inspected and sampled periodically to check if any fungal or bacterial contamination has occurred, and whether there has been any decline in water content and/or vigour and viability.

Context

For provision of planting stock for re-introductions and restoration programmes, or simply for the maintenance of seeds whilst undertaking experimentation, it is sometimes necessary to store recalcitrant seeds in the short- to medium-term (weeks to months). The basic principle for maximizing the storage life span of recalcitrant seeds is that water contents should be retained at essentially the same levels as those characterizing the newly harvested state. Thus, the seeds must not lose water either before or after being placed in storage. Even very slight degrees of dehydration can stimulate the initiation of germination, and further dehydration can initiate deleterious changes that impact on vigour and viability and shorten the period for which the seeds can be stored. Keeping recalcitrant seeds under conditions that will

maintain their water content is termed, hydrated storage, and is achieved by holding the seeds in closed conditions under saturated RH.

Technical aspects

To avoid any water loss from the seeds, hydrated storage must be carried out at saturated RH, achieved by maintaining a saturated atmosphere in the storage containers. Ideally, sealing polythene bags with an inner paper bag inside ('bag within a bag') or sealing plastic buckets of appropriate size for the seed numbers, are favoured for storage (Pasquini *et al.*, 2011). As an essential precaution, storage containers such as buckets with sealing lids, as well as internal grids, must be sterilised prior to the introduction of seeds. Irrespective of the container chosen, a means for absorbing any condensate needs to be included, and changed on becoming damp.

Storage temperatures should be the lowest that seeds of individual species will tolerate, without any deleterious effect on vigour and viability. This will slow both progress towards germination and fungal proliferation. The temperature of the store must be kept constant to minimize condensation on the interior surfaces of the storage containers. For recalcitrant seeds of temperate origin, temperatures of 6 ± 2 °C are generally suitable for storage, while for the majority of seeds of tropical/sub-tropical origin, 16 ± 2 °C is normal range. Exceptions occur, particularly for seeds of some equatorial species (Sacandé *et al.*, 2004; Pritchard *et al.*, 2004).

Under hydrated storage conditions, fungi (or less frequently bacteria) are likely to proliferate, so vigilance and appropriate action to curtail seed-to-seed infection is required. If infected seeds are not removed, they will contaminate the entire batch in a storage container. This renders the stored seeds useless and eliminates their potential for supplying explants for cryopreservation. Hence, regular inspection right from the outset, and appropriate action such as the application of fungicidal agents should be done to eliminate surface and internal contaminants from seeds at the earliest possible opportunity (Calistru *et al.*, 2000).

Seed surfaces need to be disinfected, dried of any residual sterilant, and dusted with a broad-spectrum fungicide. Internally-borne fungi, largely located immediately below the seed coverings, would be most effectively eliminated by the uptake of appropriate systemic fungicides by the seeds. However, these may well affect the seeds adversely. Thermotherapy, as applied to infected acorns (Sutherland *et al.*, 2002), is another possibility, but this can be used only when seeds are resilient to transitorily-raised temperatures – which is not always the case. To disinfect inner

surfaces directly, it is necessary to ensure that the seeds survive well in hydrated storage after removal of the coverings, and that presence of systemic fungicides in the seed tissues are not damaging.

Depending on the duration of hydrated storage, containers should be briefly and periodically ventilated to avoid development of anoxic conditions at which time the contents of containers must be inspected and any contaminated seeds discarded. Storing seeds in a monolayer is ideal, but if seeds are stored in several layers, the seeds should be mixed about during aeration. After removing any seed showing signs of contamination, the container must be emptied, all apparently uncontaminated seeds disinfected and the seed lot replaced in a sterilised container.

Stored seeds must be sampled periodically to check whether there has been any decline in water content and vigour and viability. If water content has remained essentially what it was when the seeds were placed in hydrated storage, and there is no apparent fungal (or bacterial) proliferation, but viability has declined, then the end of the useful storage period will have been reached. Similarly, if visible signs of germination of many of the seeds are apparent, the end of the useful storage period will have been reached. A decline in viability of seeds that have not lost water to any marked extent, or root protrusion by most of the seeds, gives a measure of the time for which hydrated storage is possible under the specific temperature regime used.

Contingencies

Loss of water from seeds indicates that high RH was not maintained, probably because the storage containers were not properly sealed. This leaves uncertain results for the sample, which should be discarded. Loss of viability of seeds during storage may also be the result of maintenance at inappropriate temperatures. This parameter needs to be resolved by trials testing seed responses to a range of temperatures. Seeds may have lost viability because they were originally of poor quality, or of being too immature at harvest.

In cases where there is a high incidence of internally-contaminated seeds in an accession, the contaminants should be isolated and identified, with a view to developing effective means to eliminate them from future collections. Identification of fungi, certainly to genus level, could assist in selection of fungicides that may be more efficacious in combination ('cocktails'), specifically targeting those fungi. Sometimes viruses are present in the seeds, which cannot be eliminated by any treatments. If they can cause serious diseases, the plants must be discarded as soon as viral symptoms are observed.

Contamination may prove to be intractable to any remedial treatment, in which case seeds cannot be stored in this manner, and alternate forms of conservation of the genetic resources must be sought. In such cases, seeds should be set to germinate, with seedlings developed from any seeds that are uninfected being maintained under slow-growth conditions, and/or used to provide alternative explants for *ex situ* conservation, for instance transferred and planted in field genebanks, or other gardens, as appropriate.

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6.4 Standards for *in vitro* culture and slow growth storage

Standards

6.4.1 Identification of optimal storage conditions for *in vitro* cultures must be determined according to the species.

6.4.2 Material for *in vitro* conservation should be maintained as whole plantlets or shoots, or storage organs for species where these are naturally formed.

6.4.3 A regular monitoring system for checking the quality of the *in vitro* culture in slow-growth storage, and possible contamination, should be in place.

Context

In vitro conservation is used for maintenance of plant organs or plantlets in a medium-term time frame (some months up to some years) under non-injurious, growth-limiting conditions. Generally, it is not desirable for long-term conservation (Engelmann, 2011). *In vitro* conservation is preferentially applied to clonal crop germplasm as it also supports safe germplasm transfers under regulated phytosanitary control. Technical documents provide detailed information on the possibilities offered by *in vitro* storage, on the main parameters to consider and on the links and complementarity with other storage technologies, such as field genebanks (Reed *et al.*, 2004; Engelmann, 1999a).

In vitro cultures serve as sources of disease-free materials for distribution, multiplication and a source of explants for cryo-preservation. Safe removal and disposal of infected materials is essential, as it ensures that a pathogen or pest is not released into the environment. Permanent regular monitoring is necessary to avoid accumulation of

contamination that might take place during transfers, be transmitted through the air from vessel to vessel or actively transported by vectors like mites and thrips. Breakdown by hyperhydration is another danger, which usually starts in some vessels a little earlier so that a chance exists to rescue the other material when noticed early enough.

Technical aspects

Optimal conditions for slow growth need to be identified prior to storage. These may be achieved by manipulating variables, including light-regime, temperature, and medium composition, individually, or in combination (Engelmann, 1991), but experimentation is generally required to achieve optimal results.

The type and physiological condition of explants is basic to success or failure of *in vitro* slow growth. *In vitro* culture is also used as a preparatory phase to cryopreservation as well as for recovery phases after cryopreservation. Thus, suitable media and conditions for *in vitro* growth of explants need to be developed as a first step. This involves appropriate surface disinfection procedures and germination medium (starting with a standard medium [Murashige and Skoog, 1962], which may need refining). The basal medium may be determined from the literature concerning culture of similar species. Standard protocols have been published and can be used for guidance (including George 1993; Hartmann *et al.*, 2002; Chandel *et al.*, 1995) but in many cases, detailed trials using explants media and growth conditions are critical and custom-made protocols using explants media and growth conditions are needed even if the species are closely related.

Ensuring that the materials are maintained as whole plantlets or shoots, can avoid hyper-hydricity ('vitrification'). For explants of species that naturally grow slowly, no manipulation of media or of culture conditions may be necessary.

Experimentation with a range of permutations and combinations of the means to achieve satisfactory slow growth are imperative when first working with explants of a species. For example, very variable responses to manipulations for slow growth have been recorded for different species of single genera. Maintenance of long-term genetic stability of material stored under slow-growth conditions is imperative (Engelmann, 2011). Optimal storage temperatures for cold-tolerant species may be from 0 to 5 °C or somewhat higher; for material of tropical provenance the lowest temperatures tolerated may be in the range from 15 to 20 °C, depending on the species (Normah *et al.*, 2011; ProMusa; Engelmann, 1999a; Engelmann, 1991).

Various modifications are generally made to culture media, especially reduced levels of minerals, reduction of sucrose content and/or manipulation of the type and concentration of growth regulators, while inclusion of osmotically-active substances (e.g. mannitol) may also be effective (Engelmann, 2011; Engelmann, 1999a). Activated charcoal in the medium may adsorb exuded polyphenolics (Engelmann, 1991).

The type, volume, means of closure and atmosphere in culture vessels constitute important parameters (Engelmann, 2011; Engelmann, 1991), which can be established only by experimentation when working with new material.

Although slow-growth storage is traditionally used for material cultured *in vitro*, plantlets may also be maintained *ex vitro* under growth-restricting conditions. Seedling slow growth in shaded, light-limiting conditions under natural canopies is an inexpensive alternative (Chin, 1996). Furthermore, induction of storage organs *in vitro* can be used for effectively enhancing the conservation period in natural storage organ forming crops (e. g. ginger [Engels *et al.*, 2011], taro, yam, potato etc.).

Contingencies

In vitro culture of explants of woody species may pose particular problems, especially regarding exudation of polyphenolics (Engelmann, 1999b). Associated problems include poor rooting and the explants becoming hyperhydric. Hyperhydration and leaf necrosis developed during slow growth can lead to deterioration of quality and in some cases death of the whole propagules.

In some material, accumulation of covert bacteria may become a gradually increasing obstacle for prolonged slow-growth storage. It may be counteracted by temporary removal of vitamins from the medium or addition of antibiotics, but rarely these measures will be of permanent success. Thus, it may be necessary to discard these cultures from the storage (Abreu-Tarazi *et al.*, 2010; Leifert and Cassels, 2001; Senula and Keller, 2011; Van den Houwe and Swennen, 2000; Van den Houwe *et al.*, 1998).

Within a genepool, there may be large differences in the response to *in vitro* storage between species/varieties, some responding well while others cannot be conserved using this technology, thus making its application impossible (e.g. for coffee [Dussert *et al.*, 1997]). In some species (e.g. yam), storage organs may be formed *in vitro*, but their germination is difficult to attain. This is true also for *in vitro*-derived bulblets in some accessions of a species (e.g. garlic [Keller, 2005]).

In some species, intrinsic genetic instability (e.g. sugar cane) may be enhanced by *in vitro* culture techniques, whereas in others (e.g. cassava) stability over extended

storage periods has been demonstrated (IPGRI/CIAT 1994). In the latter cases, somaclonal variation may occur in higher frequencies. In most cases, somaclonal variation is minimized by consequent use of techniques that avoid induction of adventitious shoots or any formation basal callus after cutting. Where callus has formed this needs to be cut off during transfer to the next culture period. To avoid confusion about the reasons for any genetic deviations occurring, thorough observation of uniformity of source explants is needed and chimerism should also be excluded from the donor material (or carefully maintained if needed in variegated plants). Since regular screening by means of molecular markers seems to be too expensive, regular sampling may be undertaken in cases where somaclonal variation is expected to occur.

Dormancy of organs may become a problem, when shoots stop developing (often occurring in species that form *in vitro* storage organs). Additional cutting or application of cytokinins may break dormancy. If this is not successful, then waiting for some time until spontaneous sprouting may be the only (even though uncertain) solution.

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6.5 Standards for cryopreservation

Standards

6.5.1 The explants selected for cryopreservation should be of highest possible quality, and allow onward development after excision and cryopreservation.

6.5.2 Each step in the cryo-protocol should be tested individually and optimized in terms of vigour and viability in retention of explants.

6.5.3 Means should be developed to counteract damaging effects of reactive oxygen species (ROS) at excision and all subsequent manipulations.

6.5.4 Following retrieval, explants should be disinfected using standard sterile procedures.

Context

Cryopreservation permits cells or tissue to be stored for an indefinite period in LN (–196 °C) where metabolic activities are suspended. Four steps are essential in any cryopreservation protocol: (i) selection, (ii) preculture[1], (iii) cryopreservation techniques, (iv) retrieval from storage, and, (v) seedling or plantlet establishment.

Cryo-protocols should be developed to prevent cryopreservation damages, and could include possible cryo-protection, partial drying, cooling, storage at cryogenic temperatures, rewarming and rehydration. There are two main types of cryopreservation procedures:

1 treatment for slow explants' acclimatization to dehydration/cold/freezing

conventional slow freezing, based upon freeze-induced dehydration; and, flash-freezing (vitrification), which involves dehydration prior to cooling (Engelmann, 2011a).

Technical aspects

Selection of explants

Dehydration rate, and how evenly cells and tissue dry, depend on size, and since the vast majority of recalcitrant seeds are too large to be dried rapidly and evenly, they cannot be cryopreserved intact. In addition, cells with water contents ≥ 1.0 g g^{-1}, cannot survive after exposure to cryogenic conditions. Excision and cultivation of suitable explants should be developed specifically for the purpose of cryopreservation. The explants should be as small as possible, but big enough to allow onward development after excision, and after cryopreservation. Higher cellular/tissue uniformity within the explant elevates a chance of cryoprotection of all (or majority) explant cells and its regeneration capacity without callus proliferation. Explants for cryopreservation can be produced from embryonic axes, shoot tips, meristematic and embryogenic tissues. For recalcitrant seeds, excised embryos/axes are the explants of choice for cryopreservation. In the event they are too large, do not withstand the required degree of dehydration, are sensitive to all common modes of surface disinfection, and/or are intractable to culture conditions, explants such as shoot apical meristems are a better option.

For vegetatively propagated species, explants of choice are buds, shoot tips, meristematic, and embryogenic tissue. Not all types of explants are amenable to similar cryoprotection procedures, even when the parent species are relatively closely related taxonomically (Sershen *et al.*, 2007), and responses to cryoprotection procedures need to be ascertained per species as well as genotypes. Under-developed material is generally more susceptible to excision damage; likewise, seeds that have developed/germinated to the stage of visible protrusion of radicals (or other parts of the embryo) should not be selected (Goveia *et al.*, 2004).

Whole anthers or isolated pollen grains can be used for cryopreservation as well. They represent the inherited genetic diversity like seeds, but bearing the male germ units, they usually have the haploid chromosome set only (see Ganeshan, 2008; Rajashekaran, 1994; Weatherhead *et al.*, 1978, for a review). When pollen is conserved, it needs to be embedded in gelatine capsules or paper pouches or packed on a paper strip, with some species requiring dehydration of the pollen prior to storage.

To retrieve the material, anthers or pollens are shed from capsules, pouches or strips at room temperature. The assessment of pollen germination is best undertaken in a germination medium. Viability may be tested by pollen staining, and the results are correlated to pollen germination, although the germination rate will almost always be lower. When the behaviour of a species is still not known, test pollinations are needed to confirm successful fertilization by seed set (Ganeshan *et al.*, 2008; Rajashekaran *et al.*, 1994; Weatherhead *et al.*, 1978).

Probabilistic tools are available which facilitate calculation of the number of propagules to store and retrieve, depending on the objectives, survival after cryostorage, and other parameters (Dussert *et al.*, 2003).

Cryopreservation techniques

It is important that a drying time-course of excised embryos/axes be conducted to identify the drying time required to reduce material to appropriate water content. An additional drying time-course needs to be done after any pre-growth or cryoprotectant treatment.

Cooling rate to LN temperatures is important and needs to be considered in relation to the explant water content. The cryo-protocol should be selected to ensure the water content lies within the range that prevents intracellular ice-crystal formation on cooling and warming, but also avoids desiccation damage to subcellular structure. At the higher end of the water content range to which axes are dried, the faster the cooling rate the better, as very rapid cooling of small specimens tends to be even and minimizes the duration in the temperature range that would permit ice crystallization. The embryos/axes, generally constitute only an insignificant fraction of seed mass and volume, and are suitable for flash-drying, thus overcoming the problem of metabolism-linked damage. On the other hand, cooling rate is less critical for recalcitrant axes flash-dried (using evaporative dehydration) near to their lower limits of tolerance.

Techniques based on dehydration during controlled-rate cooling, have an application when the material to be cryopreserved consists of embryogenic cultures and of shoot tips from temperate species (Engelmann, 2011a). For vegetative material, many protocols and examples of cryopreservation of a range of explants across species using one or more of the procedures, are documented (Benson *et al.*, 2007). In addition, there is a vast number of publications on cryopreservation of apices, other meristematic tissues, embryogenic tissues and dormant buds, and the journal, CryoLetters, is a good source for many of these. Once a successful protocol has been developed for a species, periodic testing of samples extracted from cryopreservation should be carried out, initially after a short storage interval.

Most plant vitrification protocols use cryoprotectants (usually a mixture of penetrating and non-penetrating types). Evaporative dehydration has generally been employed for zygotic embryos/embryonic axes. Although originally developed for apices and somatic embryos, encapsulation-dehydration and the procedure termed vitrification (employing various plant vitrification solutions [PVS]), have also been used in procedures to cryopreserve seed-derived embryos and embryonic axes. A recent overview (Engelmann, 2011b) provides the information that all vitrification protocols developed for somatic embryos, utilizing PVS2. Vitrification using PVS2 has also been used for cryopreservation of shoot tips of a wide range of species from both tropical and temperate provenances, the former including several recalcitrant-seeded and vegetatively propagated species. Another common vitrification solution is PVS3 (Nishizawa *et al.*, 1993) which does not use dimethyl sulphoxide (DMSO) and can therefore be preferred for species that are damaged by DMSO. A range of alternative loading and vitrification solutions have been developed recently, which can be efficiently used for cryopreserving materials which prove sensitive to PVS2 and PVS3 (Kim *et al.*, 2009a; Kim *et al.*, 2009b).

At the lower limits of dehydration tolerated by recalcitrant embryos and axes, generally a proportion of freezable water is retained. During both slow cooling and rewarming, ice crystallization can occur in the freezable water fraction between about -40 and -80 °C. Rewarming at ~37 to 40 °C prevents this, noting that transfer from cryogenic temperatures must be very rapid.

The main cryopreservation techniques and their crucial required parameters are given below:

- controlled-rate cooling: choosing of cryoprotectant (rarely mixture of cryoprotectants); selection of cooling rate (for avoiding crystallization inside of cells);
- encapsulation-dehydration: determination of osmotic dehydration time and it rate of treatment, determination of air desiccation time;
- vitrification: determination of kind of vitrification solution and it time of treatment (assessment of their toxicity); PVS2 should be used on ice.
- droplet freezing: determination of kind of vitrification solution and it time of treatment (assessment of their toxicity).

Retrieval from cryostorage

Rewarming of vitrified germplasm is often undertaken in two steps, the first is slow to allow for glass relaxation, usually at ambient room temperatures. This is followed by more rapid rewarming at ca. 45 °C to avoid ice nucleation (Benson *et al.*, 2011).

Specimens processed by encapsulation-dehydration[2] may be transferred directly onto recovery/germination medium for rapid rewarming, or the cryotubes containing the alginate beads may be placed in a water-bath at 40 °C for 2-3 min. Alternatively, the beads may be rehydrated by transferring them for ~10 min in liquid medium. The removal of the capsule has also been shown to be advantageous (Engelmann *et al*., 2008). Encapsulation-dehydration has proved to be consistent and successful for shoot tips of many species (González and Engelmann, 2006), somatic embryos of conifers (Engelmann, 2011b), a range of citrus species and varieties, and temperate fruit species (Damiano *et al*., 2003; Damiano *et al*., 2007).

To restore metabolic activity in the cell upon rewarming, toxic cryoprotectants must be removed from the cell and the normal water balance gradually restored as the cell is returned to a normal functioning temperature. The original composition of the recovery medium may have to be slightly modified after explants have been dehydrated or cryogen-exposed. With the use of plant vitrification solutions (PVS), after rapid rewarming, a dilution or unloading step (removal of toxic PVS) is necessary (Sakai *et al*., 2008; Kim *et al*., 2004).

All steps in cryopreservation could compromise survival, and particularly, warming and rehydration can be accompanied by a burst of reactive oxygen species (ROS)[3] (Whitaker *et al*,. 2010; Berjak *et al*., 2011). Rewarming and rehydration media should ideally also counteract the deleterious effects of ROS, but it is imperative that means are established to reduce the bursts of (ROS) accompanying excision (Whitaker *et al*., 2010; Berjak *et a.l,* 2011; Engelmann, 2011a; Goveia *et al*., 2004). Treatments with cathodic water (an electrolysed dilute solution of calcium chloride and magnesium chloride) had potent anti-oxidative properties, which counteracted the effects of ROS bursts at all stages of a cryopreservation protocol for recalcitrant embryonic axes of *Strychnos gerrardii*, and promoted shoot develo ment (Berjak *et al*., 2011). The beneficial effects of the treatment are more marked when development of embryos/axes progress during a hydrated storage period, indicating the importance of developmental status of the seeds. It appears that treatment of axes with the non-toxic anti-oxidant, cathodic water, offers both an explanation for previous failures of axes to produce shoots, and an ameliorative treatment to counteract stress-related ROS bursts. Furthermore, the instruments used for embryo/axis

2 Encapsulation-dehydration entails the explants being encapsulated in alginate beads and cultured (pre-grown) in a sucrose-enriched liquid medium for periods up to 7 d. Following this they are subjected to dehydration, using a laminar air-flow or flash-drying, or by being exposed to activated silica gel, to dry explants to a water content ~0.25 g g^{-1} (20 percent wmb), and finally cooled rapidly.

3 ROS are highly reactive molecules, often free radicals, which damage proteins, lipids and nucleic acids.

excision can exacerbate ROS production. In this regard, use of a hypodermic needle is likely to cause less trauma than will a surgical blade (Benson *et al.*, 2007). The use of DMSO, a hydroxyl radical scavenger, as a preculture step (before complete severing of the cotyledonary remnants) and as a treatment after their removal, has shown to facilitate shoot development. Other antioxidant substances are also used to counteract ROS formation, e.g. ascorbic acid and tocopherol (Chua and Normah, 2011; Johnston *et al.*, 2007; Uchendu *et al.*, 2010). Survival of plant material can also be assessed based on the enzymatic activity of living plant cells (Mikula, 2006).

Seedling and plantlet establishment

Once excised embryos/embryonic axes have been rewarmed, the next step is to generate and establish a seedling or plantlet to complete the regeneration cycle. Seedling and plantlet establishment requires two steps: (i) its establishment *in vitro* and (ii) establishment *ex vitro* and hardening-off or acclimation. The material recovered from cryostorage, must be introduced to recovery medium initially in the dark. For introduction into *in vitro* culture, the surface of explants require to be disinfected and handled with sterilised instruments, with all procedures being carried out in a laminar air-flow. In conditions where no laminar flow box (clean bench) is available, it may be possible to perform the work in closed clean rooms with thorough room and air disinfection. Embryos and embryonic axes need to be rehydrated for 30 min at ambient temperature in the dark. Where they are directly exposed to a rewarming medium, rehydration should be in a solution of the same composition. Resultant seedlings each producing both a root and a shoot are a measure of successful axis cryopreservation. For vegetatively propagated material, cryostorage is considered successful when shoots are obtained, which can be either rooted or further micropropagated.

After a precautionary culture period in the dark (Touchell and Walters, 2000), explants are usually exposed to conventional growth room lighting conditions and temperature regimes which should be established at the outset as suiting the species and its provenance. Light regimes and temperature for *in vitro* germination and seedling/plantlet development are parameters that may need to be fine-tuned, and transferring explants through several culture phases may be necessary. It is critical that the seedlings and plantlets produced *in vitro* are initially maintained under high RH, which is gradually reduced.

The establishment *ex vitro* and its hardening-off essentially involves transfer of the seedlings/plantlet from slow growth culture or cryopreservation of vegetative material from the heterotrophic *in vitro* condition to a sterile soil-based medium

in which the autotrophic condition will develop. Recovery media must contain macro- and micro-nutrients, essential minerals and a carbon source, but may also require addition of growth regulators. Media must have been autoclaved during preparation, and any heat-labile components (if required) filter-sterilised and added subsequently. Suitable germination media for embryos/axes of a variety of species are based on MS (Murashige and Skoog, 1962): however, the MS nutrient medium may be utilized at full-strength, or half- or quarter-strength, as indicated by explant responses when first working with seeds of particular species. Depending on the objective sought, explants recovered from cryopreservation are directly grown into a seedling/plantlet for acclimatization, or a multiplication phase can occur before acclimatization, thus offering the possibility of producing the desired number of copies of the retrieved accession.

Contingencies

It should be noted that protocol development can require more than a single collection and may spread over two or more years due to the seasonal nature of seed availability.

It should be noted that material can be conserved either in LN or above LN in the vapour phase. Storage in the vapour phase is much more expensive and less safe than storage directly in LN. Even if some microbes are suspended in LN, there is not essentially the consequence that they would contaminate the samples, because they pass some washing procedures under sterile conditions upon rewarming. Even if spores may adhere to the surface of the explants, microbes cannot enter them in LN because all such processes are stopped at such low temperatures.

Excised axes may not germinate because of their maturity status. Hence, the collected propagule needs to be placed in hydrated storage and sampled periodically for germination and for performance of excised axes. In the event that neither seed/propagule nor excised embryos/axes geminate, the material may be dead, or dormant. Performing a tetrazolium test will determine whether or not the seeds are viable. If so, then dormancy may be assumed, and investigations to break the dormant condition need to be undertaken.

In the case of most recalcitrant-seeded species, regeneration as practised for orthodox-seeded species is not an option. If there is an unacceptable decline in quality of cryo-stored embryos/axes, the only option would be re-sampling of seeds from the parent population(s) and refining of the procedures. In cases where embryos/embryonic axes continue to be intractable to cryopreservation, then attention must

be focused on the development of suitable alternative explants, ideally derived from seedling/plantlets established *in vitro*.

Material from prolonged *in vitro* culture or *in vitro* storage may no longer be suitable for extracting shoot tips for cryopreservation, since this material may contain or may have accumulated covert bacteria (endophytes) which will break out during recovery from cryopreservation and, thus, hamper cryopreservation entirely. There are instances where explants (e.g. nodal segments) of source material from long-term *in vitro*-maintained cultures are excessively hydrated. In such cases, source material should be cultured *de novo*.

Cultures that have become infected need to be immediately removed from the growth room and destroyed. The most devastating contingency in any growth room is infestation by mites. After removing any cultures showing 'mite tracks', rapid response by disinfesting the facility is required. This is followed by inspection of each culture vessel and removal and destruction of any left which show evidence of mites (which bite through Parafilm™, and spread fungal spores from any infected culture to others).

Depletion of LN in a cryostorage vat or LN freezer would lead to irretrievable loss of all samples. If not detected, electrical or other failure of the temperature control system in a growth room could cause overheating with consequent loss of *in vitro* material.

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6.6 Standards for documentation

Standards

6.6.1 Passport data for all accessions should be documented using the FAO/Bioversity multi-crop passport descriptors. In addition, accession information should also include inventory, orders, distribution and data user feedback.

6.6.2 Management data and information generated in the genebank should be recorded in a suitable database, and characterization and evaluation data (C/E data) should be included when recorded.

6.6.3 Data from 6.6.1. and 6.6.2 should be stored and changes updated in an appropriate database system and international data standards adopted.

Context

Comprehensive information about accessions is essential for genebank management. Passport data is a minimum, but additional information including geographical (GPS coordinates) environmental (overlaid climate and soil maps) data of the collection site and historical information as well as data on characterization and evaluation of the material are all very useful.

Technical aspects

Due to advances in information technology, it is now relatively simple to record, manage and share information about accessions. All genebanks should use compatible data storage and retrieval systems. The FAO/Bioversity multi-crop passport descriptors (Alercia *et al.*, 2012) should be used by all genebanks as it facilitates data exchange.

Characterization and Evaluation data are produced by users. Such data are useful to the genebank in the management of their collections (Filer, 2012) and to facilitate the consecutive use. Genebanks are recommended to request information feedback on these data.

Management data should be as complete as possible to enable an effective handling of the collection. Most management data are only of internal use to the curator and of limited or no value to others, users and/or recipient genebanks. Therefore, management data should be restricted for use of the collection holder only; a set of the accession history, life form and availability can be extracted for public use. Beside the key data for the accession (passport and characterization data) they should contain the following:

- History (date of acquisition, preliminary numbers, date of changing the numbers, taxonomical determination, name of the specialist who determined the material, cultivation of any donor material in field or greenhouse, way of extracting the *in vitro*- and cryo-material from this donor material).
- Type of storage (*in vitro* or cryopreservation, or hydrated storage in the case of recalcitrant seeds).
- Place of the stored material (cultivation rooms, cryo-tank with concrete placement in rack and box).
- Splitting of the accession in several parties (when material is split in sub-clones, several cryopreservation sets, number of stored tubes).
- Safety duplication (duplication date, duplicated in which institution/country, responsible person there, reference to duplication agreement documents).
- Reference to the protocol used for *in vitro* culture and/or cryopreservation.
- Labelling of the culture vessels (colour codes, barcodes). LN-resistant labels are available, which, if necessary, can be wrapped around already frozen cryotubes.

Further advances in biotechnology will allow phenotypic data to be complemented by molecular data. Bar coding of accessions will be helpful in managing the information and the material and reduces the possibility of making mistakes.

A majority of genebanks now have access to computers and the internet. Computer-based systems for storing data and information allow for comprehensive storage of all information associated with the management of *in vitro* and cryopreservation collections. Germplasm information management systems such as GRIN-Global (2011) have specifically been developed for universal genebank documentation and information management. The adoption of data standards which today exist for most aspects of genebank data management helps make the information management easier and improves use and exchange of data. Sharing accession information and making it publicly available for potential germplasm users is important to facilitate and support the use of the collection. Ultimately, conservation and usability of conserved germplasm are promoted through good data and information management

Contingencies

Loss or incomplete documentation reduces the value of an accession, to the point of making it unusable. Inappropriate material (e.g. not LN-resistant labels) can cause loss of data. In large collections, skill of the workers becomes a very important factor. Risks of inadequate data entries must be clearly indicated. In complicated collections, active access to management data should be limited to the responsible persons only.

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6.7 Standards for distribution and exchange

Standards

6.7.1 All germplasm should be distributed in compliance with national laws and relevant international conventions.

6.7.2 All samples should be accompanied by a complete set of relevant documents required by the donor and the recipient country.

6.7.3 The supplier and recipient should establish the conditions to transfer the material and should ensure adequate re-establishment of plants from *in vitro*/ cryopreserved material.

Context

Germplasm distribution is the supply of a representative sample from a genebank accession in response to requests from germplasm users. There is a continuous increase in demand for genetic resources to meet the challenges posed by climate change, by changes in virulence spectra of major insect pests and diseases, by invasive alien species and by other end-users needs. This demand has led to wider recognition of the importance of using germplasm from genebanks, which ultimately determines the germplasm distribution. It is important that distribution of germplasm across borders adheres to international norms and standards relating to phytosanitary regulations and according to provisions of relevant international treaties and conventions on biological diversity and plant genetic resources.

Technical aspects

The two international instruments that govern the access of genetic resources are the ITPGRFA and the CBD. The ITPGRFA facilitates access to PGRFA, and provides for the sharing of benefits arising from their utilization. It has established a multilateral system for PGRFA for a pool of 64 food and forage crops (commonly referred to as Annex 1 crops to the Treaty), which are accompanied SMTA for distribution. SMTA can also be used for non-Annex 1 crops; however, other models are also available. Access and benefit-sharing under CBD is according to its Nagoya Protocol. Both the ITPGRFA and CBD emphasize the continuum between conservation and sustainable utilization, along with facilitated access and equitable sharing of benefits arising from use.

All accessions should be accompanied with the required documentation such as phytosanitary certificates and import permits, as well as passport information. The final destination and the latest phytosanitary import requirements for the importing country (in many countries, regulations are changed frequently) should be checked before each shipment. Germplasm transfer should be carefully planned in consultation with the national authorized institute, for the appropriate documentation, such as an official phytosanitary certificate, that complies with the requirements of the importing country. The recipient of the germplasm should provide the supplying genebank with information concerning the documentation required for the importation of plant material, including phytosanitary requirements.

Most recalcitrant-seeded species are long-lived perennials that do not reproduce until they are several years old. Thus, regeneration is not a rapid way to bulk up sample sizes to meet demand. If the sample is in the form of alternate explants *in vitro*, multiplication before the production of independent plantlets is possible, but a request must be made in advance.

Germplasm should reach its destination in good condition and so adverse environmental conditions during transport and clearing customs should be minimized. A reliable courier service having experience in dealing with customs is recommended. The time span between receipt of a request for germplasm and the dispatch of the materials should be kept to a minimum to enhance the efficiency of the genebank function. If the sample is in the cryopreserved state and is being transferred to another genebank where it will continue to be cryopreserved, the sample must be shipped in a LN 'dry shipper'.

If the sample will be set out to grow immediately on receipt, it can be rewarmed, rehydrated and encapsulated in a calcium alginate bead prior to dispatch. Such

synthetic seeds were originally developed for somatic embryos, but can successfully maintain in good condition cryopreserved excised embryos/axes that have been rewarmed and rehydrated, for at least 10 days at 16 °C without germination (radicle protrusion) being initiated. Germination and seedling/plantlet establishment of synthetic seeds is possible both *in vitro* and could succeed in sterile seedling mix. It is an option also for other small explants from cryopreservation, but the technique is applied in few cases only.

Plantlets derived from *in vitro* slow-growth storage or cryopreservation should be sent in appropriate containers. Recipients of *in vitro*/cryopreserved material need to have the possibility to transfer the material to pots or to the field, or be able to make such arrangements.

Sterile plastic bags, which may contain special aeration zones, are recommended for sending of *in vitro* plantlets. If glass is used, sufficient stuffing of the containers and declaration of fragility need to be ensured. In cases of glass and plastic vessels, also the right orientation of the containers needs to be indicated.

Contingencies

Poor handling, including inappropriate packaging or delay in shipment, can lead to loss of viability and the loss of material. Thus, it is very important that the supplier and recipient have established the condition under which the material is transferred and that the prerequisite for adequate re-establishment of plants is ensured.

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6.8 Standards for security and safety duplication

Standards

6.8.1 A risk management strategy should be implemented and updated as required that addresses physical and biological risks identified in standards including issues such as fire, floods and power failures.

6.8.2 A genebank should follow the local Occupational Safety and Health requirements and protocols. The cryo-section of a genebank should adhere to all safety precautions associated with using LN.

6.8.3 A genebank employs the requisite staff to fulfil all routine responsibilities to ensure that the genebank can acquire, conserve and distribute germplasm.

6.8.4 A safety duplicate sample of every accession should be stored in a geographically distant genebank under best possible conditions.

6.8.5 The safety duplicate sample should be accompanied by relevant documentation.

Context

It is of the utmost importance that the physical infrastructure of any genebank as well as the safety of its staff be protected so as to ensure that the conserved germplasm is safe from any threatening external factors. To manage a germplasm collection successfully, a genebank also requires skilled and trained staff. Management involves not only the maintenance of the collection and its data but also an assessment of risks from human activity or those naturally caused. There are particular hazards associated with the use of LN.

The physical security of the collections also requires a safe duplication of the collections in a geographically distant location, under the same conditions. In case of natural/physical catastrophe (fire, flood), this duplication might be used to re-build the collections. In addition to the sample itself, safety duplication involves the duplication of information that implies database backup.

Technical aspects

A genebank should implement and promote systematic risk management that addresses the physical and biological risks in the every-day environment. It should have in place a written risk management strategy on actions that need to be taken whenever an emergency occurs in the genebank concerning the germplasm or the related data. This strategy and an accompanying action plan must be regularly reviewed and updated to take account of changing circumstances and new technologies, and well publicized among their genebank staff.

The occupational health and safety of the staff should also be considered. The cryostorage area should be well ventilated with forced air extraction, and oxygen monitors should be in place. Leakage of LN into cryovials is potentially dangerous; therefore, appropriate vessels that are specifically designed for the purpose should be used, and the manufacturers' instructions should be strictly adhered to. To reduce risk of personal injury operators should wear protective clothing, gloves and face masks.

Supplies of LN must always be available, and it is vital that levels of LN are maintained. The cryogenic storage tanks are supposed to be placed in an appropriated location: aerated and with temperature less than 50 °C. Maintenance of the level of LN in storage containers is absolutely critical; if all the LN evaporates, the entire contents of the storage container must be discarded.

For the maintenance of viability of samples, the temperature of the tissue must be kept below the glass transition temperature[1]. Care must be taken that when removing a vial from a cryo-cane or from a cryo-box that the temperature of the remaining vials does not increase to the glass transition temperature. Vials should not be labelled with conventional adhesive labels, as they will come off at LN temperatures. The use of a dedicated PC-operated label printer allows specific cryovial labels to be printed, recording information and a unique barcode. The manufacturer's recommendations about which vial to use for which particular purpose should be adhered to.

Active genebank management requires well-trained staff, and it is crucial to allocate responsibilities to suitably competent employees. A genebank should therefore, have a plan in place for personnel, and a corresponding budget allocated regularly so as to guarantee that a minimum of properly trained personnel is available to fulfil the responsibilities of ensuring that the genebank can acquire, conserve and distribute germplasm. Access to disciplinary and technical specialists in a range of subject areas is desirable. Staff should have adequate training acquired through certified training and/or on-the-job training and training needs should be determined as they arise.

For the physical security of the collections, safe duplication of the collections in a geographically distant location under the same conditions should be considered. In case of natural/physical catastrophe (fire, flood), this duplication might be used to re-build the collections. The duplicating bank should be located somewhere that is politically and geologically stable, and at an elevation that rising sea levels will not be a problem. The storage conditions for the safety duplicate should be as good as those of the initial collection.

1 In PVS2, one of the most commonly-used cryo-protectant solutions, glass transitions occur at about -115 °C.

Safety duplication requires a signed legal agreement between depositing and storing or repository genebank. The latter has no entitlement to the use and distribution of the germplasm. The access to the collections should be controlled to avoid unauthorized usage.

Samples for the safety duplicate should be prepared in the same way as the initial collection. It is the responsibility of the depositor to ensure that the safety duplicate is of good quality. To prevent deterioration in transit to the receiving bank, cryopreserved samples should be dispatched in a LN dry shipper, and transit should be as rapid as possible.

Contingencies

When suitably trained staff is not available, or when there are time or other constraints, a solution might be to outsource some of the work or call for assistance from other genebanks.

Unauthorized entry to the genebank facilities can result in direct loss of material, and jeopardize the collections through introduction of pest and diseases.

LN containers are often contaminated with fungi or bacteria. If samples are stored in the liquid phase of the nitrogen, contamination of the sample can occur.

Liability issues may arise if material deteriorates in transit. Therefore, all eventualities need to be adherent to the consignment agreement.

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