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A protocol to determine seed storage behaviour

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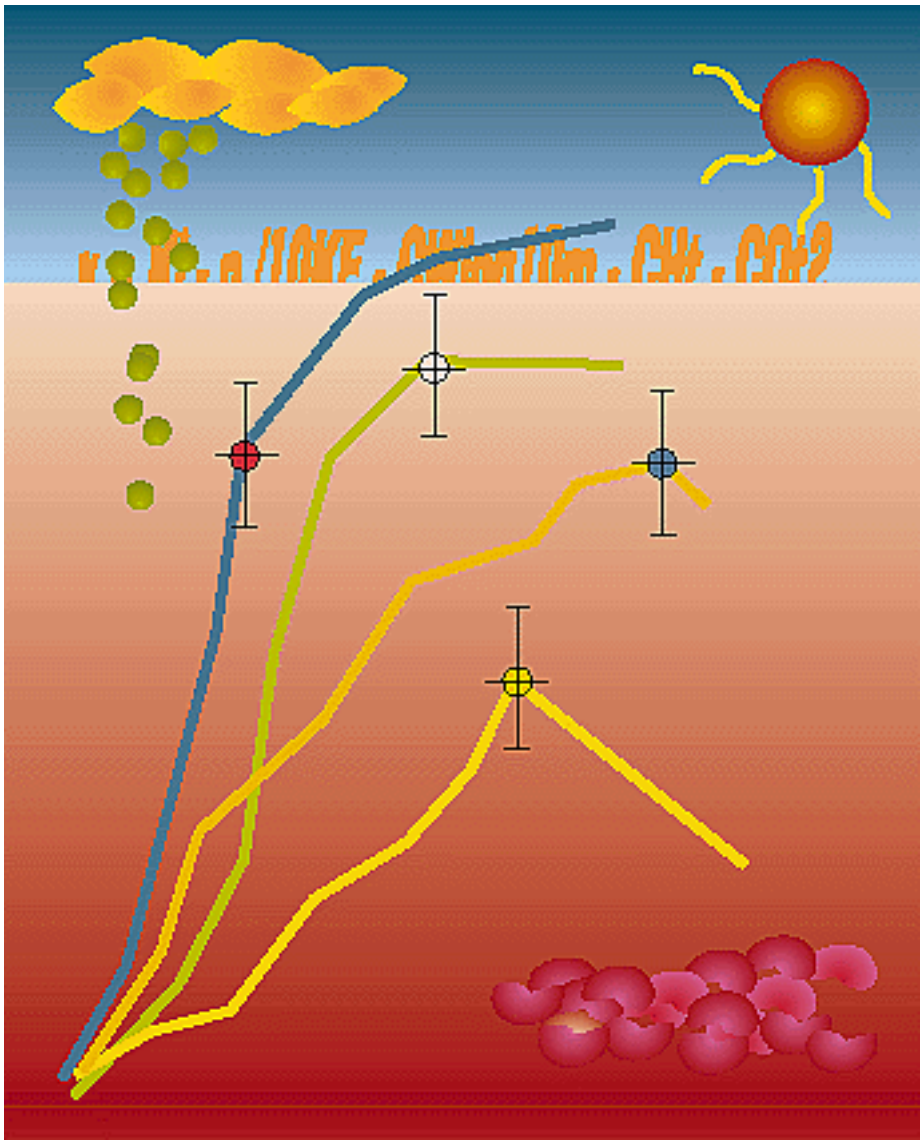

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Introduction to the series

The concept of the Technical Bulletin series was developed by Dr J.M.M. Engels and Ms J. Toll of the Germplasm Maintenance and Use Group of IPGRI. The Series as a whole is targeted at scientists and technicians managing genetic resources collections. Each title will aim to provide guidance on choices while implementing conservation techniques and procedures and in the experimentation required to adapt these to local operating conditions and target species. Techniques are discussed and, where relevant, options presented and suggestions made for experiments. The Technical Bulletins are authored by scientists working in the genetic resources area and IPGRI would appreciate receiving suggestions of topics for future volumes. In addition, IPGRI would encourage, and is prepared to support, the exchange of research findings obtained at the various genebanks and laboratories.

Masa Iwanaga
Chair, IPGRI Publications Committee and
Deputy Director General (Programme)

Contents

Introduction to this volume

The editors are pleased with the publication of this volume since it is dealing with an important subject area in the conservation of plant genetic resources. Because of the increased interest to conserve a wide range of species, including forestry species, the need to know how to handle and conserve the seeds of a given species in an optimum and cost-efficient way has become very pertinent. It is hoped that this publication will help genebank staff and other scientists in their duties to conserve the dwindling resources in a more secured and effective way.

The **Protocol to Determine Seed Storage Behaviour** should be read in conjunction with the related IPGRI publication **Seed Storage Behaviour: a Compendium**. The latter handbook provides an introduction to seed storage physiology, a selective summary of the literature on seed survival in storage, and thus on seed storage behaviour for over 7000 species.

Since the **Protocol** is meant to provide the reader with a guide for experimental work in determining seed storage behaviour under local conditions, ample space has been left in the margins for personal notes and observations. IPGRI would very much welcome receiving these results for inclusion in the aforementioned **Compendium**, to make them available to the plant genetic resources community. In addition, readers are invited to provide IPGRI with any feedback on possible improvements to the **Protocol**.

Summary

Ex situ conservation through long-term storage of seed is, in principle, possible for a significant proportion of higher plants. Where feasible, long-term seed storage serves as a safe and relatively inexpensive method of plant genetic resources conservation. At present, however, information on seed storage behaviour (i.e. survival and longevity of seed under various storage conditions) is available for only about 3% of higher plants. This publication provides an approach by which conservationists can determine whether or not long-term seed storage is feasible for a particular species, i.e. whether or not that species shows orthodox seed storage behaviour.

A two-stage procedure is outlined. The protocol not only enables determination of seed storage behaviour, but also allows for the determination of suitable environments for medium-term and short-term seed storage depending on whether the species under investigation shows intermediate or recalcitrant seed storage behaviour.

This Technical Bulletin provides advice on the implementation of the protocol, examples of ways in which the results from seed storage studies could be misinterpreted due to confounding factors, as well as several alternative approaches for estimating seed storage behaviour prior to carrying out actual investigations with the seeds. In particular, the latter section introduces the concept of a multicriteria approach for estimating seed storage behaviour.

1. INTRODUCTION

The importance of seed storage has been recognized ever since humans began to domesticate plants. The duration of successful storage depends upon both the objectives and the species concerned. Since agriculture began, farmers have had to maintain viable seeds from one growing season to the next (i.e. short-term seed storage, typically 3 to 9 months but occasionally up to 18 months). It may also be desirable to maintain "carry-over stock" for several years (medium-term seed storage, typically 18 months to 5 or 6 years). In both these cases, conventional practices have developed from previous experience of problems and successes with seed storage. In contrast, the goal of genebanks is essentially the maintenance of seed viability in a much wider range of species for indefinite periods (long-term seed storage, typically considered as 10 to 100 years or more), a much more difficult task which requires both specific facilities and a great deal of information if failures in *ex situ* genetic resources conservation by seed storage are to be avoided.

Seed longevity (i.e. the period of survival) varies greatly among species. It may also vary among accessions within a species because of differences in genotype and provenance. Influences of provenance on potential longevity result from the cumulative effect of environment during seed maturation, harvesting, drying and the pre-storage environment, and the time of seed harvest, duration of drying and the subsequent period before seed is placed in store. How much of the potential longevity established through the effects of genotype and provenance, etc. is realized depends on subsequent storage conditions.

Not all species' seeds respond to the environment before and during storage in the same way. Three main categories of seed storage behaviour are now recognized (although each may be further subdivided). Roberts (1973) defined two categories of seed storage behaviour: orthodox and recalcitrant. More recently, a third category intermediate between the orthodox and recalcitrant categories has been identified (Ellis *et al.* 1990a). Seeds of species with orthodox seed storage behaviour can be maintained satisfactorily *ex situ* over the long term in appropriate environments (but it should be noted that the absolute longevity of seed accessions can differ markedly among species even in the same environment). The maintenance of the viability of seeds of species with intermediate or recalcitrant seed storage behaviour is problematic, however. In general, medium-term storage is feasible for seeds of species with interme-

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diate seed storage behaviour provided the storage environment is well-defined (and well controlled), but short-term storage is usually the best that can be achieved with seeds which show recalcitrant seed storage behaviour (and, again, only under well-defined and well-controlled environments). The precise definitions of these three categories of seed storage behaviour are provided elsewhere (see section 6), but it follows from the above that if an accession of seeds is to be conserved then it is essential to know whether the species shows orthodox, intermediate or recalcitrant seed storage behaviour in order to determine first, the most suitable storage environment(s), and second, the likely duration of successful storage.

We have collated information on seed survival during storage for over 7000 species in a *Compendium* (Hong *et al*. 1996). However, published information on seed storage behaviour, particularly in non-crop species, is meagre in the context of the task of *ex situ* biodiversity conservation. The *Compendium* summarizes published information, but is limited to only 2.5% of the 250 000 or so species of flowering plants (Hong *et al*. 1996).

The objective of this publication is to suggest a protocol by which (i) seed storage behaviour can be determined for species in which information is currently lacking and (ii) appropriate environments can be determined for medium-term and short-term seed storage for species with intermediate or recalcitrant seed storage behaviour, respectively. This text is directed at those who wish to conserve seed accessions (e.g. foresters, seed producers, genebank managers and technicians, etc.) with the **minimum** of preliminary work and with **limited** equipment. With this objective in mind we begin by introducing the protocol. Supporting text (for example, the definitions of seed storage behaviour, and approaches to estimating seed storage behaviour before storage data are available) and caveats are then provided. Note that throughout this text, terms specific to botany and plant genetic resources are defined in *Elsevier's Dictionary of Plant Genetic Resources* (IBPGR 1991); seed moisture contents are expressed on the fresh weight basis (or wet weight basis, w.b.), according to the Rules of the International Seed Testing Association (see section 3.5). At the same moisture content seeds of contrasting species may differ in equilibrium relative humidity (and thus water activity and water potential) (see Cromarty *et al.* 1982; Roberts and Ellis 1989). These differences are largely the result of differences in seed composition. From the point of view of the status of the water within seeds of contrasting species it would be preferable

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to refer to equilibrium relative humidity, but existing practices in seed research concentrate on moisture content. Accordingly both are given herein.

2. DESIGN OF INVESTIGATIONS TO DETERMINE SEED STORAGE BEHAVIOUR AND TO SUGGEST APPROPRIATE ENVIRONMENTS FOR STORAGE

For practical seed storage purposes, particularly genetic resources conservation, discrimination among the orthodox, intermediate and recalcitrant categories of seed storage behaviour (see section 6) enables one to determine whether the species can be maintained successfully over the long term (e.g. at –20°C with 5±1% moisture content), the medium term (e.g. at +10°C at moisture contents in equilibrium with 40-50% RH, i.e. about 7-11% moisture content, depending upon species), or only the short term (e.g. imbibed seeds at +15°C), respectively. Simplistically, then, it could be argued that only three treatments (storage at conditions similar to the examples given in parentheses above) are required in a protocol to determine seed storage behaviour. However, we believe it is preferable (in order to reduce mistakes in classification) to use a two-stage procedure with more treatment combinations.

Although the definitions of orthodox and intermediate seed storage behaviour (in particular) are based on the response of seed longevity to air-dry storage environments, a minimum degree of desiccation tolerance is required if seeds are not to die as soon as they are first exposed to air-dry environments. Accordingly, the first step of the protocol to determine seed storage behaviour considers desiccation tolerance to low moisture contents.

Using ambient relative humidity and temperature to dry seed to about 12-18% moisture content (depending on season, location and species) has been commonly practised (Willan 1985). Although tolerance of desiccation to these levels of moisture content is usually sufficient to differentiate recalcitrant seed storage behaviour from orthodox and intermediate seed storage behaviour, it is insufficient to distinguish between the latter two categories. Desiccation to a wider range and to lower values of moisture content is therefore required for practical decisions on seed storage. This, of course, has long been recognized. For example, in order to investigate desiccation tolerance in seeds of *Citrus* spp., Honjo and Nakagawa (1978), Mumford and Grout (1979) and King and Roberts (1980b) dried seeds from about 40 to 3.5% moisture content by reducing seed moisture content in steps of 5-

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9% and then determined viability. Similarly, in *Quercus* spp., seed viability was determined after reducing seed moisture content in 5% steps (Tamari 1978; Gosling 1989; Pritchard 1991; Finch-Savage 1992a), while reducing seed moisture content in 5% and 2% steps from >40 to 8% was advised by F.T. Bonner for tropical tree species (Schaefer 1990). Finally, determining viability after reducing seed moisture content in steps of 5-10%, or even 2-5%, from fresh seed values to 5% moisture content and below has been used frequently in tree seed investigations (King and Roberts 1980b; Chin *et al*. 1981; Tompsett 1984a, 1987; Corbineau and Côme 1988; Hong and Ellis 1990, 1992a, 1992c; Ellis *et al*. 1990a, 1991a, 1991b, 1991c; Berjak *et al*. 1990; Dickie *et al*. 1991, 1992; Chaudhury and Chandel 1994; Pritchard *et al*. 1995).

2.1. The degree of desiccation tolerance

On receiving (or harvesting) a sample of seeds of a species in which seed storage behaviour is unknown it is necessary (for reasons, see section 6.2.7) first to note the degree of maturity of the seeds (or fruits), and to take two subsamples; the first to estimate seed moisture content and the second to estimate seed viability.

Since germination tests are carried out after sampling from a larger population of seeds, they are subject to sampling error. This can be considerable, particularly if sample sizes are very small. Where the number of seeds available for such tests is not a limiting factor, it is suggested that all germination tests be carried out on samples of 400 seeds each.

Estimates of seed moisture content are also subject to error, of course, and the most appropriate way to minimize problems arising from such sources of error when determining desiccation tolerance is to determine viability after a large number of different desiccation treatments. Ideally (i.e. when the number of seeds available is not a limiting factor), samples of fresh seeds should be taken, followed by further samples taken at 5% moisture content steps as moisture content is reduced to 15%, but subsequently in 2.5% steps to around 2.5% moisture content (the latter may be difficult to achieve in very starchy seeds, in which case the lowest value should be about 4% moisture content). For example, if initial seed moisture content is 58%, the ideal number of subsamples is 13 (i.e. samples drawn at 50, 45, 40, 35, 30, 25, 20, 15, 12.5, 10, 7.5, 5 and 2.5% moisture content). If 400-seed samples were used for the germination tests for each of the above, then 5600 seeds would be required, plus about 140 g (or 260-325 large seeds) for the moisture content determinations.

However, it is very often the case, especially in large seeds, that only a few seeds are available for experimental work. In such cases, it is preferable to reduce the germination test sample sizes rather than the number of treatments imposed. However, if sample sizes have been reduced to between 50 and 100 seeds per test but sufficient seeds are still not available then it is necessary to also reduce the number of desiccation treatments. In this case, we suggest reductions in the desiccation treatments to 50, 40, 30, 20, 15, 12.5, 10, 7.5, 5 and 2.5% moisture content; if seed supply is still insufficient then moisture content should be reduced to 40, 20, 15, 10 and 5%. In the case of a very limited supply of seeds, the number of desiccation treatments (i.e. in addition to the control) might be reduced to two, at 12% and 5% moisture content, but of course this would be very much a preliminary investigation.

It is preferable to determine the degree of desiccation tolerance and also seed storage behaviour within a species on results from **several different seed lots, not on one seed lot alone**. Ideally the different seed lots should be obtained from as many different sources as possible. **On no account should different seed lots be mixed, however**.

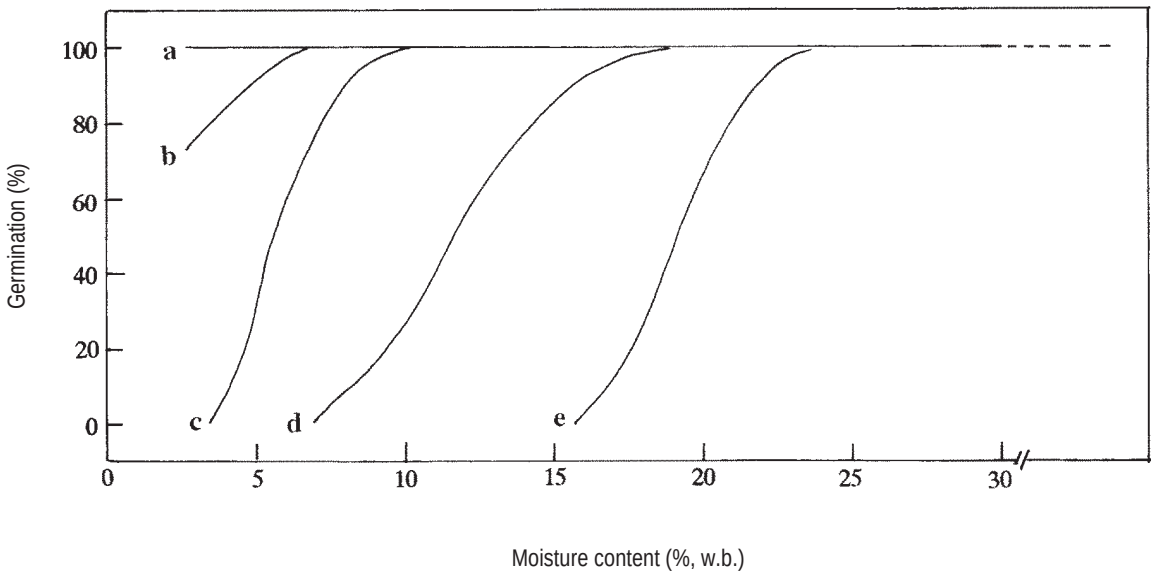

Fig. 1. The typical patterns of desiccation tolerance (normal germination, %, plotted against moisture content, % w.b.) are shown for species with orthodox (a), intermediate (c) and recalcitrant (e) seed storage behaviour. Two further patterns (b and d) are also shown which may be found in species with orthodox and intermediate seed storage behaviour, respectively, where the seed lot has been harvested prematurely or too late, or has been wrongly pretreated (see sections 4, 6.2.7 and 6.4). Seeds of some species with recalcitrant seed storage behaviour can be much more intolerant of desiccation than those shown by pattern e.

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After the samples have been dried, their viability should then be determined. This will normally be estimated by a germination test, in which case it is important to avoid imbibition injury to the dry seeds (see section 6.2.6), and to avoid confounding seed dormancy with viability (see Ellis *et al*. 1985a or section 4.7).

The results for normal germination against seed moisture content should be plotted. It is then necessary to interpret these results. There are three main possible outcomes:

(a) all seeds tolerate desiccation (i.e. show no loss in viability) to about 5% moisture content and below (and in particular to levels of moisture content in equilibrium with 10-13% RH at 20°C), in which case they are *likely* to show orthodox seed storage behaviour;

(b) most or all seeds tolerate desiccation to about 10-12.5% moisture content (i.e. in equilibrium with about 40-50% RH at 20°C), but further desiccation to lower moisture contents reduces viability, in which case they are *likely* to show intermediate seed storage behaviour;

(c) most or all seeds are killed by desiccation to 15-20% moisture content (i.e. values in equilibrium with >70% RH at 20°C), in which case they are *likely* to show recalcitrant seed storage behaviour.

Note that these comments are probability statements; the determination of desiccation tolerance alone does not enable determination of seed storage behaviour.

These three patterns of response are shown in Figure 1 (patterns a, c and e respectively). Two further patterns of response to desiccation, b and d, are also shown in which preliminary conclusions on seed storage behaviour are more uncertain. Pattern b is found, for example, both in species with orthodox seed storage behaviour where the seed lot has been harvested slightly prematurely (see section 6.2.7) or has been pretreated (e.g. prechilling, see section 6.2.8), and in some of the best seed lots of species with intermediate seed storage behaviour. Pattern d is found, for example, in species with intermediate seed storage behaviour where the seed lot has been harvested prematurely (see section 6.4) or exposed to high moisture contents for long periods (e.g. soaking, fermentation, moist storage, prechilling).

In other words, conclusions based on desiccation tolerance alone (particularly in cases where investigations are limited to a single seed lot) can sometimes be erroneous. Hence, the second step in this algorithm comprises investigations of seed survival

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during storage in different environments. Depending upon whether orthodox, intermediate, or recalcitrant seed storage behaviour is *suspected* from the results of step 1, go to 2.2.1, 2.2.2, or 2.2.3, respectively.

2.2. Determination of suitable environments for seed storage

The second step of the protocol requires investigations of the survival of seeds following storage in different environments. A simplified outline of the two steps is presented in Figure 2.

2.2.1. Confirmation of orthodox seed storage behaviour

Seeds which tolerate desiccation to 5% moisture content do not necessarily show orthodox storage behaviour. For example, seeds of *Cattleya aurantiaca* (Orchidaceae) tolerated desiccation to 3.7 and 2.2% moisture content (94% germination), but only 10% germinated following 90 days of hermetic storage at –18°C with 3.7% moisture content, while 36% germinated after 6 years of storage at 5°C with this moisture content (Seaton and Hailes 1989; Pritchard and Seaton 1993). Hence investigations of seed survival in different storage environments are required to determine seed storage behaviour.

A factorial combination of three levels of moisture content (10, 7.5 and 5%), four different values of temperature (10, 5, 0 and –20°C) and at least two different durations (3 and 12 months or more) of hermetic storage (i.e. a total of 27 subsamples) is suggested. The subsamples include controls: for each of the three levels of moisture content before experimental storage + three moisture content levels x four temperatures x two durations. This particular combination of treatments can be useful in delineating optimum air-dry storage environments if, in fact, the seeds show intermediate rather than orthodox seed storage behaviour. If the number of seeds available is considerable then include more subsamples for additional longer-duration treatments, for example, to 10-15 years, as Barton (1961) often used in her studies. Where only a limited supply of seeds is available, it is possible to store seeds in only one environment (*viz*, –20°C with 5% moisture content). If all or most seeds survive the pre-storage desiccation treatment but nevertheless many die during 12 months of subsequent storage then the species probably shows intermediate seed storage behaviour. If no loss in viability is evident during this period then the species probably shows orthodox seed storage behaviour. This is because, in our experience, even the very best quality seed lots of species with intermediate seed storage behaviour have shown some loss in viability during 12 months of storage under such conditions.

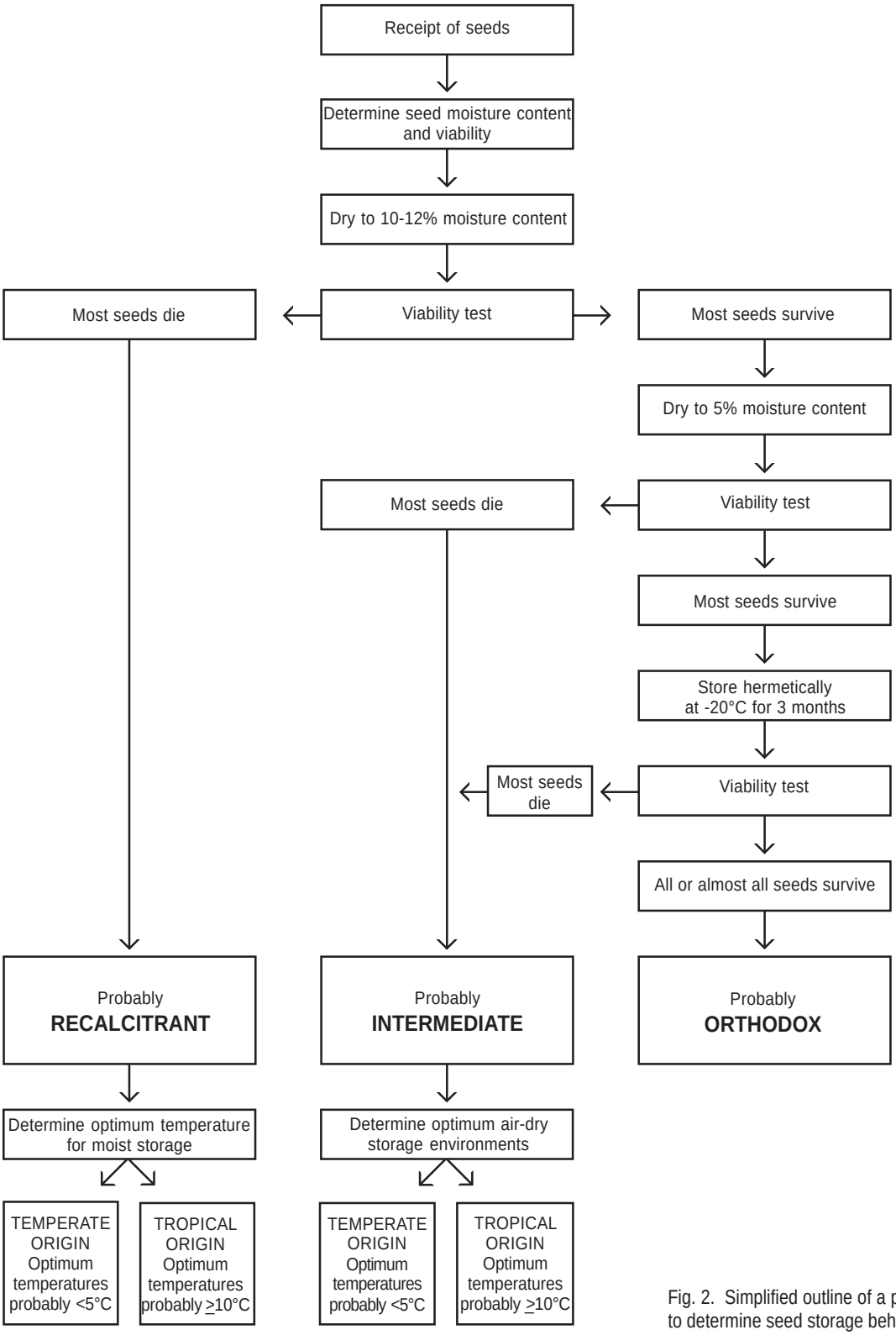

Fig. 2. Simplified outline of a protocol to determine seed storage behaviour

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Seeds of species which show orthodox seed storage behaviour can be stored in a wide range of different environments, although their longevity will vary greatly depending upon environment. The variation of the longevity of orthodox seeds with environment in air-dry storage is described by the seed viability equation (see section 6.2.2). The preferred conditions for the storage of seeds of species with orthodox seed storage behaviour is –18°C or less with 5±1% moisture content (Cromarty *et al.* 1982).

2.2.2. Determination of optimum air-dry storage environments for species believed to show intermediate seed storage behaviour

Subsamples should be stored hermetically in a total of 105 treatment combinations comprising a factorial combination of five moisture contents (x+4, x+2, x, x-2 and x-4, where x is the moisture content, identified in the initial desiccation tolerance investigation, beyond which further desiccation reduced viability significantly), and seven storage temperatures (20, 15, 10, 5, 0, –10 and –20°C) and at least three storage durations (3, 12 and 24 months). If sufficient seeds are available, extra treatments can enable viability tests to be made after storage durations of 5 years or more. If the number of seeds is limited, a minimum of 18 treatment combinations including three levels of moisture content (x+2, x and x-2), three temperature levels (15, 10 and 5°C for species which originate in the lowland tropics; 10, 5 and 0°C for species adapted to the high altitude tropics, or 10, 0 and –10°C for species adapted to temperate latitudes) and two different storage durations (6 and 24 months). In both the above cases, control treatments (without experimental storage) at each moisture content are also required.

Comparison of the results of viability tests after these different seed storage treatments should indicate which of the storage environment(s) used minimizes loss in viability during storage; this environment should be selected for the storage of seeds of that species. However, further investigations of seed survival in storage with different seed accessions of that species, longer periods of storage, and factorial combinations of different storage temperatures and moisture contents close to the initial estimate of the optimum combination are required in order to (a) maximize longevity in storage and (b) estimate likely maximum storage periods before regeneration is required.

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2.2.3. Determination of optimum storage environments for species with recalcitrant seed storage behaviour

This procedure is unlikely to provide results which challenge the conclusion drawn from the initial investigation of desiccation tolerance. Rather, the procedure has the objective of determining a suitable environment for short-term seed storage assuming the species shows recalcitrant seed storage behaviour.

In general, the longevity of recalcitrant seeds is maximal when stored fully or almost fully imbibed with oxygen freely available. However, germination is either **prevented** or **reduced** to a very slow rate. In other words, treatments are akin to slow-growth treatments in tissue culture. It is, therefore, easier to store recalcitrant species with dormant seeds than those with non-dormant seeds under such conditions, because seeds of the latter tend to germinate during storage. Low temperatures can reduce the rates of both seed deterioration and germination provided that they remain above the value which results in chilling damage (if applicable) or the lower value at which ice crystallization occurs (see section 6.3.2). Determination of the optimum temperature for imbibed seed storage is the principal objective of this procedure, because in species where chilling damage does not occur, seed longevity can be extended considerably at low temperature. In short, moist storage for recalcitrant seeds should be at moisture content levels between the "lowest safe moisture content" (see section 6.3) and the "fully-imbibed" level at the coolest temperature which is not damaging to seed viability. Here, we describe two methods of estimating optimum storage environments.

2.2.3.1. Method 1

This method has several advantages in that it requires (i) small number of seeds and (ii) often a short period of time. The main disadvantage is that it cannot provide any estimate of the duration of seed survival. This method comprises two steps.

Step 1. Determination of the minimal and optimal temperatures for germination

Freshly harvested seeds are tested for germination at constant temperatures ranging from –5 to 30°C for species which are adapted to temperate climates, or from 5 to 40°C for species which are adapted to warmer climates, in 5°C increments (i.e. –5, 0, +5, 10, 15, 20, 25, 30, 35 and 40°C). The duration of this test must be long enough (at least 3 months) to allow the seeds at

relatively cool temperatures to germinate (or die); in certain tree species, in particular, these durations may be considerable. At the end of the germination test, the total numbers of rotten (i.e. dead) seeds as well as firm, fresh seeds should be recorded.

Step 2. Determination of chilling sensitivity

When the above germination tests are concluded, the firm, fresh ungerminated seeds from each temperature treatment should be transferred to the optimum temperature (estimated from the results of step 1) for germination and the progress of germination recorded.

The objective of this test is to determine the coolest temperature at which the seeds can be stored without either chilling or freezing damage. Thus, the ideal temperature for imbibed seed storage would be that at which no seeds germinated or died during step 1, and from which all fresh seeds germinated after transfer to the optimum temperature for germination. This ideal is unlikely to be achieved. In practice it is necessary to select that regime in which the results most closely match this ideal. It is probable that this temperature is at, or close to, the minimum temperature at which there is some progress in germination.

2.2.3.2. Method 2

Fungal growth during moist seed storage is a problem, particularly with fully imbibed seeds (i.e. seeds at the minimum moisture content at which they will germinate). This problem can be reduced considerably by storage of seeds at moisture contents at or slightly less than the value at which they are shed. (Fungicides or antibiotics will further reduce fungal growth.) A factorial combination of two levels of moisture content (fully imbibed and subimbibed, i.e. about 2-5% below fully imbibed), four different levels of temperatures (20, 15, 10 and 5°C for species adapted to the lowland tropics; 15, 10, 5 and 0°C for species adapted to the highland tropics; and 10, 5, 0 and -5°C for species adapted to temperate latitudes), and at least four durations (1, 3, 6 and 12 months, with possible extension to 24 months for seeds of species adapted to temperate climates) are recommended for storage with viability being determined subsequent to these treatments. Those test results should indicate the most suitable environment (of those tested) for the short-term storage of recalcitrant seeds. Moist seed storage (e.g. at the moisture content at which they are shed) in a moist medium is preferable to imbibed storage in aerated polythene bags.

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3. PROCEDURES TO BE ADOPTED IN SEED STORAGE RESEARCH

The preceding text, which describes the protocol itself, is deliberately short. This is because it aims only to give an outline of the protocol which is discussed in further detail in this section. Those with considerable experience of seed storage research will recognize many caveats and concerns which are essential in such research but which, for reasons of brevity, have not yet been mentioned. The remainder of this text introduces some of these concerns, but the reader is also referred to the various relevant IPGRI (formerly IBPGR) publications, and especially the *Handbooks of Seed Technology for Genebanks*.

The following points should help to reduce the possibility of investigations providing results which result in erroneous conclusions being drawn regarding seed storage behaviour.

3.1. Harvesting or collection of fruits or seeds

Fruits or seeds should normally be harvested at maturity, i.e. when they are ready to disperse naturally. (There are exceptions to this generalization, however, such as where viviparous germination occurs or where seeds are not readily shed.) The criteria of maturity for forest tree seeds (a particular problem) have been described elsewhere (Willan 1985; Bonner *et al*. 1994). Monitoring the moisture content and dry weight of seeds during their development can often be useful in helping to decide when to harvest. In the case of trees and shrubs, seeds should ideally be collected from a single plant. If this does not provide sufficient samples, seeds at the same stage of maturity should be collected from neighbouring plants of the same age. Seeds of different maturity or those from different locations should not be mixed together; such samples should be treated as different accessions.

3.2. Transport

Where seeds are harvested at a high moisture content within fruits, it is generally safer to transport fruits rather than first extracting the seeds. Moist extracted seeds may germinate, or begin to germinate, during transport. Also, extracted seeds may lose moisture rapidly, which can be deleterious for those with recalcitrant seed storage behaviour, or they may rot. Strong perforated plastic or bamboo containers (or similar non-airtight containers), are generally suitable for transport. If temporary storage cannot be avoided, fruits of species of unknown seed storage behaviour should be stored at 15-20°C in perforated polythene bags for as short a period as possible before beginning the protocol.

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3.3. Extraction of seeds

Since relatively small quantities of seeds are required for research, extracting seeds from fruits by hand is feasible, and often desirable. Try to avoid using machines (blender, macerator), soaking in water, fermentation treatments and chemical treatments (such as hydrochloric acid, sodium carbonate, or enzyme digestion, etc.). Washing and cleaning seeds in running tap water under pressure, after macerating the flesh by hand, is normally sufficient to remove gelatinous coverings from seeds.

3.4. Drying

Seeds should be dried (in order to determine desiccation tolerance, section 2.1) immediately after extraction from fruits. Drying to low moisture contents should be comparatively quick, but at a cool temperature, in order to minimize seed deterioration during drying. To achieve this, the drying atmosphere must be dry, and ideally below 10% relative humidity (which will allow desiccation to about 3% moisture content for oily seeds and to about 4-6% moisture content for most starchy seeds). A temperature of about 15°C is safe for most tropical species (Table 1), even if desiccation is damaging. Delay in drying or slow drying (i.e. at a comparatively high relative humidity), together with high temperature (above 25°C) will tend to reduce viability considerably in orthodox seeds, particularly oily seeds. The rate of drying also depends on the amount of seed (particularly the depth of the layer of seeds), the circulation of dry air within the drying cabinet and the species. The seeds should be placed in a thin layer on top of a metal mesh to maximize air circulation. Details of techniques for drying seeds have been described elsewhere (Cromarty *et al.* 1982; Cromarty 1984). If seed-drying equipment (which has the advantage of forced-air circulation) is not available, silica gel is a very effective desiccant, particularly for drying seeds to very low moisture content. Silica gel is also useful even where environments of around 10% RH and 15°C are available in order to dry starchy seeds below about 8% moisture content.

Methods of drying seeds using silica gel have been described elsewhere (Hanson 1985; Cheng *et al.* 1990), but a few points are noted below. Dry (newly regenerated) silica gel is in equilibrium with about 5% RH at 20°C. The first change of colour of the indicator (diminishing intensity of blue) occurs when the silica gel moistens to equilibrium with about 12-13% RH. By the time it has become pale blue it is in equilibrium with about 49% RH (and thus very moist); 100 g of regenerated silica gel (in equilibrium with about 5% RH) can absorb about 7 g of moisture

Table 1. Optimum seed storage temperature and comments on plant ecology (references are shown in brackets)

Species	Seed storage behaviour	Optimum storage temperature	Origin and ecology
Artocarpus heterophyllus	Recalcitrant	15°C (10)	Native to SE Asia and Polynesia, dominant in rain forests of tropical lowland (13).
Camellia sinensis	Probably Intermediate	1°C (1)	Native to N Burma, grows naturally at altitudes up to 2000 m (13); the trees can tolerate subzero temperatures from –5 to –20°C (14).
Carica papaya	Intermediate	10°C (4)	Native to S Mexico and Costa Rica, up to 1700 m altitudes, but the plant does not withstand frost (13).
Citrus limon	Intermediate	5°C (3)	Native to drier monsoon areas of SE Asia (13); the tree can withstand light frost (13), or even –10°C for short periods (21).
Coffea arabica	Intermediate	10°C (11)	Native to highlands of 1300-1900 m altitudes of Ethiopia, where climate is dominated by 4-5 months of dry season with extreme temperatures of 4 and 31°C (8); the tree cannot survive at 0°C (13), but can survive 2°C for 6 hours (7), and 4°C for longer periods (5, 8).
Dipterocarpus baudii	Probably Recalcitrant	14°C (16)	Native to evergreen tropical lowland rain forests of SE Asia, where annual rainfall is about 2000 mm (2).
Dipterocarpus intricatus	Intermediate	2°C (18) to 6°C (17)	Native to dry dipterocarp forests of savanna zones of SE Asia, naturally grown at altitudes up to 1400 m (2).
Hevea brasiliensis	Recalcitrant	7°-10°C (6)	Native to Amazon basin, in evergreen tropical lowland rainforests, with annual rainfall of 2000-4000 mm. Despite tropical lowland origin, the plant can tolerate low temperatures; winter injury observed only when temperature is 4-5°C, and some clones can withstand –1°C (19).
Mangifera indica	Recalcitrant	15°C (9)	Native to rain forests of Indo Burma, India; naturally grown from lowland to altitudes of 1300 m (13); the plant is killed by 2°C, and can tolerate extreme temperatures of 5-10°C and 43°C for short periods (15).
Theobroma cacao	Recalcitrant	15°C (12)	Understorey trees of tropical rain forests of S America; also grown at 1300 m altitude in the Venezuelan Andes (13). Low temperature limit for cocoa-growing is a mean monthly minimum of 15°C, and an absolute minimum of 10°C (20).

References: (1) Amma and Watanabe 1983; (2) Ashton 1983; (3) Barton 1943; (4) Bass 1975; (5) Bauer *et al*. 1990; (6) Beng 1976; (7) Cambrony 1992; (8) Coste 1992; (9) Fu *et al*. 1990; (10) Hanson 1984; (11) Hong and Ellis 1992c; (12) Mumford and Brett 1982; (13) Purseglove 1968; (14) Sakai and Hakoda 1979; (15) Singh 1968; (16) Tamari 1976; (17) Tompsett 1987; (18) Tompsett 1992; (19) Webster and Baulkwill 1989; (20) Wood 1973; (21) Yelenosky 1978.

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from seeds until the first colour change is apparent (in equilibrium with 13.5% RH). At this point, the silica gel should be regenerated. Often, only a 1-cm layer of silica gel (i.e. the layer closest to the seeds) changes colour initially. For example, a drying box 10 x 10 x 12 cm containing 250 g dry silica gel can dry 250 g of rice (*Oryza sativa*) seeds (about 10 000 seeds) from 14-15% to about 5% moisture content in 21 days at 20°C. Such drying boxes can be placed in incubators maintained at suitable temperatures.

Silica gel should be regenerated when the colour first begins to change, i.e. two to four times a day when seeds are very moist, once a day when seed moisture content is reduced to 10-15% and every 3-7 days when moisture content is reduced below 7%. Silica gel is regenerated by drying in an oven maintained at about 130°C for 3-4 hours. It should then be stored in a sealed container overnight to cool to ambient temperature before being used to dry the seeds.

To determine whether seeds have reached the desired moisture content (DMC%), seed moisture content can be monitored by weighing before (initial seed weight, g) and during drying. The following formula can be applied:

Weight of seed (g) at DMC% =

$$\frac{(100 - \text{initial MC\%})}{(100 - \text{DMC\%})} \times \text{initial seed weight (g)}$$

Example: 125.3 g of seeds at 52.1% moisture content. What is the weight once this sample of seeds is dried to 15% moisture content?

Weight of seed at 15% moisture content =

$$\frac{(100 - 52.1)}{(100 - 15)} \times 125.3 = 70.61 \text{ g}$$

It should be noted that some seeds may be lost during monitoring. Hence, the final value must be determined directly (see below). However, before this is done, seed moisture content should be allowed to equilibrate by maintaining the seeds sealed at 15°C for 1 day if moist (above 20% moisture content), or for 2-3 days if dry (<20%), before samples are drawn to determine moisture content and germination ability.

3.5. Determination of seed moisture content

The procedures prescribed by the International Seed Testing Association (ISTA) (1993a, 1993b) should be followed. Information on the determination of seed moisture content for tree seeds can be found elsewhere (Bonner 1972; Bonner *et al*. 1994; Krisnapillay *et al*. 1991).

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For very large tree seeds, the determination of the moisture content of as many as 20-25 individual seeds (for each moisture content treatment) is recommended. Separate determination of the moisture content of embryo, or embryonic axis, cotyledons or endosperm, and the seed-covering structures may also be helpful when interpreting results.

Seed moisture content should be expressed on the wet basis (%, w.b.) and reported to the nearest 0.1% (e.g. ISTA 1993a, 1993b).

3.6. Germination tests

The conduct of seed germination tests has been described at length in *Handbook of Seed Technology for Genebank. Volume I.* (Ellis *et al*. 1985a). However, several points require emphasis here.

3.6.1. Humidification of dry seeds

To avoid imbibition injury, seeds at moisture contents below about 8-12% (depending on species and accession) need to be humidified until their moisture content has been increased to about 15-17% (estimate by weight). The objective is to raise seed moisture content while avoiding contact with liquid water. Such treatments can usually be completed within 12-48 hours at 20°C, depending on species. Small seeds can be spread uniformly within a Petri dish (empty and without lids). Three very moist paper towels are placed flat inside a large polythene box. The dishes containing the seeds are then placed on top of the moist paper and the tight-fitting box lid replaced. The box can then be placed in an incubator at 20°C. Thus, the atmosphere inside the box is very humid, but the seeds are not in contact with liquid water. Larger seeds can be placed in a cloth bag (mosquito net material) and placed on top of a gauze (to avoid any risk of seeds dropping through) above water in a desiccator or similar container (e.g. large polythene box) at 20°C (care should be taken to avoid direct contact of seeds with water). The seed layer should be only one seed deep, ideally, to enable all seeds to take up moisture equally rapidly from the atmosphere.

3.6.2. Dealing with dormancy and hard seeds

Specific germination test recommendations can be found in *Handbook of Seed Technology for Genebanks. Volume II*. (Ellis *et al*. 1985b), *Germination of Australian Native Plant Seed* (Langkamp 1987), and *Seeds of Woody Plants in North America* (Young and Young 1992). However, for seed storage research, it is often preferable not to pretreat seeds unless it can be confirmed that the method is not

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damaging to seeds of the accession being investigated. For example, we recommend that seeds should not be scarified with sulphuric acid, boiling water or by soaking as is commonly practised in nursery sowing. Scarification by hand (on sandpaper) for hard seeds (e.g. Leguminosae, Malvaceae), and removal of seed-covering structures (exocarp, mesocarp, endocarp, pericarp) by hand are preferred procedures in seed storage research. Prechilling (for species of temperate climates, including high altitude in the tropics), gibberellic acid (GA_3 at low concentrations, <100 ppm), potassium nitrate (concentration <0.1%) and light are acceptable in storage research provided that the treatment is not damaging to seeds. Dormancy-breaking research should be carried out before the start of seed storage research.

3.6.3. Duration of germination tests

The germination test should continue until seeds germinate or rot. This duration can be as long as 18 months for some species. Removal of the seed-covering structures, filing or chipping seeds with a scalpel, or nicking with a needle may help to promote germination during prolonged tests.

Although viability can be evaluated by the topographical tetrazolium test and the excised embryo test, a word of warning is necessary. The evaluation of viability by these tests is difficult, time-consuming, subjective and the techniques rely on considerable practical expertise (Justice 1972; Buszewicz and Gordon 1973; Ellis *et al*. 1985a; Bonner *et al*. 1994). Moreover, for many species no established or well-described techniques are available. It is suggested that these tests be applied to evaluate the sound and fresh seeds at the end of the germination tests, i.e. those seeds which remain intact and have not been severely damaged by fungal or bacterial growth. The goals of seed storage are successful germination and seedling establishment. Results of rapid viability tests are therefore best used to complement germination test results when investigating seed longevity and seed storage behaviour.

3.6.4. Dealing with fungal development during the germination test

Fungal development sometimes occurs during germination tests, especially for species with a mucilaginous layer subjected to prolonged duration germination tests. Fungi also develop profusely when seeds are dead. It is preferable to avoid using biocides before or during germination tests, but mucilaginous seeds can be disinfected by soaking in a 1% sodium hypochlo-

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rite solution (commonly comprised of 1 part of domestic bleach, i.e. 10% sodium hypochlorite, with 9 parts of distilled water) for 2-5 minutes, washed/rinsed thoroughly with tap water and blotted dry with paper towels. If fungal development occurs during tests, put the seeds in a sieve and wash with tap water under pressure to remove mycelia and dead tissue. The seeds can then be blotted surface dry with paper towels; rotted seeds should be discarded. The sound and fresh seeds can be moved to fresh germination media.

3.6.5. Evaluation of germinating seeds

Germinating seeds are counted at intervals during tests. The criterion of germination should be normal seedling development (ISTA 1993a, 1993b). After counting, germinating seeds are discarded. At the end of tests, record the number of fresh seeds present; these may be viable but dormant, and the use of the tetrazolium test or excised embryo test is helpful in confirming this.

4. FACTORS LEADING TO THE MISINTERPRETATION OF SEED STORAGE BEHAVIOUR

Roberts *et al.* (1984) recognized at least nine identifiable ways in which experimental results might be misinterpreted or confounded with other factors so that orthodox seeds might be wrongly classified as recalcitrant. We list below ten possible reasons for the misinterpretation of seed storage behaviour.

4.1. Investigations with immature seeds

Immature seeds, e.g. with moisture content above 60%, can be very sensitive to damage from desiccation even though the mature seeds show orthodox (Dasgupta *et al.* 1982; Ellis *et al.* 1987, 1993; Fischer *et al.* 1988; Hong and Ellis 1990, 1992a) or intermediate seed storage behaviour (Ellis *et al.* 1991a; Hong and Ellis 1995). Similarly, at later stages of seed development such as at the end of the seed-filling phase many orthodox species may still show damage upon desiccation to very low moisture content levels (see section 6.2.7, Fig. 6) (Hong and Ellis 1992a; Ellis *et al.* 1993; Ellis and Hong 1994). Thus, tests on immature seeds of a species with orthodox seed storage behaviour could result in erroneous classification as intermediate or recalcitrant. Similarly, the desiccation of immature seeds of a species with intermediate seed storage behaviour (see section 6.4, Fig. 8) may result in the erroneous classification as recalcitrant. This is a potential problem in tree species where

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losses of ripe seeds by wind, insects, animals and pests are anticipated, or in species in which hardseededness occurs during ripening, and in which losses are traditionally avoided by premature harvesting (Willan 1985).

4.2. Delayed harvesting

Delaying harvests beyond harvest maturity may also render seeds sensitive to damage from desiccation to very low moisture contents. For example, delaying the harvest of seeds of a *japonica* rice grown in a hot (stressful) seed production environment resulted in reduced desiccation tolerance, compared with those harvested at harvest maturity (Ellis and Hong 1994). The seeds produced and aged in a hot environment showed certain characteristics similar to those of intermediate seeds (Ellis and Hong 1994).

4.3. Seed processing methods

Methods of seed extraction from fruits may also influence desiccation tolerance. Any method involving long durations of exposure to high seed moisture content at temperatures at which some progress towards germination (not necessarily visible germination) is possible can increase desiccation sensitivity. Such treatments include prolonged soaking, fermentation of fleshy fruits and long durations of "temporary" storage of moist or freshly extracted seeds (Hong and Ellis 1992b).

4.4. Pretreated seeds

In temperate regions, in particular, seeds of species which exhibit strong dormancy (such as tree seeds) are commonly stored moist and cool in order to break dormancy (prechilling) prior to sowing. Such treatments may result in loss in desiccation tolerance (because germination *sensu strictu* may occur). Such effects may explain certain reports of **considerable** desiccation sensitivity in wild rice (*Zizania palustris*) since it is a common practice to store seeds of this species in water at 3°C.

4.5. Improper drying methods

Since the rate of loss of viability in orthodox seeds is a function of time, temperature, and moisture content (Roberts *et al*. 1984), inappropriate drying regimes, for example the use of high temperatures (particularly in seeds harvested/shed at high moisture contents), may result in considerable loss in viability during seed drying.

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4.6. Desiccation tolerance to a limited range of air-dry moisture contents

It is comparatively easy in many climates to dry seeds to 10-15% moisture content using ambient relative humidity. However, tolerance of desiccation to such levels of moisture content does not necessarily mean that the seeds are orthodox. For example, seeds of arabica coffee (*Coffea arabica*), papaya (*Carica papaya*) and oil palm (*Elaeis guineensis*) show intermediate seed storage behaviour and are able to tolerate desiccation to 10-12% moisture content. This also implies that current catalogues of orthodox seeds which have been derived from reports of longevity in open storage may include species with intermediate seed storage behaviour.

4.7. Short-duration viability tests

Fresh seeds of many tropical trees germinate readily during 14-day germination tests. However, seeds of other species require much longer to germinate. For example, seeds of *Persoonia comata* require one year or more (Fox *et al.* 1987) and seeds of *Acrocomia sclerocarpa* require 2.5 years to germinate (Ellis *et al.* 1985b). Furthermore, although fresh seeds may germinate readily, once dried they may be more difficult to germinate because of hardseededness. This is often observed with seeds of Leguminosae, Malvaceae, Cannaceae, Rhamnaceae and Tiliaceae (Ellis *et al.* 1985b). Unless the hardseededness is overcome, seeds which do not germinate due to hardseededness could be confounded with dead seeds.

Apart from hardseededness, dormancy is sometimes induced by drying. Specific treatments are required to remove this dormancy, and the seeds require more time to germinate. For example, dry seeds of *Corylus avellana* (Bradbeer 1968; Jarvis 1975), *Citrus* sp. (King and Roberts 1980b), several genera of the Rosaceae (*Malus*, *Pyrus*, *Prunus*) (see Ellis *et al.* 1985b), and *Fagus sylvatica* (Poulsen 1993) are much more difficult to germinate than the fresh seeds, and this has led to their mistaken classification as recalcitrant.

Young and Young (1992) collated information on seed germination for about 386 genera of woody plants in North America, and they observed that only 40% of these genera contain species whose seeds will germinate readily without pretreatment. The remaining genera (60%) require specific pre-treatments, such as prechilling, warm stratification followed by prechilling and scarification, and in most cases prolonged germination test durations are also required (Young and Young 1992). According to

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Soepadmo (1989), about 3-10% of the tree species native to Malaysia possess seeds which require a period of more than 26 weeks to attain even 50% germination, among which seeds of *Anisophyllea griffithii*, *Hydnocarpus woodii* and *Barringtonia macrostachya* require 95, 83 and 65 weeks, respectively, to attain 50% germination. Seeds of about 70% of the tree species native to Malaysia germinate within 10 weeks, and only in the few remaining species is less than 2 weeks required to achieve 50% germination (Soepadmo 1989).

4.8. Rapid loss of viability during storage under ambient conditions

Rapid loss in viability during storage under ambient environments does not necessarily mean that seeds are recalcitrant. For example, seeds of *Salix* spp. were reported to lose viability during 2 days of open storage (Campbell 1980). However, seeds of 24 species of *Salix* have been maintained without loss in viability during 3 years in hermetic storage at –19°C with 6-10% moisture content (Zasada and Densmore 1977) or 5 years when stored over a desiccant at –8°C (Sato 1955).

4.9. Quick tests in liquid nitrogen

Dry orthodox seeds at 2-18% moisture content often survive exposure to liquid nitrogen. A quick method for "identifying" orthodox seeds, which has been used to some extent, is the determination of survival following short-duration exposures to liquid nitrogen (from 1 hour to 6 days). However, this method is not always reliable since both the moisture content and the rates of cooling and rewarming need to be optimized for survival in each species separately. For example, whole seeds of *Juglans*, *Prunus* and *Populus* (with orthodox seed storage behaviour) were reported to be damaged by exposure to temperatures cooler than –40°C (Wang *et al.* 1994). Moreover, such quick tests cannot distinguish between orthodox and intermediate seeds. For example, papaya seeds at 9-10% moisture content survived 24 hours of exposure to liquid nitrogen (Becwar *et al.* 1983; Chin and Krishnapillay 1989) but papaya shows intermediate seed storage behaviour and seeds stored hermetically at -20°C with 7.6-9.4% moisture content for 365 days showed 80-90% loss in viability (Ellis *et al.* 1991b).

4.10. Imbibition damage to dry seeds

This phenomenon may also be the cause of reports of apparent desiccation sensitivity in very dry seeds. Imbibition damage is

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particularly likely when seeds are soaked in water, as often occurs in tree seedling nurseries, for example, but can also occur in standard laboratory tests (Ellis *et al.* 1990b). Hence, the desirability of preconditioning dry seeds at 100% RH before germination tests, as recommended in section 6.2.6.

5. APPROACHES TO PREDICT SEED STORAGE BEHAVIOUR

In cases where seeds are in short supply thus making it difficult to apply the protocol in full, it is worth first taking stock of what is known about the variation in seed storage behaviour among different species of flowering plants. Hence in this section we describe certain approaches to predict seed storage behaviour in species for which experimental results are currently not available.

5.1. Association between plant ecology and seed storage behaviour

Roberts and King (1980) suggested that there is an association between plant ecology and seed storage behaviour. According to this hypothesis, orthodox species originate from environments subjected to occasional or seasonal drought in which desiccation tolerance of the seeds is essential for seed survival and the continued regeneration of the species. On the other hand, recalcitrant species tend to originate from moist ecosystems in which seeds are subjected to high humidity during seed development, maturation and after shedding.

In accordance with this hypothesis, all 115 shrub species of 29 families native to the Mojave Desert show orthodox seed storage behaviour (Kay *et al.* 1988), while seeds of those species of the genus *Dipterocarpus* which are native to dry habitats show some desiccation tolerance but those species inhabiting moist evergreen areas tend to be intolerant of desiccation (Tompsett 1987, 1992). Similarly, arabica coffee shows intermediate seed storage behaviour and is native to the dry and cool regions of Ethiopia, while liberica coffee (*Coffea liberica*) shows recalcitrant seed storage behaviour and is native to the hotter and more humid regions of Liberia (Hong and Ellis 1995). Dickie *et al.* (1992) also observed that species within the Palmae that show orthodox seed storage behaviour are native to dry habitats whereas those species that show recalcitrant seed storage behaviour are native to relatively moist habitats. Moreover, although seeds of most oak (*Quercus*) species show recalcitrant seed storage behaviour (King and Roberts 1979, 1980a), seeds of

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Q. emoryi, which is native to savanna, are not recalcitrant (Nyandiga and McPherson 1992).

From the information on seed storage behaviour that has now been collated for over 7000 species from 251 families of flowering plants (Hong *et al*. 1996), it is evident that species which show recalcitrant seed storage behaviour do not occur naturally in arid habitats, desert and savanna. In these environments, the majority of plant species show orthodox seed storage behaviour, while a few may show intermediate seed storage behaviour. It is clear, however, that further generalizations are not possible. In particular, not all species native to moist habitats, rain forests or flooded forests show recalcitrant seed storage behaviour. Species native to moist environments produce all three categories of seed storage behaviour. There is perhaps, however, a greater likelihood of recalcitrant behaviour in species associated with climax vegetation.

While all orthodox seeds, whether tropical or temperate, store best at subzero temperatures, this is clearly not true of species showing intermediate or recalcitrant seed storage behaviour, and information on their geographical distribution and ecology also helps in the determination of optimum storage temperatures. Table 1 shows that there may be an association between the optimum seed storage temperature and the minimum air temperature at which the plants can survive without chilling injury. From this observation it appears that the cool temperatures that cause chilling injury to the growing plants also reduce seed longevity and in some cases result in almost immediate seed death. In general, fresh (or moist) seeds of species with intermediate or recalcitrant seed storage behaviour which are adapted to tropical lowlands tend to show chilling injury at 10-15°C, while seeds of those adapted to high latitudes or high altitudes are able to tolerate exposure to cooler temperatures. For example, Sasaki (1980) divided *Shorea* species into two groups in relation to the effect of storage temperature on seed survival; seeds of the Yellow Meranti group tolerate 4°C, while seeds of the White Meranti, Red Meranti and Balau groups show chilling injury at 10-15°C; the species listed in the Yellow Meranti group by Sasaki (1980) are those adapted to high altitudes (Ashton 1983).

5.2. Association between taxonomic classification and seed storage behaviour

In general, species in Chenopodiaceae, Combretaceae, Compositae, Labiatae, Solanaceae and Pinaceae show orthodox

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seed storage behaviour, while species in Rhizophoraceae (in which vivipary predominates) are recalcitrant. Species within Leguminosae, Gramineae, Cucurbitaceae, Cruciferae and Rosaceae show orthodox seed storage behaviour, but with several notable exceptions. On the other hand, it appears that no member of the Dipterocarpaceae shows orthodox seed storage behaviour; most show recalcitrant and a few intermediate seed storage behaviour. Similarly, although all members of the Sapotaceae were thought to be recalcitrant, in fact, some show recalcitrant and others intermediate seed storage behaviour. All three categories of seed storage behaviour can be found among the members of Meliaceae.

Seed storage behaviour can also differ among species within a genus, e.g. most species in the genus *Acer* are orthodox but there are some that are not. Tompsett (1983) suggested that differences in desiccation tolerance among *Araucaria* species could be geographical and taxonomic in origin; for example, *A. angustifolia* and *A. araucana* from South America and *A. bidwillii* from Australia in the Colymbea section of *Araucaria* mostly show recalcitrant seed storage behaviour, whereas species such as *A. heterophylla*, *A. cunninghamii*, *A. columnaris* and the numerous New Caledonian species in the Eutacta section are rather more tolerant of desiccation.

5.3. Association between plant, fruit or seed characters and seed storage behaviour

Orthodox seed storage behaviour is shown by species which produce achenes, many-seeded berries, many-seeded dehiscent capsules, many dry-seeded pods (but not arillate), many dry-seeded follicles, schizocarps and utricles. Most species which produce siliques (one known exception being *Wasabia* spp.) and caryopses (three known exceptions are *Porteresia coarctata*, *Spartina* spp. and *Zizania* spp.) also produce orthodox seeds. On the other hand, all three categories of seed storage behaviour can be found among the species which produce:

- drupes containing 1-4 seeds or many arillate seeds;
- pods containing 1-5 large seeds or many arillate seeds;
- berries containing 1-10 seeds;
- capsules containing 1-5 seeds;
- single-seeded nuts.

In particular, and in contrast to some views, not all single-seeded nuts show recalcitrant seed storage behaviour.

The arillate character, present in seeds with short lifespans in open storage, is not associated with seed storage behaviour; this

characteristic is present in species which produce orthodox, intermediate and recalcitrant seeds.

The deciduous character is a mechanism by which plants avoid damage from either drought or cold. This character is not associated with desiccation tolerance in the seeds, but may be associated with chilling tolerance. For example, seeds of the deciduous rubber tree (*Hevea brasiliensis*) are recalcitrant, but despite the tropical lowland origin, the seeds are best stored at 7-10°C (Beng 1976). Seeds of deciduous Dipterocarps (e.g. *Dipterocarpus alatus, D. intricatus, D. obtusifolius, D. tuberculatus*) can tolerate cool temperatures, as low as 6°C, while seeds of

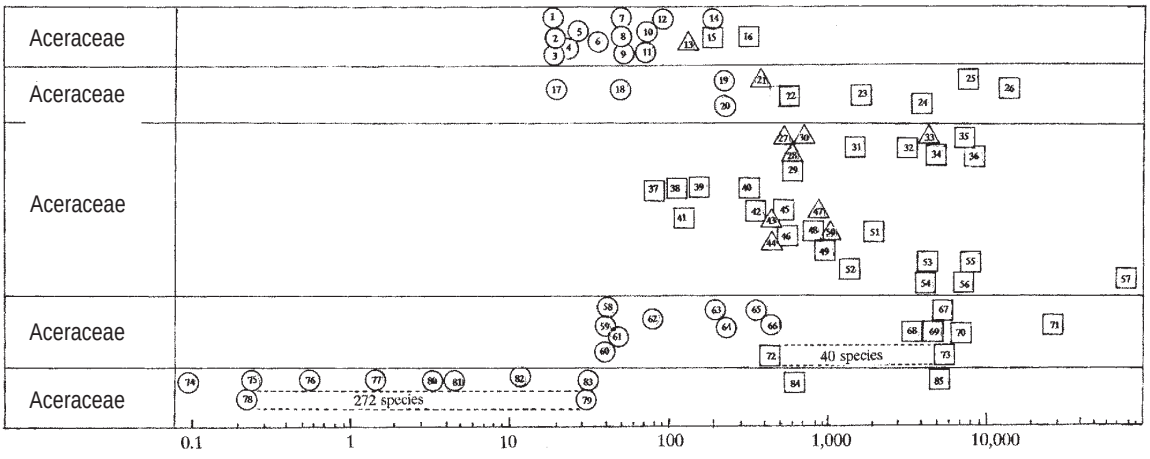

Fig. 3. Distribution of thousand-seed weight (TSW, g) for 92 species (number within symbol) within 27 genera of 5 families. Circles indicate orthodox, triangles intermediate, and squares recalcitrant seed storage behaviour. TSW values are from Hong *et al*. (1996).

Aceraceae: 1, *Acer rubrum*; 2, *A. spicatum*; 3, *A. sieboldianum*; 4, *A. rufinerve*; 5, *A. ginnala*; 6, *A. pensylvanicum*; 7, *A. japonicum*; 8, *A. negundo*; 9, *A. tataricum*; 10, *A. saccharum*; 11, *A. palmatum*; 12, *A. campestre*; 13, *A. macrophyllum*; 14, *A. platanoides*; 15, *A. pseudoplatanus*; 16, *A. saccharinum*. **Araucariaceae**: 17, *Agathis australis*; 18, *Agathis robusta*; 19, *Agathis macrophylla*; 20, *Araucaria cunninghamii*; 21, *A. scopulorum*; 22, *A. rulei*; 23, *A. columnaris*; 24, *A. hunsteinii*; 25, *A. heterophylla*; 26, *A. araucana*; 27, *A. angustifolia*; 28, *A. bidwilli*. **Dipterocarpaceae**: 29, *Dipterocarpus intricatus*; 30, *D. tuberculatus*; 31, *D. turbinatus*; 32, *D. crinitus*; 33, *D. baudii*; 34, *D. alatus*; 35, *D. zeylanicus*; 36, *D. obtusifolius*; 37, *D. pilosus*; 38, *Hopea helferi*; 39, *H. odorata*; 40, *H. wightiana*; 41, *Shorea acuminata*; 42, *S. parviflora*; 43, *S. leprosula*; 44, *S. dasyphylla*; 45, *S. bracteola*; 46, *S. pauciflora*; 47, *S. ovalis*; 48, *S. macroptera*; 49, *S. assamica*; 50, *S. platyclados*; 51, *S. roxburghii*; 52, *Anisoptera glabra*; 53, *Balanocarpus heimii*; 54, *Dryobalanops aromatica*; 55, *Dryobalanops oblongifolia*; 56, *Parashorea densiflora*; 57, *Vatica* sp. **Fagaceae**: 58, *Nothofagus obliqua*; 59, *N. procera*; 60, *Fagus sylvatica*; 61, *Fagus grandifolia*; 62, *Castanopsis sempervirens*; 63, *Castanopsis chrysophylla*; 64, *Lithocarpus densiflorus*; 65, *Castanea dentata*; 66, *C. mollissina*; 67, *C. crenata*; 68, *C. sativa*; 69, *Quercus vaccinifolia*; 70, *Q. falcata*; 71, *Q. emoryi*; 72, *Q. velutina*; 73, *Q. serrata*; 74, *Q. robur*; 75, *Q. rubra*; 76, *Q. borealis*; 77, *Q. alba*; 78, *Q. acutissima*; 79, *Q. suber*; 80, *Q. semecarpifolia*. **Myrtaceae**: 81, *Metrosiderox polymorpha*; 82, *Callistemon lanceolatus*; 83, *Melaleuca leucadendron*; 84, *Leptospermum scoparium*; 85, *Eucalyptus tereticornis*; 86, *Eucalyptus saligna*; 87, *Tristania conferta*; 88; *Syncarpia laurifolia*; 89, *Psidium guajava*; 90, *Myrtus communis*; 91, *Eugenia jambolana*; 92, *E. grandis*. The TSW of *Eucalyptus* spp. are shown by the two species with extreme TSW values.

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evergreen Dipterocarps must be stored at 10°-15°C or warmer to avoid damage (Tompsett 1992).

5.4. Association between seed size and storage behaviour

Seed size alone does not determine seed storage behaviour. Nevertheless, on average recalcitrant seeds do tend to be larger than intermediate seeds, which in turn tend to be larger than orthodox seeds. Figure 3 gives some indication of the association between thousand-seed weight (TSW, g) and seed storage behaviour among species within a genus (*Acer*), and among genera within families. The heaviest TSW recorded for orthodox seeds are 6300 g in *Hardwickia pinnata* Roxb. (Leguminosae) and 5000-8000g (at 10% moisture content) in cashew (*Anacardium occidentale* L.) (Anacardiaceae). If cashew is harvested at the end of the seed-filling phase, the fresh nut weight will be 13 g / nut (circa 45% moisture content). Therefore, species which produce seeds with TSW >13 000 g are unlikely to show orthodox seed storage behaviour. On the other hand, seeds with TSW between 30 and 13 000 g may show orthodox, intermediate or recalcitrant seed storage behaviour. Thus, seed size *per se* provides no indication of seed storage characteristics within this range. Similarly, although species with smaller seeds (TSW <25 g) are likely to show orthodox seed storage behaviour, there are sufficient exceptions (e.g. intermediate seed storage behaviour within Orchidaceae and *Wasabia japonica* within Cruciferae) to indicate that this generalization is based on probability and is not prescriptive.

5.5. Association between seed moisture content at maturity or shedding and seed storage behaviour

In Figure 4 we have collated information on the moisture content of seeds at maturity or at shedding (termed MCS, seed moisture content at shedding) for species showing orthodox, intermediate or recalcitrant seed storage behaviour. While some discrimination is apparent, there is also considerable overlap. For species with recalcitrant seed storage behaviour these moisture contents are distributed between 36 and 90%, for intermediate between 23 and 55%, and for orthodox between <20 and 50%. Thus, it appears that seeds shed at moisture contents above 60% are likely to show recalcitrant seed storage behaviour, and that those shed or harvested at moisture contents of around 20% or below are very likely to show orthodox seed storage behaviour. However, while species with MCS <35% are unlikely to show recalcitrant seed storage

behaviour, if MCS is between about 25 and 55% then no generalization is possible. Of course, as more data become available this picture may change.

5.6. The use of several criteria combined to indicate likely seed storage behaviour

In sections 5.1-5.5 we have shown that most generalizations on probable seed storage behaviour based on a single criterion are subject to rather too many exceptions to be helpful. Nevertheless, it may be possible to develop a multiple-criteria predictive framework to assess seed storage behaviour. In Figure 5 we have combined information on seed weight, seed moisture content at shedding and seed storage behaviour. In this presentation all species which combine a TSW of 2500 g and below with

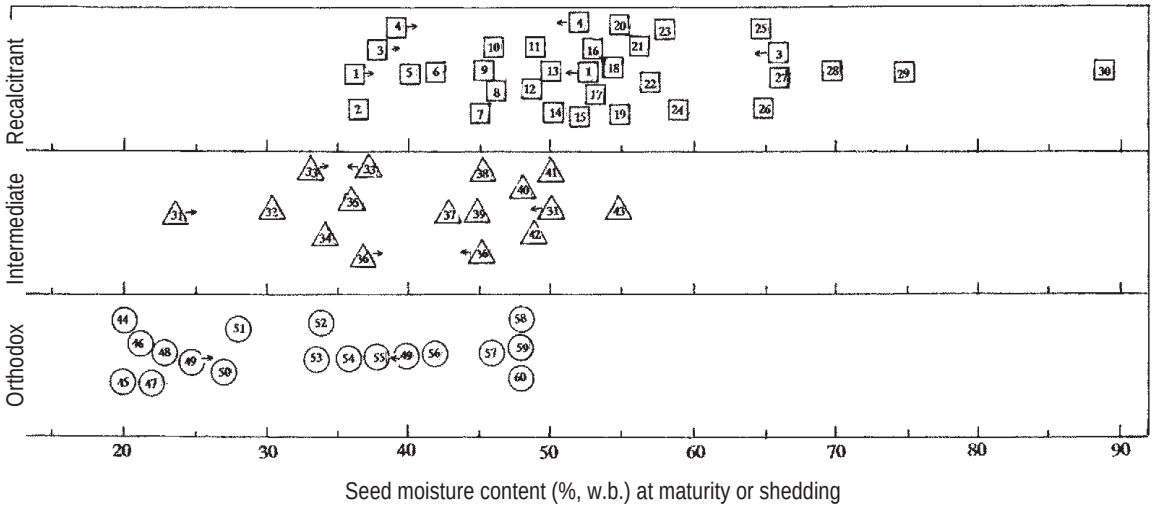

Fig. 4. Seed moisture content (%, w.b.) at maturity or shedding (MCS) for 60 species (number within symbol) and seed storage behaviour. Circles represent orthodox, triangles intermediate, and squares recalcitrant seed storage behaviour. MCS values are from Hong *et al*. (1996).

1, *Hevea brasiliensis*; 2, *Nephelium lappaceum*; 3, *Symphonia globulifera*; 4, *Durio zibethinus*; 5, *Hopea hainanensis*; 6, *Shorea acuminata*; 7, *Theobroma cacao*; 8, *Dimocarpus longan*; 9, *Shorea roxburghii*; 10, *Dryobalanops aromatica*; 11, *Aesculus hippocastaneum*; 12, *Quercus robur*; 13, *Shorea robusta*; 14, *Eugenia jambos*; 15, *Lansium domesticum*; 16, *Araucaria hunsteinii*; 17, *Artocarpus heterophyllus*; 18, *Dipterocarpus baudii*; 19, *Hancornia speciosa*; 20, *Myristica fragrans*; 21, *Balanocarpus heimii*; 22, *Coffea liberica*; 23, *Acer pseudoplatanus*; 24, *Calamus scipionum*; 25, *Persea americana*; 26, *Garcinia mangostana*; 27, *Avicennia marina*; 28, *Artocarpus champeden*; 29, *Telfairia occidentalis*; 30, *Sechium edule*; 31, *Elaeis guineensis*; 32, *Zizania palustris*; 33, *Shorea platyclados*; 34, *Azadirachta indica*; 35, *Araucaria columnaris*; 36, *Citrus limon*; 37, *Dacrycarpus dacrydioides*; 38, *Veitchia merillii*; 39, *Citrus reticulata*; 40, *Coffea arabica*; 41, *Coffea canephora*; 42, *Oreodoxa regia*; 43, *Carica papaya*; 44, *Oryza sativa*; 45, *Lens culinaris*; 46, *Glycine max*; 47, *Carya illinoensis*; 48, *Triticum aestivum*; 49, *Fagus sylvatica*; 50, *Annona muricata*; 51, *Acer platanoides*; 52, *Annona cherimolia*; 53, *Vitis vinifera*; 54, *Araucaria cunninghamii*; 55, *Cucumis melo*; 56, *Prunus avium*; 57, *Cucurbita moschata*; 58, *Punica granatum*; 59, *Lycopersicon esculentum*; 60, *Capsicum annum*.

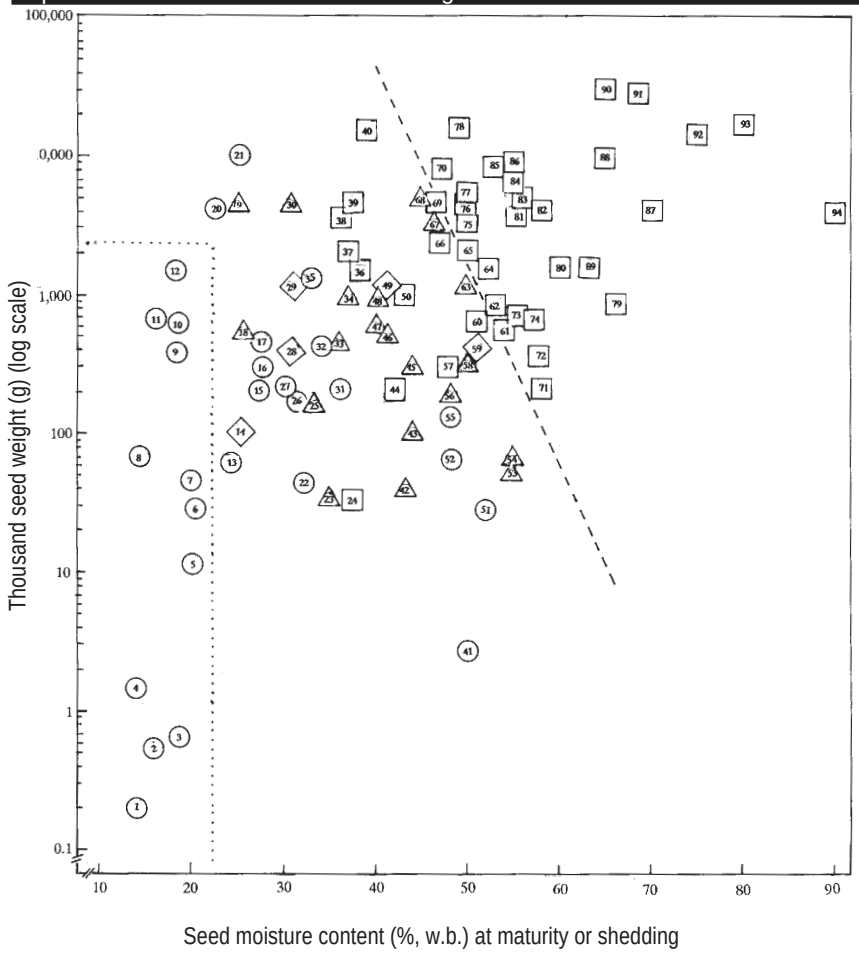

Fig. 5. Seed storage behaviour of 94 contrasting species (number within symbol) in relation to seed moisture content (w.b.) at harvest/natural shedding (MCS) and thousand seed weight (TSW, log scale). Circles represent orthodox, triangles intermediate, and squares recalcitrant seed storage behaviour. Data on MCS and TSW values are from Hong *et al*. (1996). The dotted and broken lines are discussed in the text.

1, *Saccharum officinarum*; 2, *Trifolium repens*; 3, *Lactuca sativa*; 4, *Brassica juncea*; 5, *Psidium guajava*; 6, *Oryza sativa*; 7, *Hordeum vulgare*; 8, *Albemoschus esculentus*; 9, *Glycine max*; 10, *Phaseolus vulgaris*; 11, *Ricinus communis*; 12, *Vicia faba*; 13, *Fraxinus excelsior*; 14, *Michelia champaca*; 15, *Acer platanoides*; 16, *Zea mays*; 17, *Annona muricata*; 18, *Carya illinoensis*; 19, *Elaeis guineensis*; 20, *Anacardium occidentale*; 21, *Vitis vinifera*; 22, *Piper nigrum*; 23, *Zizania palustris*; 24, *Porteresia coarctata*; 25, *Acer macrophyllum*; 26, *Terminalia brownii*; 27, *Fagus sylvatica*; 28, *Arum maculatum*; 29, *Araucaria cunninghamii*; 30, *Annona cherimolia*; 31, *Araucaria columnaris*; 32, *Shorea platyclados*; 33, *Phoenix dactylifera*; 34, *Dipterocarpus alatus*; 35, *Nephelium lappaceum*; 36, *Hevea brasiliensis*; 37, *Calophyllum maria*; 38, *Durio zibethinus*; 39, *Theobroma cacao*; 40, *Lycopersicon esculentum*; 41, *Punica granatum*; 42, *Podocarpus dacrydioides*; 43, *Malus domestica*; 44, *Citrus reticulata*; 45, *Shorea acuminata*; 46, *Citrus aurantium*; 47, *Azadirachta indica*; 48, *Manilkara achras*; 49, *Chrysophyllum cainito*; 50, *Quercus emoryi*; 51, *Shorea macroptera*; 52, *Dimocarpus longan*; 53, *Carica papaya*; 54, *Averrhoa carambola*; 55, *Cucurbita maxima*; 56, *Citrus limon*; 57, *Baccaurea motleyana*; 58, *Coffea arabica*; 59, *Citrus sinensis*; 60, *Diospyros kaki*; 61, *Shorea pauciflora*; 62, *Roystonea regia*; 63, *Landolphia kirkii*; 64, *Shorea roxburghii*; 65, *Veitchia merrillii*; 66, *Acer pseudoplatanus*; 67, *Acer saccharinum*; 68, *Araucaria hunsteinii*; 69, *Dysoxylum cauliflorum*; 70, *Nectandra ambigens*; 71, *Couepia polyandra*; 72, *Guilfoylia monostylis*; 73, *Eugenia jambos*; 74, *Araucaria araucana*; 75, *Dryobalanops aromatica*; 76, *Araucaria angustifolia*; 77, *Dipterocarpus obtusifolius*; 78, *Aesculus hippocastanum*; 79, *Diospyros confertiflora*; 80, *Coffea liberica*; 81, *Podocarpus henkelii*; 82, *Balanocarpus heimii*; 83, *Artocarpus heterophyllus*; 84, *Myristica fragrans*; 85, *Garcinia mangostana*; 86, *Dipterocarpus crinitus*; 87, *Quercus cerris*; 88, *Castanea sativa*; 89, *Persea americana*; 90, *Mangifera indica*; 91, *Castanospermum australe*; 92, *Telfairia occidentale*; 93, *Areca catechu*; 94, *Sechium edule*.

Notes

MCS of 23% and below (below and to the left of the dotted line) show orthodox seed storage behaviour, while those at the combinations of weights and moisture content levels at shedding greater than those shown by the broken line show recalcitrant seed storage behaviour. However, evidence of all three types of seed storage behaviour can be detected between the two lines shown in Figure 5.

Clearly, while the development of a multiple-criteria decision framework for seed storage behaviour is worth pursuing, it will be necessary to involve more than two criteria in such schemes.

6. SEED STORAGE BEHAVIOUR

This section provides definitions of and further details on the three categories of seed storage behaviour already mentioned. The orthodox and recalcitrant categories of seed storage behaviour were first defined more than 20 years ago by Roberts (1973). The intermediate category was introduced more recently (Ellis *et al.* 1990a). These three terms are used here in the context of their original definitions. Note that some authors have used the terms orthodox and recalcitrant rather more loosely—and, as a consequence, sometimes erroneously—than Roberts' definition, while other authors have subdivided the categories, in part to help distinguish among species with recalcitrant seed storage behaviour but which differ considerably in the degree of desiccation which results in seed death. There are two practical aspects to contrasting patterns of seed storage behaviour: first, the effect of desiccation on seed viability; second, the response of seed longevity (period of seed survival) to the storage environment.

6.1. Desiccation tolerance

Anhydrous biology has emerged as an important area in the biological sciences. Desiccation tolerance in certain living organisms provides one mechanism of adaptation for survival (Leopold 1986). Tolerance to desiccation permits metabolic activity to be suspended during periods of stress and occurs in a wide variety of organisms, e.g. seeds (Roberts 1972, 1973; Bewley and Black 1994), pollen (Hoekstra 1986), spores of Bryophyta (Chalaud 1932 cited by Sussman and Halvorson 1966) and Pteridophyta (Keilin 1959), lichens (Bewley 1972), resurrection plants (Gaff 1977), viruses (Spector 1956), spores of bacteria (Murrell and Scott 1957) and fungi (Keilin 1959), cysts of several Protozoa (Doflein and Reichenow 1953), dry ova of several *Ascaris* spp. (Witenberg 1961 cited by Sussman and Halvorson 1966) and dry larvae of several nematodes

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(Demeure and Freckman 1981) and insects (Hinton 1960).

Desiccation tolerance in seeds has recently been reviewed by Leprince *et al*. (1993), Bewley and Black (1994) and Vertucci and Farrant (1995). Desiccation tolerance often confers considerable longevity on living organisms in the quiescent state, particularly seeds; dry seeds of certain plant species have been reported to survive for centuries (Ewart 1908; Harrington 1972; Priestley 1986) and hence the potential for *ex situ* genetic resources conservation through long-term seed storage.

Seed development and maturation can be divided into several different stages (Galau *et al*. 1991). It is important to note that during their early development seeds are intolerant of desiccation. During their development and maturation, seeds develop the ability to survive desiccation, although the maximum degree of desiccation tolerance attained, and the rate at which it develops, vary substantially among species.

6.2. Orthodox seed storage behaviour

6.2.1. Definition

Orthodox seeds can be dried, without damage, to low levels of moisture content and, over a wide range of environments, their longevity increases with decrease in seed storage moisture content and temperature in a quantifiable and predictable way (Roberts 1973). The latter is defined by the improved seed viability equation (Ellis and Roberts 1980a). In essence, in order for seed storage behaviour to be defined as orthodox two conditions must be satisfied.

- Mature seeds survive desiccation to low moisture contents, at least to 2-6% depending on the species. Above this value (but within the air-dry range) there is a negative logarithmic relation between seed moisture content and longevity (Ellis and Roberts 1980a, 1980b; Ellis 1988).
- With regard to the effect of temperature on longevity, there is a negative relation between temperature (at least between –20 and 90°C) and seed longevity at a constant moisture content (Roberts 1973). The precise form of this relation is a negative semi-logarithmic relation modified by a quadratic term such that the relative benefit to longevity of a reduction in temperature declines the cooler the temperature (Ellis and Roberts 1980a; Ellis 1988; Dickie *et al*. 1990).

6.2.2. The seed viability equation

Relations between seed survival and storage duration, tempera-

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ture and moisture content have been quantified by the equation:

$$v = K_i - p / 10^{K_E - C_W \log_{10} m - C_H t - C_Q t^2}$$

where v is probit percentage viability after p days in storage at m% moisture content (w.b.) and t°C, K_i is a constant specific to the seed lot, and K_E, C_W, C_H and C_Q are species viability constants (Ellis and Roberts 1980a). This equation indicates the (considerable) extent to which the longevity of orthodox seeds in air-dry storage can be altered by manipulating the environment in which they are stored. The implications of the equation for genetic resources conservation by long-term seed storage have been discussed in detail elsewhere (Cromarty *et al*. 1982).

6.2.3. Moisture content limits to the seed viability equation

There are two limits to the negative logarithmic relation between seed moisture content and seed longevity (Roberts and Ellis 1989): an upper limit, beyond which seed longevity in hermetic storage is no longer reduced with further increase in moisture, and beyond which seed longevity in aerated storage increases with further increase in moisture content (Roberts and Ellis 1982); and a lower limit below which further reduction in moisture content no longer increases longevity in hermetic storage (Ellis *et al*. 1988, 1989, 1990c, 1990d, 1992). These two limits are clearly pertinent to any discussion of contrasting types of seed storage behaviour.

The upper moisture content limit is about 15% in lettuce (*Lactuca sativa*) (Ibrahim and Roberts 1983), about 18% in onion (*Allium cepa*) (Ellis and Roberts 1977), 22% in elm (*Ulmus carpinifolia*) (Tompsett 1986), 22% in niger (*Guizotia abyssinica*) (Zewdie and Ellis 1991b), 24% to 28% in tef (*Eragrostis tef*) (Zewdie and Ellis 1991b), and about 26% in durum wheat (*Triticum durum*) (Petruzzelli 1986). Despite wide variation among species in terms of moisture content, these values coincide with a seed water potential of about –14 MPa (Roberts and Ellis 1989; Zewdie and Ellis 1991b), i.e. the upper moisture content limit to the viability equation occurs atlevels of seed moisture content in equilibrium with about 85-90% RH at 20°C.

The lower moisture content limit to the seed viability equation also varies substantially among species. For example, investigations of seed longevity in hermetic storage at 65°C suggest values of about 6% for pea (*Pisum sativum*) and mung bean (*Vigna radiata*), 4.5% for rice and tef, and 2% for sunflower (*Helianthus annuus*) (Ellis *et al*. 1988, 1989, 1992). These variant levels of moisture content coincide with 10-12% equilibrium

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relative humidity at 20°C (Ellis *et al*. 1988, 1989, 1992), however, or with a seed water potential of about –350 MPa at this temperature (Roberts and Ellis 1989). The value of this low moisture content limit may not be identical at cooler storage temperatures, however (Ellis *et al*. 1989; Vertucci and Roos 1990). For example, one suggestion is that this value is provided by levels of moisture content in equilibrium with 19-27% RH at the cooler storage temperature of 35°C (Vertucci and Roos 1990). This important topic is discussed elsewhere (see Vertucci *et al*. (1994) and Ellis *et al*. (1995) and references therein).

6.2.4. Longevity of moist orthodox seeds

Above the upper moisture content limit to the application of the viability equation in orthodox seeds, the trend of seed longevity in relation to moisture is reversed in aerated storage (i.e. where oxygen is readily available to the seeds) whereby longevity increases with further increase in moisture content (Roberts and Ellis 1982, 1989; Ibrahim *et al*. 1983). When orthodox seeds are fully hydrated, they tend to germinate, but if this can be prevented by maintaining the seeds in a dormant condition they can often remain viable for many years (Villiers 1974, 1975). For example, Barton (1961) reported that 25 and 37% of the seeds of *Amaranthus retroflexus* and *Rumex obtusifolius*, respectively, stored on moist glass wool at constant temperatures of 20 and 30°C germinated during 8 years of moist storage, while the remaining seeds were viable and germinated when transferred to suitable environments.

6.2.5. Temperature limits to the seed viability equation

There are no obvious temperature limits to the application of the seed viability equation in normal use. For example, the temperature term of the seed viability equation has been shown to apply between those subzero temperatures used in conventional seed genebanks and the very high temperatures used in certain heated-air seed driers (Dickie *et al*. 1990). However, the application of the seed viability equation at temperatures cooler than about –20°C or so is not advised (Dickie *et al*. 1990), but this is unlikely to be of concern to most readers because most seed genebanks do not operate at temperatures cooler than this at present. For information on seed storage at cooler temperatures, and especially in liquid nitrogen (–196°C) or in the vapour above, see Stanwood and Bass (1981) and Stanwood (1984).

Between –20 and 0°C, however, problems can arise if seeds are too moist. If seeds are too moist when they are exposed to

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subzero temperatures then ice crystals can form and cause seed death. For example, soyabean (*Glycine max*) seeds at 26% moisture content will be killed when exposed to –65°C, but seeds at 20% moisture content will survive at least a brief exposure to this temperature (Leopold and Vertucci 1989).

In theory, the water present within seeds would not be expected to freeze when they are cooled to –20°C if seed moisture content is in equilibrium with about <85% RH (Roberts and Ellis 1989). In practice, small losses in viability during storage have sometimes been detected in orthodox seeds in equilibrium with 70% RH (at ambient temperature prior to hermetic storage), and consequently it has been suggested that it would be prudent to first dry seeds at 15-20°C until moisture contents have been reduced to values in equilibrium with ≤65% RH at 15-20°C before beginning hermetic storage at –20°C in order to avoid any possibility of freezing damage (Zewdie and Ellis 1991a). Thus, safe levels of seed moisture content (i.e. those at which freezing damage is avoided) for storage at –20°C are about 12.5-13.5% for cereals but lower moisture content is necessary for species with oily seeds.

6.2.6. Imbibition injury

Water can also influence orthodox seed survival during imbibition; the rapid uptake of water by dry seeds can result in imbibition injury (Powell and Matthews 1978). Although the phenomenon is particularly pronounced if seeds are immersed (soaked) in water, it can also occur in standard laboratory germination tests (Ellis *et al*. 1982 1990b). Seeds are more likely to be damaged the lower their initial moisture content (Ellis *et al*. 1990b) and the cooler the temperature at which they imbibe water (Pollock 1969; Powell and Matthews 1978). Imbibition injury is pronounced in Leguminosae and Malvaceae (see Ellis *et al*. 1985b), but it is now clear that the problem can also occur in other species. For example, imbibition injury was reported in *Populus alba*, particularly in seeds at less than 8% moisture content (Polya 1961). Imbibition injury can be avoided by conditioning (humidifying) the seeds in a moist atmosphere (close to 100% RH) in order to raise seed moisture contents to 16-18% before the seeds are set to germinate in contact with liquid water (Ellis *et al*. 1985a). This conditioning takes about 24 hours or more depending on initial moisture content (Ellis *et al*. 1990b) and species (Ellis 1987). It is suggested that seeds at 8% moisture content and below, irrespective of species, should routinely be humidified before germination tests; some seed accessions at higher moisture contents (8-12%) may also benefit from humidification.

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6.2.7. Seed development, desiccation tolerance and the potential longevity of orthodox seeds

Since both the desiccation tolerance and the potential longevity of seeds of species with orthodox seed storage behaviour change during seed development and maturation, it is essential to take note of the maturity of the seed accession when using the protocol. In this section, we provide a few examples of such changes.

There is now considerable evidence that developing and maturing orthodox seeds do not attain maximum potential longevity until some time after the end of the seed-filling phase (Kameswara Rao *et al*. 1991; Demir and Ellis 1992a, 1992b; Ellis and Pieta Filho 1992; Ellis *et al*. 1993; Zanakis *et al*. 1994). Provided seeds have reached the stage in development at which potential longevity is maximal then it appears that desiccation tolerance to low moisture content (below 5%) will also be maximal (Ellis and Hong 1994).

Orthodox seeds do not tolerate desiccation at all stages of their development and maturation. For example, the change from desiccation intolerance to desiccation tolerance has been reported to occur about halfway through seed development in *Phaseolus vulgaris* (Dasgupta *et al*. 1982) and *Sinapis alba* (Fischer *et al*. 1988). In six grain legumes, the onset of desiccation tolerance to about 10% moisture content occurred when maturation drying had reduced the seed moisture content on the mother plant to about 60% (Ellis *et al*. 1987). This coincided more or less with the end of the seed-filling phase, defined as physiological

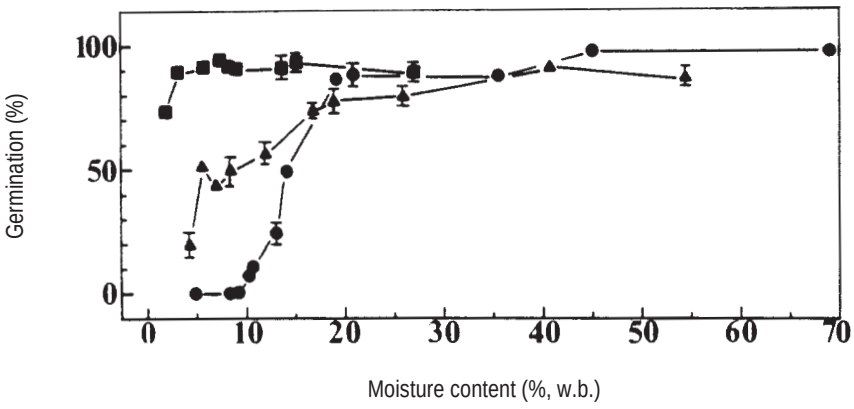

Fig. 6. Effect of rapid enforced desiccation to different moisture contents (%, w.b.) on the germination of three seed lots of Norway maple (*Acer platanoides*) harvested at different stages of development in 1991. Seeds were harvested on 3 September (●), 24 September (▲) and 31 October (■). From Hong and Ellis (1992a).

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maturity by Shaw and Loomis (1950), now termed "mass maturity" (Ellis and Pieta Filho 1992).

Immature seeds of Norway maple (*Acer platanoides*), which shows orthodox seed storage behaviour, harvested at 68% moisture content, i.e. before mass maturity, are damaged by desiccation, particularly below 20% moisture content (Fig. 6). Seeds harvested at mass maturity (54% moisture content, 24 September) tolerated desiccation to 12-15% moisture content, but further desiccation reduced viability. The developing seeds did not attain desiccation tolerance to very low moisture contents (3%) until 3 to 4 weeks after mass maturity (31 October) when maturation drying had reduced seed moisture content on the mother plant to about 27-30% (Hong and Ellis 1990, 1992a). Similarly, the achievement of maximum desiccation tolerance to low levels of moisture content (4%) in rice did not occur until some 2-3 weeks after mass maturity, when maturation drying on the mother plant had naturally reduced seed moisture contents to levels below 32% (Ellis and Hong 1994).

These examples, however, should not be regarded as generalizations. The development of desiccation tolerance to very low moisture contents may occur at different developmental stages in different species, and may also be influenced by the seed production environment. One important difference among species may be that of seed moisture content at seed or fruit shedding or at harvest maturity. For example, seeds of tomato (*Lycopersicon esculentum*) and sweet pepper (*Capsicum annuum*), both of which show orthodox seed storage behaviour, attain desiccation tolerance when seed moisture content is reduced naturally during maturation drying to 52-55%, and fruits are ripe when seed moisture content is about 42-48% (Demir and Ellis 1992a, 1992b). Rice is an example wherein an effect of seed production environment on the development of desiccation tolerance to low moisture content has been detected (Ellis and Hong 1994). Seeds of a *japonica* rice produced in a cool environment of 28° / 20°C showed consistently greater desiccation tolerance at each stage of maturation drying than those produced at 32° / 24°C. Finally, we have noted that seeds of the Leguminosae tend to develop desiccation tolerance relatively later during their development and maturation compared with seeds of the Gramineae.

6.2.8. Reduction of desiccation tolerance

Once orthodox seeds begin to germinate they start to lose desiccation tolerance, thus becoming more sensitive to desiccation the further germination is allowed to progress (Akalehiywot and Bewley

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1980; McKersie and Stinson 1980; McKersie and Tomes 1980; Dasgupta *et al.* 1982; Senaratna and McKersie 1983; Koster and Leopold 1988). Moreover, it is also important to note that both desiccation tolerance to very low moisture content and potential longevity can be reduced by short-duration imbibition treatments which do not result in visible germination (i.e. radicle emergence) (Hong and Ellis 1992b).

Consequently, there is some possibility that any treatment of seeds involving high seed moisture content before storage may reduce desiccation tolerance. Presoaking, even for as few as 3 hours, results in deleterious effects on the ultrastructure of rye (*Secale cereale*) embryos when subsequently dehydrated to their initial moisture content (Sargent *et al.* 1981). Similarly, mung bean which had been imbibed for 8 hours lost 5% viability when dried back to between 4.3 and 6.4% moisture content, despite the fact that no seeds germinated during this imbibition treatment (Hong and Ellis 1992b).

Cold moist storage (stratification) methods have commonly been practised as a method for short-term storage when dealing with very dormant seeds of trees and shrubs. For example, the benefit of moist storage at low temperatures of 3-5°C of orthodox seeds of temperate species (e.g. *Fagus*, *Fraxinus*, *Liriodendron*, *Magnolia*, *Prunus*) (Schopmeyer 1974; Young and Young 1992) is that the seeds are ready to germinate immediately after storage, while seeds stored air dry require a long duration of prechilling before germination will occur. There have been several reports that reduction in desiccation tolerance and subsequent longevity results from drying orthodox seeds which have been stored in a moist condition. Haut (1932) reported a deleterious effect of drying after a prechill treatment on the germination of *Prunus* seeds, whereas drying before prechilling was not deleterious. Prechilled seeds of beech (*Fagus sylvatica*) were less tolerant of subsequent desiccation compared with those which had not been prechilled (Muller and Bonnet-Masimbert 1989). Similarly, seeds of barley (*Hordeum vulgare*) stored moist at 15°C for 14 days lost 20% viability when dried to 3.6% moisture content (Hong and Ellis 1992b).

Seed priming (also known as osmotic priming, and osmoconditioning), which aims to promote faster, more uniform seedling emergence (Heydecker 1977) and may also increase longevity in certain species (Probert *et al.* 1991), can also reduce desiccation tolerance when seeds are dried to low moisture content. For example, Carpenter and Boucher (1991) reported that reducing the moisture content of unprimed seed of the orthodox

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pansy (*Viola* x *wittrockiana*) from 10.5 to 5.8% caused no damage to germination, but the germination of primed seed was decreased when dried to levels of moisture content below 10%, and even more so when dried to 5.8% moisture content.

Thus, pretreatments to seeds which involve exposure to high levels of seed moisture content run the risk of being deleterious to desiccation tolerance and subsequent longevity. It is suggested, therefore, that for long-term storage, i.e. at low moisture content and cool temperatures, dormancy-breaking and priming treatments should not be used before storage unless they have been proven not to damage desiccation tolerance to low moisture content and subsequent longevity.

6.3. Recalcitrant seed storage behaviour

Recalcitrant seeds cannot be dried without damage and so they cannot conform to the viability equation which describes relations between longevity and air-dry seed storage environments (Roberts 1973). When fresh recalcitrant seeds begin to dry, viability is first slightly reduced as moisture is lost, but then begins to be reduced considerably at a certain moisture content termed the "critical moisture content" (King and Roberts 1979, 1980a) or "lowest safe moisture content" (Tompsett 1984a). If drying continues further, viability is eventually reduced to zero. Hence, the relationship between the ability to germinate when tested following desiccation (and rehydration) and moisture content is typically S-shaped (Fig. 7).

The water status of recalcitrant seeds has been extensively studied in *Avicennia marina* (Berjak *et al*. 1990), *Landolphia kirkii* (Pammenter *et al*. 1991, 1993; Berjak *et al*. 1992), *Quercus robur* (Finch-Savage 1992b; Finch-Savage *et al*. 1993; Grange and Finch-Savage 1992) and many other species (Pammenter *et al*. 1993).

Critical moisture content levels vary greatly among species (King and Roberts 1979; Chin 1988), and even among cultivars and seed lots (King and Roberts 1979; Chin 1988). They may also vary with the method of drying (Farrant *et al*. 1985; Pritchard and Prendergast 1986; Pritchard 1991). The values of the "lowest safe moisture content" vary between extremes of 23% for cocoa (*Theobroma cacao*) (Mumford and Brett 1982) and 61.5% for *Avicennia marina* (Farrant *et al*. 1986). Despite great variation in the lowest safe moisture content value among species, these moisture content levels are equivalent to relative humidities of 96-98% (or a seed water potential of about –1.5 to –5 MPa) (Roberts and Ellis 1989; Probert and Longley 1989; Pritchard 1991; Dickie *et al*. 1991; Poulsen and Eriksen 1992), although Vertucci and Farrant (1995) have suggested a drier value of –11 MPa.

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6.3.1. Factors influencing desiccation sensitivity in recalcitrant seeds

Desiccation tolerance in recalcitrant seeds increases during seed development on the mother plant (Fig. 7); however, unlike orthodox seeds, maturation drying to low moisture contents does not occur (Hong and Ellis 1990; Finch-Savage 1992a), and fresh recalcitrant seeds have high levels of moisture content at maturity / shedding, between, for example, 36% for rubber (Chin *et al.* 1981) and 90% for choyote (or chayote) (*Sechium edule*) (Ellis 1991).

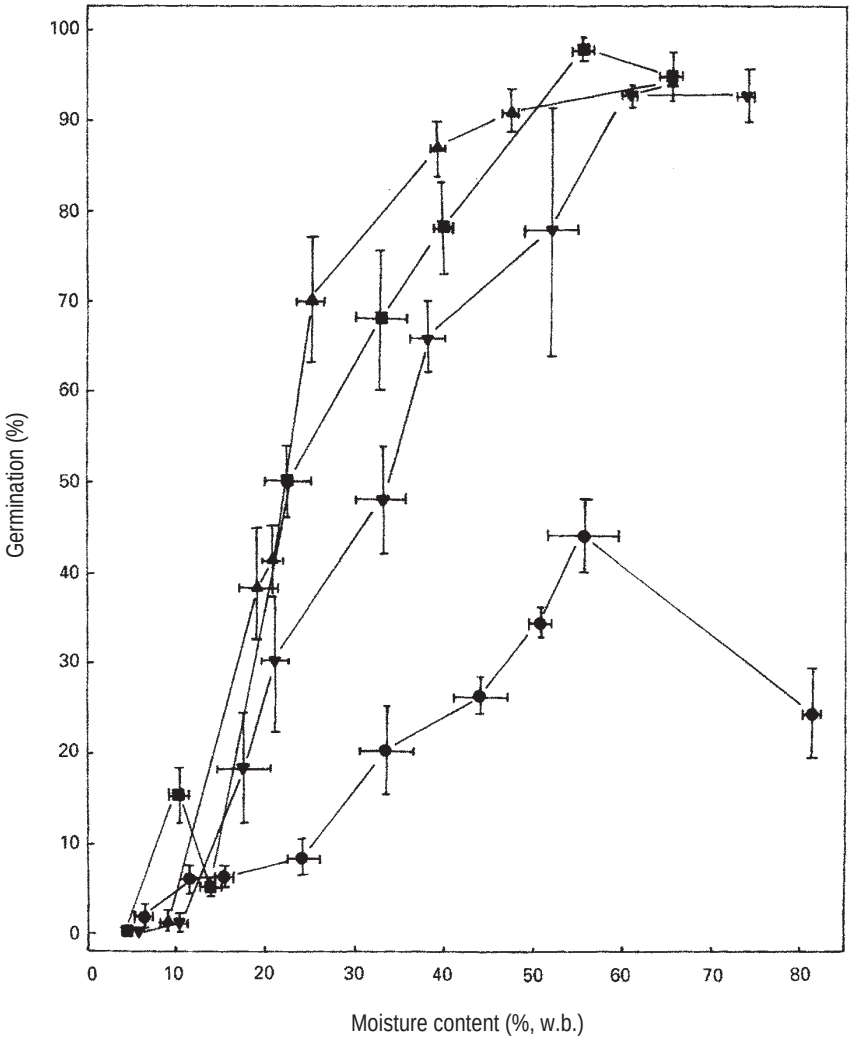

Fig. 7. Relations between germination (%) and moisture content (%, w.b.) for sycamore seeds (*Acer pseudoplatanus*) harvested on 12 July (●), 16 August (▼), 20 September (■) or 18 October 1988 (▲), dried to the moisture contents shown. From Hong and Ellis (1990).

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Considerable differences in moisture content can be detected among tissues within a particular recalcitrant seed. For example, Grabe (1989) reported that, with the exception of durian (*Durio zibethinus*) and jackfruit (*Artocarpus heterophyllus*), the storage tissues of recalcitrant seeds are always at a lower moisture content than the embryonic axis. Desiccation of excised embryos or embryonic axes has considerable practical potential for the *in vitro* conservation of recalcitrant embryos, since embryos are able to survive desiccation to lower moisture contents than whole seeds (Chin 1988). For example, fresh seeds (36% moisture content) of *Hevea brasiliensis* tolerated desiccation to 20% moisture content but no seeds survived further desiccation to 15% moisture content (Chin *et al*. 1981). However, 50-80% of their excised embryos (55% moisture content) survived desiccation to 14% moisture content when cultured *in vitro* (Normah *et al*. 1986). Reports of survival of excised embryos or embryonic axes to a lower moisture content than their intact seeds are numerous (Finch-Savage 1992b; Chandel *et al*. 1995).

While there are several reports that fast drying allows intact recalcitrant seeds to survive desiccation to lower moisture contents than slow drying (Farrant *et al*. 1985; Pritchard 1991), Finch-Savage (1992b) showed that drying rate does not affect the desiccation sensitivity of whole seeds of *Quercus robur*. However, fast drying allowed excised embryos of *Araucaria hunsteinii*, *Hevea brasiliensis*, *Landolphia kirkii*, *Quercus robur* and *Quercus rubra* to survive desiccation to lower moisture contents than similar embryos dried more slowly within intact seeds (Normah *et al*. 1986; Pritchard and Prendergast 1986; Pritchard 1991; Pammenter *et al*. 1991; Finch-Savage 1992b). Fu *et al*. (1993) reported that drying excised embryonic axes by silica gel or an aseptic air current allowed excised embryonic axes to survive desiccation to a lower value than that achieved by the vacuum method. For example, although the vacuum drying method provided more rapid drying, no excised embyonic axes of *Artocarpus heterophyllus* survived desiccation to 44% moisture content, while the excised embryonic axes dried with an aseptic air flow and silica gel tolerated desiccation to 26 and 16% moisture content, respectively (Fu *et al*. 1993).

6.3.2. Longevity of recalcitrant seeds in moist storage

There is no satisfactory method for maintaining the viability of intact recalcitrant seeds over the long term. This is because they cannot be dried; neither can they be stored at subzero temperatures because they would then be killed by freezing injury re-

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sulting from ice formation. In addition, some tropical recalcitrant seeds are also damaged by chilling injury at temperatures of 10-15°C and below. The longevity of recalcitrant seeds is generally short, particularly for species adapted to tropical environments, typically from a few weeks to a few months (King and Roberts 1979, 1980a). However, the longevity of seeds of species adapted to temperate environments can be maintained for much longer periods, e.g. more than 3 years for oak (*Quercus* spp.) seeds stored moist at –3°C (Suszka 1971-1974).

In practical terms, species with recalcitrant seeds can therefore be subdivided into those of tropical origin, and those adapted to temperate climates (temperate latitudes, or high altitudes in the tropics); the latter can be stored at cooler temperatures and for longer. This subdivision tallies with the classification of Bonner (1990).

6.3.3. Cryopreservation of excised embryos

Cryopreservation of somatic and zygotic embryos has been reported to be successful for many species which show orthodox, intermediate and recalcitrant seed storage behaviour (Engelmann *et al.* 1995b).

For successful cryopreservation, excised embryos from seeds with recalcitrant seed storage behaviour must survive desiccation below the threshold freezable moisture content (Hor *et al.* 1990) of about 22-27% for *Landolphia kirkii* (Pammenter *et al.* 1991) to 30-33% for *Acer saccharinum*, *Nephelium lappaceum*, *Durio zibethinus*, and *Artocarpus heterophyllus* (Becwar *et al.* 1983; Hor *et al.* 1990), below which value there is no freezable water for ice formation by cooling to ultra-low temperatures. For example, embryonic axes of *Aesculus hippocastanum* survived desiccation to 12% moisture content and subsequent cryostorage in liquid nitrogen (Pence 1992). Similarly, 40% of excised embryonic axes of longan (*Dimocarpus longan*) survived both desiccation to 18% moisture content and subsequent storage for 24 hours in liquid nitrogen (Fu *et al.* 1993). Method of drying and rate of drying are two important factors infuencing the desiccation tolerance of excised embryos (or embryonic axes).

Results reported for the following species have shown the feasibility of the cryopreservation of excised embryos or embryonic axes of recalcitrant seeds: *Aesculus* spp. (Pence 1990, 1992), *Araucaria hunsteinii* (Pritchard and Prendergast 1986), *Artocarpus heterophyllus* (Krishnapillay 1989 cited by Engelmann *et al.* 1995b, Chandel *et al.* 1995), *Castanea sativa* (Pence 1990, 1992), *Coffea liberica* (Normah and Vengadasalam 1992; Hor *et al.* 1993), *Cocos*

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nucifera (Chin *et al*. 1989; Assy-Bah and Engelmann 1992), *Dimocarpus longan* (Fu *et al*. 1990, 1993), *Hevea brasiliensis* (Normah *et al*. 1986), *Landolphia kirkii* (Vertucci *et al*. 1991), *Quercus* spp. (Pence 1990, 1992; Jorgensen 1990; Gonzalez-Benito and Perez-Ruiz 1992) and *Theobroma cacao* (Pence 1991). However, these approaches remain "experimental" and genebanks wishing to apply such approaches should contact IPGRI for advice.

6.4. Intermediate seed storage behaviour

For the majority of species, it was previously a relatively simple matter to clarify seed storage behaviour as either orthodox or recalcitrant in accordance with Roberts' definitions. This was also convenient because long-term seed storage for plant genetic conservation was feasible for the former category only. Of course, mistakes in classification occurred on occasion, but more importantly evidence began to accumulate that these two categories did not account satisfactorily for all observations on seed storage behaviour. For example, Teng and Hor (1976) showed that seeds of both star fruit (*Averrhoa carambola*) and papaya withstood desiccation to around 10-12% moisture content and could be stored successfully in hermetic containers at these levels of moisture content. In contrast to orthodox seeds, however, they lost viability much more rapidly in air-dry storage at 0°C than at warmer temperatures of 12-21°C. And then Tompsett (1984a, 1984b) showed that the longevity of seeds of *Araucaria columnaris* was increased in air-dry storage by reduction in storage temperature or moisture content, but only within limited ranges, because viability was reduced with desiccation below about 12% moisture content. In both these cases then it was clear that the seeds could be stored in certain air-dry environments successfully but their behaviour did not satisfy the definition of orthodox seed storage behaviour provided by Roberts (1973).

We have investigated this topic extensively, initially with seeds of arabica coffee, using a factorial combination of several storage temperatures and moisture contents and have shown that seeds of arabica coffee (Ellis *et al*. 1990a, 1991a, Hong and Ellis 1992c), robusta coffee (*Coffea canephora*) (Hong and Ellis 1995), oil palm (Ellis *et al*. 1991c), papaya (Ellis *et al*. 1991b) and several *Citrus* species (Hong and Ellis 1995) show a type of seed storage behaviour intermediate between the orthodox and recalcitrant categories defined by Roberts (1973). The essential feature of intermediate seed storage behaviour is that the negative relation between seed longevity in air-dry storage and mois-

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ture content is reversed at values below those in equilibrium (at 20°C) with about 40-50% RH. This type of seed storage behaviour is also found in neem (*Azadirachta indica*), star-apple (*Chrysophyllum cainito*), sapodilla (*Manilkara achras*) (Hong and Ellis, unpublished data), wild rice (*Zizania palustris*) (Vertucci *et al.* 1994), and in many other species (Hong *et al.* 1996).

The main feature of intermediate seed storage behaviour described above is often (but not always) also associated with damage immediately after desiccation to a relatively low moisture content, about 7-12% moisture content depending on species (Fig. 8). The critical levels of moisture content of intermediate seeds at which more rapid loss in viability occurs during hermetic storage or desiccation damage to germination is immediately evident varies considerably with species, degree of maturity and method of seed extraction/handling. In general, seeds which are extracted at maturity tolerate desiccation to moisture contents in equilibrium with about 40-50% RH, i.e. about 10% moisture content for arabica coffee, and 7% moisture content for certain *Citrus* spp. Another feature of intermediate

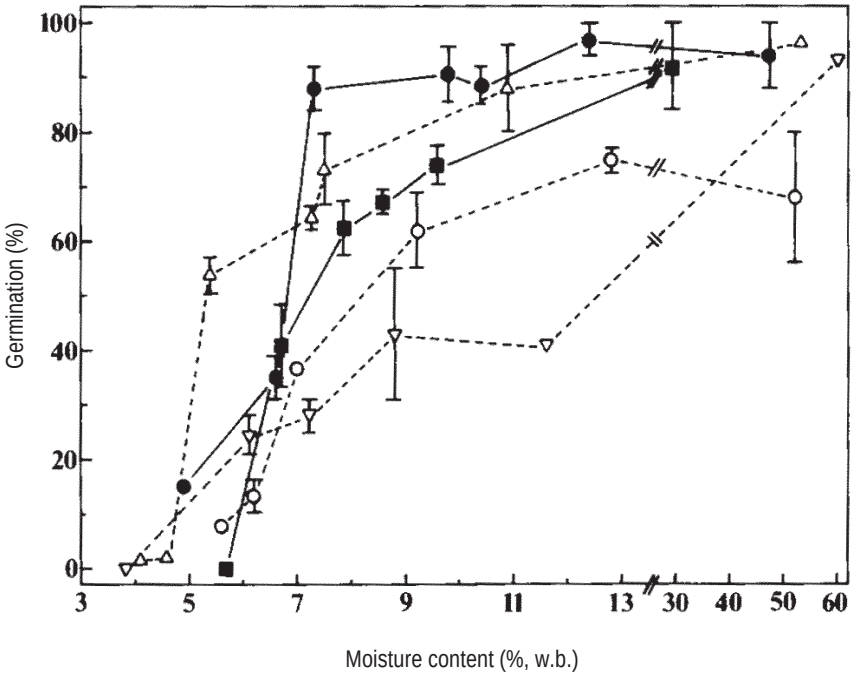

Fig. 8. Desiccation sensitivity (normal germination, %, versus moisture content, % w.b.) in seed lots of arabica coffee (*Coffea arabica*) cv. SL28 from Kenya (■) and South Africa (●) extracted from ripe (red) fruits, and seeds extracted at Reading from green (▽), yellow (△), or red fruits (○) from South Africa. From Ellis *et al.* (1991a).

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seeds of tropical origin is the fact that the longevity of dry seeds (7-10% moisture content) is reduced with reduction in storage temperature below about 10°C (Ellis *et al*. 1990a 1991a, 1991c; Hong and Ellis 1992c). In such cases, then, there is an optimum air-dry storage environment for the maintenance of seed viability. In arabica coffee this is about 10°C with 10-11% moisture content (Hong and Ellis 1992c). Moreover, although an increase in seed storage moisture content and temperature above these values reduces longevity, it is by no means certain that these relations conform to the seed viability equation for orthodox seeds (Hong and Ellis, unpublished).

From the point of view of optimum air-dry seed storage environments, it may be helpful to distinguish between species with intermediate seed storage behaviour adapted to tropical environments and those adapted to temperate environments (including high altitudes in the tropics). For example, intermediate seeds of tropical origin such as arabica coffee (Wellman and Toole 1960; Bendana 1962), and papaya (Bass 1975) can be stored at moisture contents in equilibrium with 50% RH (9-10% moisture content) and 10°C for up to 5 and 6 years, respectively, without loss in viability. The viability of intermediate seeds of temperate origin is also maintained well at moisture contents in equilibrium with about 50% RH but at cooler temperatures of 5 to –20°C. For example, wild rice seeds can be maintained in hermetic storage at –2 or 3°C with 9-11.5% moisture content for 9-12 months without loss in viability (Oelke and Stanwood 1988; Oelke *et al*. 1990), or at –18°C with 5.4-6.8% moisture content for 15-16 months for embryonic axes with loss in viability limited to about 11-15% (Berjak *et al*. 1994).

Seed maturity also affects desiccation tolerance in intermediate seeds (Ellis *et al*. 1991a; Vertucci *et al*. 1994). For example, seeds of arabica and robusta coffee extracted from fruits of intermediate maturity (yellow) were able to tolerate greater desiccation than those from either ripe (red) or immature (green fruits) (Ellis *et al*. 1991a; Hong and Ellis 1995) (Fig. 8). The method of seed extraction and handling may also influence desiccation tolerance. For example, seeds of arabica coffee imbibed at 30°C for 3-10 days showed greater sensitivity to desiccation (Ellis *et al*. 1991a). It is suggested that seed processing methods involving high seed moisture contents, e.g. soaking, fermentation, moist storage and storage in cold water (e.g. *Zizania*) will tend to reduce subsequent desiccation tolerance and seed longevity in intermediate seeds (Hong and Ellis 1992c).

Intermediate seeds of species of tropical origin die more

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rapidly when the temperature is lowered below about 10°C. In some cases, temperatures just below 0°C kill whole seeds immediately (Ellis *et al.* 1990a, 1991a, 1991b, 1991c). Nevertheless, there is some possibility that the cryopreservation of seeds with intermediate seed storage behaviour may be feasible with more research. Despite reports of the immediate death of (whole) seeds following cryostorage in liquid nitrogen, e.g. *Coffea arabica* (Becwar *et al.* 1983), *Corylus avellana* (Normah *et al.* 1994), *Corylus cornuta* (Stanwood and Bass 1981), *Elaeis guineensis* (Grout *et al.* 1983) and *Roystonea regia* (Ellis *et al.* 1991c), there have been several reports of the survival of seeds of species with intermediate seed storage behaviour following immersion in liquid nitrogen, e.g. *Camellia sinensis* (Hu *et al.* 1993, 1994), *Carica papaya* (Becwar *et al.* 1983; Chin and Krishnapillay 1989), *Elettaria cardamomum* (Chaudhury and Chandel 1995), *Musa violascens* (Chin and Krishnapillay 1989), *Passiflora edulis* (Becwar *et al.* 1983), and *Piper nigrum* (Chaudhury and Chandel 1994).

Since the whole seeds of species which show intermediate seed storage behaviour tolerate desiccation to relatively low moisture contents (7-10%), excised embryos may have a greater chance to survive cryostorage in liquid nitrogen than the recalcitrant seeds. For example, oil palm shows intermediate seed storage behaviour (Ellis *et al.* 1991c) but excised embryos of oil palm which had been dried to 10.4% moisture content showed no loss in viability during 8 months of storage in liquid nitrogen whereas intact seeds were killed (Grout *et al.* 1983). Similar results for oil palm have been reported by others (Hor *et al.* 1992; Engelmann *et al.* 1995a, 1995b).

Reports of successful cryostorage of excised embryos of *Camellia sinensis* (Chaudhury *et al.* 1990, 1991; Wesley-Smith *et al.* 1992; Chandel *et al.* 1993, 1995), *Citrus sinensis* (Radhamani and Chandel 1992), *Coffea arabica* (Abdelnour *et al.* 1992), *Corylus avellana* (Pence 1990; González-Benito and Pérez 1994; Normah *et al.* 1994; Reed *et al.* 1994), *Howea forsteriana* (Chin *et al.* 1988; Chin and Krishnapillay 1989), *Musa* spp. (Abdelnour-Esquivel and Mora 1992), *Poncirus trifoliata* (Radhamani and Chandel 1992) and *Veitchia merrilli* (Chin *et al.* 1988; Chin and Krishnapillay 1989) have shown some potential for long-term storage under such conditions.

These comments on the possibility of the cryopreservation of seeds (or embryos) with intermediate seed storage behaviour are indicative of our suspicion that a concerted future programme of research may enable the development of advice for the long-term storage of intermediate seeds. Until this is done, however,

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medium-term storage is likely to be the best that can be achieved; the protocol indicates how such storage environments can be determined.

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