Biotechnological aspects of non-orthodox seeds: an African perspective

P Berjak* and NW Pammenter

School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban 4041, South Africa
* Corresponding author, e-mail: berjak@ukzn.ac.za

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Storage of seeds at low water content and sub-zero temperature in genebanks is the most convenient way of conserving the genetic resources of most spermatophytes. However, the seeds of many, particularly tropical, species are desiccation sensitive and are not amenable to storage under these conditions. Not only is long-term conservation of this germplasm difficult, but the characteristic of desiccation sensitivity also places limits on short-term storage of the seeds. This paper briefly reviews the physiology of these 'non-orthodox' seeds, and considers the progress that has been made in extending storage lifespan of the seeds, and in the cryopreservation of the germplasm. Short- to medium-term storage lifespan of hydrated seeds can be extended by reducing temperatures as low as possible — where chilling sensitivity permits — although not below zero. Long-term cryopreservation of whole seeds is precluded because of ice crystal damage to the hydrated tissue, but a novel approach in dealing with this problem is described. Excised embryonic axes are dried very rapidly (flash dried) to water contents low enough to reduce ice crystal formation on freezing, but not so low as to introduce desiccation damage, and the partially dried axes are frozen very rapidly by plunging in sub-cooled liquid nitrogen ('nitrogen slush') to reduce potential ice crystal damage. Subsequent storage in liquid nitrogen affords the means for long-term conservation of the zygotic axes (and buds, apical meristems, callus or somatic embryos) and hence the genetic resources of species producing even the most recalcitrant of non-orthodox seeds.

Introduction

The subject of recalcitrant seeds was reviewed in this journal recently (Berjak and Pammenter 2001) and some agricultural implications have been covered in Berjak and Pammenter (2004). Thus we have not reviewed the literature on the phenomena of desiccation tolerance and sensitivity in this current contribution; instead, we have focused on the practical problems posed by desiccation sensitivity in seeds, and the biotechnological approaches taken to deal with these problems. Nevertheless, some background on non-orthodoxy, and particularly recalcitrance, is presented to provide the physiological and biophysical basis underlying the necessity of biotechnological manipulations, instead of simple seed storage, for conservation of the genetic resources of such species.

Seeds provide food and feedstocks, but they are also stored to provide planting material for subsequent seasons, and, as early as the first century of the Roman Empire, Pliny was advising farmers to store the best seeds for the next year's crop (Liversidge 1976). Considerably more recently there has been growing interest in the long-term storage of seeds for the maintenance of valuable genetic resources. Originally the focus was almost exclusively on agricultural crops, but with the growing awareness of the importance of conservation of biodiversity, increasing attention has been paid to the storage of seeds of wild species, as maintaining seeds in genebanks is the simplest way of long-term conservation of the germplasm of most spermatophytes. Perhaps the foremost example of this is the Millennium Seed Bank (MSB) Project of the Royal Botanic Gardens, Kew (www.rbgkew.org.uk/msbp/) which aims to have in long-term storage by 2010 the seeds of over 24 000 species. This represents some 10% of the world's spermatophyte flora, principally dryland species, among which African species are well represented.

Conventional seed banking facilities that include low-temperature, low-relative humidity (RH) storage for orthodox seeds, do exist in Africa: among these are the Southern African Development Community (SADC) Plant Genetic Resources Centre (SPGRC), in Zambia; the South African National Department of Agriculture; The National Plant Genetic Resources Centre, Namibia; the National Genebank of Kenya; the National Centre for Genetic Resources and Biotechnology, Nigeria; the Centre National de Semences Forestières (CNSF), Burkina Faso; the Ethiopian National Tree Seed Project; the National Tree Seed Programme, Tanzania; and the Projet d’Appui Silo National, Madagascar. Much of the material stored in these seedbanks is of agricultural value:
However, considering the plant biodiversity of Africa, the extent of these facilities and range of species stored should be expanded considerably.

Furthermore, in Africa — where there is a wealth of species producing non-orthodox seeds and where considerable relevant progress has been made and expertise developed — there is an urgent need also for an extensive cryobanking facility along the lines of those abroad, which include CATIE in Costa Rica, NBGPR in India, NIAR (Japan), the MSB (UK) or the National Centre for Genetic Resources Preservation (formerly the NNSL), USA.

The conservation of seeds in conventional gene banks makes the assumption that they are storable in the first place, which in turn, is based on the premise that they show orthodox post-harvest behaviour (Roberts 1973). By this is meant that the seeds can be successfully dried to low water contents and that they will remain viable in this condition for long periods (of the order of decades or more), their longevity being increased, within limits, with decreasing water content and temperature (Ellis and Roberts 1980). Orthodox seeds are, or can be, dehydrated to low water contents because they acquire the property of desiccation tolerance relatively early during their pre-shedding development (e.g. Bewley and Black 1994, Kermode and Finch-Savage 2002).

‘Non-orthodox’ Seeds

Not all seeds are orthodox, however, and those of many species will lose viability if they are dried. The extent to which these ‘non-orthodox’ seeds can be dried before viability is lost varies among species, and is difficult to quantify (see below), but in practical terms, this lack of desiccation tolerance means that the seeds cannot be stored under the conventional conditions of low water content and low (below freezing) temperatures. Roberts (1973) termed such seeds ‘recalcitrant’. Since then investigations have shown that there are a number of species that produce seeds that are able to tolerate a greater degree of dehydration than the desiccation-sensitive recalcitrant seeds, but not as much as orthodox seeds, and which lose viability fairly rapidly when stored at low water content: such seeds have been termed ‘intermediate’ (Ellis and Roberts 1980). Orthodox seeds are, or can be, dehydrated to low water contents because they acquire the property of desiccation tolerance relatively early during their pre-shedding development (e.g. Bewley and Black 1994, Kermode and Finch-Savage 2002).

Physiological characteristics of recalcitrant seeds

Stated most simply, the underlying characteristic of recalcitrant seeds is that they are sensitive to desiccation. However, the extent of desiccation that can be tolerated is extremely variable, and is, in fact, difficult to compare among species, because the desiccation sensitivity within a species is itself variable (reviewed in Pammenter and Berjak 1999, Berjak and Pammenter 2001). Although desiccation sensitivity varies with both pre- and post-shedding stage of development, there is no time at which recalcitrant seeds will tolerate much dehydration without deleterious effects. Recalcitrant seeds do not undergo the maturation drying phase of seed development (Finch-Savage and Blake 1994), and do not go through the reduction in metabolism associated with this developmental phase (Farrant et al. 1997). Another characteristic of recalcitrant seeds, then, is that they are metabolically active when shed, and it is this metabolism that is considered to be the major factor underlying their response to desiccation (Berjak et al. 1989, Pammenter and Berjak 1999). Metabolic activity and further subcellular development will continue after shedding without provision of additional water, and germination in storage is often observed. The data available for a range of species indicate that desiccation sensitivity decreases during the development of the seed prior to shedding, and increases with storage time subsequent to shedding.

Another aspect of variability in the response to desiccation is the rate at which recalcitrant seeds are dried; the faster they are dehydrated, the lower the water content that can be tolerated before viability is lost (Normah et al. 1986, Pammenter et al. 1991, 1998, Pritchard 1991, Kundu and
Kachari 2000, Potts and Lumpkin 2000), although for *Theobroma cacao* (cocoa), Liang and Sun (2000), have identified an optimal drying rate, with faster drying being detrimental. The rate of drying has an effect on apparent desiccation sensitivity because many of the deleterious processes leading to desiccation damage are aqueous based, and the more slowly material is dried the longer it remains in the water content range where this damage can accrue; if tissue is dried rapidly it reaches lower water contents before sufficient aqueous-based damage to kill the tissue can accumulate. There is, nevertheless, a limit to the desiccation that can be tolerated no matter how fast the drying; this is generally in the range 0.18–0.23 g water g\(^{-1}\) dry mass, depending on species (Pammenter et al. 1991). The damage accruing at higher water contents is a consequence of deranged metabolism, whilst the lower limit is probably a consequence of biological damage associated with removal of water from macromolecular (including membrane) surfaces; desiccation tolerant tissue can withstand the removal of this water but sensitive tissue cannot (Walters et al. 2001).

Most recalcitrant seeds are actually too large to dry sufficiently rapidly for this effect of drying rate to be manifested. However, if embryonic axes are excised from the seeds, then they can be dried rapidly (over a period of tens of minutes to a few hours) in a dry air stream. This has led to the development of the process of flash drying (Berjak et al. 1990), which has important implications in the development of techniques for the long-term cryopreservation of this material (see below). It must be emphasised that flash drying does not induce any form of desiccation tolerance; material that has been dried to low water contents without loss of viability does not remain viable for more than a few days (Walters et al. 2001). It should be noted, however, that very rapid water removal from excised axes is likely to lead to non-uniform drying, and the inner germinative cells of the tissue may not actually suffer a severe dehydration stress in the short term (Wesley-Smith et al. 2001a) — which might partly account for the success of flash drying.

Another complicating issue is that of shedding water content. Not only does this vary among species, but within a species there is both inter- and intra-seasonal variation. Indeed, even within a single collection there can be considerable differences among individual seeds. Because the response to desiccation of a seed lot depends on so many factors it is not possible to determine a ‘critical’ water content (or more correctly, water activity) corresponding to viability loss, although there may be specific water activities below which particular processes can no longer occur (Walters 1999). Pammenter et al. (2003) attempted to model the combined effects of water content and drying rate to quantify desiccation sensitivity, but the method did not have sufficient resolution to differentiate between species with similar responses.

**Storage of non-orthodox seeds**

Seeds can be stored either in the short- to medium-term, for maintenance of planting stock, or in the long-term for the conservation of genetic resources. Orthodox seeds are desiccation tolerant, and in the dry state are chilling and freezing tolerant, consequently short-term storage presents few problems. For most species, successful long-term storage is difficult to accomplish unless this is at reduced, preferably, sub-zero temperatures, when survival of high-quality seeds can generally be achieved for periods of decades. Further discussion on this aspect of orthodox seed storage can be found in Ellis and Roberts (1980), Roberts and Ellis (1989), Vertucci and Roos (1990, 1993) and Walters (1998).

However, because recalcitrant seeds show on-going metabolism and desiccation sensitivity, they can be stored for short periods only, ranging from weeks to, at best, for temperate species, a year or two (King and Roberts 1980). This places constraints on normal seed handling and certainly precludes conventional low water content, sub-zero temperature storage for genetic conservation.

**Short-term storage**

There are two problems associated with storage of recalcitrant seeds. One is the hydrated and metabolic state of the tissue; the other is the invariable presence of micro-organisms, particularly fungal, contaminants. To maximise storage life it is important that the seeds be of good quality; seeds of initial poor quality or those that have been stressed during collection and transport lose viability rapidly in storage. This was graphically illustrated by a consignment of *Hevea brasiliensis* (rubber) seeds from a provenance in Malawi (Berjak 1989): during the nine day transit period when only a modest water loss occurred, the embryos sustained visible sub-cellular damage that appeared to be repairable when water was made available. However, in this ‘sub-imbibed’ condition the seeds had become more desiccation sensitive, and when rehydrated, their storage lifespan was considerably shorter than is the case for freshly-harvested *H. brasiliensis* seeds.

Berjak et al. (1989) suggested that storage lifespan of recalcitrant seeds is determined by the rate of germinative development in storage, and it has been suggested that slight reductions in water content, to prevent germination in storage (so-called ‘sub-imbibed’ storage), may be advantageous (Hong and Ellis 1996). However, this treatment has been found to be deleterious, certainly for seeds of tropical species (Corbineau and Côme 1988, Xia et al. 1992, Pritchard et al. 1995, Drew et al. 2000, authors’ unpublished observations). The most likely explanation for viability loss in storage is that ongoing germinative metabolism creates a requirement for extra water, subjecting the seeds to a relatively mild, but prolonged water stress (Pammenter et al. 1994). If this is the case, sub-imbibed storage would only exacerbate the situation, and so seeds should not be allowed to lose water before or during storage.

One approach to reducing metabolic rate and post-shedding development, thereby extending storage lifespan, is to reduce the temperature, but not below 0°C, at which ice crystal damage will occur. This appears to be successful with temperate and some tropical recalcitrant seeds, which are chilling tolerant. However, there are number of tropical seeds (e.g. cocoa) that are damaged at temperatures below about 15°C (King and Roberts 1980), and so care must be exercised when selecting low storage temperatures, which generally have to be determined experimentally for each species. For example, seeds of *Trichilia* species which store well at 16°C, are lethally damaged at 6°C (Kioko 2003) as
were hydrated neem (*Azadirachta indica*) seeds from Mombasa (Berjak et al. 1995). The case of neem, however, offers a good example of the further vagaries of non-orthodox seeds: depending on water content, seeds of *A. indica* may or may not withstand cold storage (Sacandé et al. 1998). Those authors found that *A. indica* seeds from both Burkina Faso and Sri Lanka were chilling-sensitive at water contents $\geq 0.11 g g^{-1}$ (which actually represents a higher water activity for these oily seeds) and, although the seeds could best be stored at a mean water content of 0.087 g g$^{-1}$, they were damaged upon imbibition at 22°C or below. It should also not be forgotten that a low temperature that may not appear damaging in the short-term, may become so on long-term exposure.

The other problem associated with storing hydrated recalcitrant seeds is the ubiquitous presence of fungal contaminants (Mycock and Berjak 1990, Berjak 1996). Even if all the storage parameters are optimised, the associated fungi can rapidly and irreversibly damage the seeds, so obviously, deterioration by fungal degradation and toxin production needs to be prevented, or at least minimised. As surface applications of fungicide are not effective in curtailing the activity of mycelium below the pericarp/testa, recourse to treatments with systemic fungicides (authors’ unpublished data) may be necessary. However, as most fungicides target particular fungal species or groups, it is necessary to assess the efficacy of systemic fungicides in curtailing proliferation of the specific fungi involved, as well as ensuring that the treatment itself does not damage the seeds.

The effects of mycoflora in curtailing hydrated storage life have been clearly demonstrated with seeds of *Avicennia marina* (Calistrue et al. 2000), periodic aerosol application of fungicide to seeds after removal of the pericarp and surface sterilisation more than doubled storage periods. We have found for *Trichilia dregeana* that while seed-associated fungi will curtail the hydrated storage of the seeds to a few weeks, when the mycoflora has been eliminated storage periods of up to eight months can be achieved. Much of this work has been reviewed in Sutherland et al. (2002). Work in our laboratory is now proceeding on the evaluation of systemic fungical ‘cocktails’ and other pre-treatments before hydrated storage of recalcitrant seeds. The as-yet unpublished results show for several species, that storage longevity can be significantly extended. However, elimination of the effects of seed-associated fungi will not confer indefinite storability on recalcitrant seeds; ultimately it is the demands of ongoing germinative metabolism in the absence of additional water that will curtail longevity.

**Long-term cryopreservation**

There has been extensive interest for a considerable time in the cryopreservation of a wide range of biological tissues, and although much attention has been paid to vegetative tissue, it is only recently that studies have been undertaken on non-orthodox seeds. Because of the short storage lifespan of these seeds, preservation at cryo temperatures ($< -130^\circ C$) is the only feasible means of long-term genetic conservation (Engelmann 2000). However, this is no simple task.

A key issue for successful cryopreservation is the avoidance of lethal intracellular ice crystal formation during cooling to cryogenic temperatures (and on subsequent warming). This is achieved by changing the phase of the free water in the tissue to that of a ‘glass’ (a very viscous amorphous phase) — a process known as vitrification. A glass has extremely high viscosity and so will effectively suspend all chemical reactions that require molecular diffusion, and it will also fill space, preventing tissue collapse. Thus vitrified tissue, particularly at low temperature, should have an extremely long storage lifespan. The water remaining in ‘dry’ orthodox seeds is considered to be in a vitrified state (Leopold et al. 1994), or at least in highly viscous solution (Buitink et al. 2000), and this contributes to their long-term survival and the ability to be stored at sub-zero temperatures. Vitrification of the water in hydrated tissue can be brought about by exposing it to concentrated cryoprotectant media, which effectively dehydrate the tissue osmotically, after which the material can be cooled to the temperature of liquid nitrogen. However, for several species we have found cryoprotectant treatment to be damaging to recalcitrant zygotic axes. Under appropriate conditions, air drying of hydrated tissue can also induce vitrification.

Intermediate seeds do not store well under conventional seed bank conditions, but they are sufficiently desiccation tolerant to permit the low water contents necessary for cryopreservation, and success has been achieved with e.g. neem (Berjak and Dumet 1996) and coffee (Dussert et al. 2002). The case of *Warburgia salutaris* is interesting; whole seeds of this highly endangered African species are not tolerant of slow air drying (Albrecht 1993), but if the seeds are very rapidly dried (by burying in silica gel), they can tolerate water contents as low as 0.1 g g$^{-1}$ and have been successfully frozen (Kioko et al. 2003).

Most recalcitrant seeds are large and dehydration, both by using cryoprotectants and by air-drying, is relatively slow and is lethal (e.g. Pammenter et al. 1998). Excised embryonic axes, on the other hand, are generally sufficiently small to be flash dried to levels where there is little or no freezeable water in the tissue. Advantage has been taken of this to develop cryopreservation procedures for germplasm from recalcitrant seeds, and flash dried excised axes of *Landolphia kirkii* have been successfully frozen to $-70^\circ C$ and stored at this temperature for 6 months (Vertucci et al. 1991). It should be noted that at that time the experiment was terminated to assess viability retention of the axes, and that six months does not represent the limit of the survival period. Excised cryopreserved axes should, theoretically, survive for indefinite periods.

The difficulty with flash drying is that it is easy to remove too much water from the axes, thus killing them by desiccation prior to freezing. A novel approach has been developed in our laboratory to overcome this problem (Wesley-Smith et al. 1992, 2001b, 2004). The method uses ultra-rapid cooling (freezing) — of the order of several hundred of degrees per second — achieved by plunging the axes directly into sub-cooled liquid nitrogen — so-called nitrogen slush — or into just-melted isopentane held in a liquid nitrogen reservoir. Such ultra-rapid cooling induces vitrification of any free water in the tissue. Under these conditions, it is not necessary to dry the tissue to the extent required if freezing is much slower, thereby reducing the problem of desiccation damage. However, the higher the water content of the embryonic axis the greater the thermal mass and hence the slower the cool-
ing. There is thus a trade-off between reducing desiccation damage (relatively high water contents required) and avoiding ice-crystal damage (rapid cooling rates, low water contents required), but ultra-rapid cooling widens the window of water contents that permits successful exposure to cryogenic temperatures. This topic is discussed more fully in Wesley-Smith et al. (2001b, 2004). Using this approach, success has been achieved with axes of tea, Camellia sinensis, (Wesley-Smith et al. 1992), horse chestnut, Aesculus hippocastanum, (Wesley-Smith et al. 2001b) and trifoliate orange, Poncirus trifoliata (Wesley-Smith et al. 2004).

Achieving successful cryopreservation of recalcitrant seed material is not as straightforward as it might seem from the discussion above. One of the problems frequently encountered is that on recovery from the cryogen, the material produces callus tissue, rather than developing into young plantlets. In any cryopreservation protocol there are three major steps; preparing or conditioning the plant material, the actual freezing process itself (cryogenic phase), and re-establishment of an actively growing plant. Inadequate or inappropriate processes utilised in any of these steps can jeopardise success. This has been clearly demonstrated by the study of Berjak et al. (1999) who compared their successful method for cryopreservation of zygotic axes of the pedunculate (English) oak, Quercus robur, with two other published protocols which had achieved minimal success, and demonstrated the cumulative deleterious effects of inappropriate treatments in those protocols in all three steps.

It is also noteworthy that to date the best results have been achieved with species from the temperate zone, tropical species presenting more difficulties. Problems can arise in the initial step of preparing the material. The dehydration step must be sufficiently rapid to prevent desiccation damage, and this requires excision of the axis from the seed. However, we have found that excising the axis from the cotyledons of newly-shed Trichilia dregeana seeds causes considerable physical damage to the shoot apex, and for successful flash drying, portions of the cotyledons must be left attached to the axis. This in turn creates a ‘down-stream’ problem in that the specimen is now larger than the optimum to cool sufficiently rapidly to prevent freezing damage during the cryogenic phase (Kioko et al. 1998). Similar problems have been observed with seeds of T. emetica and Ekebergia capensis, although E. capensis axes are small enough that even with cotyledonary segments attached, successful cryopreservation can be achieved (unpublished results). A further ‘down-stream’ problem with T. dregeana axes is that the presence of the cotyledonary blocks markedly retards root growth in vitro (Goveia et al. 2004). This phenomenon is thought to be because of the exudation of inhibitory substances (possibly polyphenolics) into the medium from the cut cotyledonary surfaces. The solution to the problem with T. dregeana (and probably other species) appears to reside in allowing the shoot apex to elongate within the wet-stored seeds, after which axes can be excised without cotyledonary segments (Goveia et al. 2004).

It is also frequently observed that ‘success’ might be thought to have been achieved in that ‘germination’ (assessed as root growth) has been reported, but there is no subsequent shoot development; the shoot meristem in most axes is not as well protected as the root meristem and appears to be more susceptible to damage. This illustrates the fact that one cannot consider merely the axis response (e.g. enlargement or greening) as a sign that the various manipulations required for cryostorage have been successful. Rather, in developing protocols, attention must be directed to the effects of the manipulations on both poles of the embryonic axis which require to retain a critical proportion of undamaged cells in order that normal seedlings be produced.

Problems can also arise in the re-establishment phase. For example, excised axes have been separated from the seed reserves and so must be germinated and the seedlings initially grown on nutrient medium; selection of the correct medium is an essential first step in the development of any cryopreservation protocol. Because of the fact that the seed production season is usually curtailed, and hydrated recalcitrant seeds can seldom be stored for very long, successful cryostorage is difficult to achieve within a single year. We have also observed another problem; although both roots and shoots might develop, the roots may not show a gravitropic response. This is ascribed to a failure of starch deposition and of the cytoskeleton to reassemble after the drying and/or drying-freezing treatment (Berjak et al. 1999, 2000, Berjak and Mycock 2004) and has been shown to be the outcome of the inappropriate water-rehydration procedure conventionally used. Based on the work of Mycock (1999) on somatic embryos, we have thawed and rehydrated cryopreserved axes in a solution containing Ca2+ and Mg2+, this treatment having being suggested to assist in the re-assembly of the cytoskeleton. Encouraging results have been achieved with Q. robur (Berjak et al. 1999), T. dregeana (Berjak and Mycock 2004) and E. capensis (unpublished results). The consequences of rehydration in the Ca2+/Mg2+ solution have been analysed in detail for T. dregeana, where normal starch deposition and disposition of the actin cytoskeleton in root cap columella cells have been correlated with downwards curvature of the roots and their growth into the germination medium (Berjak and Mycock 2004).

If the ultimate objective is to cryopreserve material for subsequent planting out, this material has to be transported to, and re-established at, the site where it is required. Transporting frozen material, or established seedlings presents difficulties, and facilities for re-establishing frozen axes may not be available. Ideally, one would like to be able to ship the material in a state that can be readily planted out. To this end we have currently had preliminary success with gel encapsulation of axes retrieved from cryostorage in a manner akin to the ‘synthetic seeds’ produced by encapsulating somatic embryos (Bajaj 1995).

Although for most ‘recalcitrant species’, the embryonic axes are very small (of the order of 1–3mm long), and constitute only an insignificant fraction of the mass and volume of the seed, this is not invariably the case. At the other extreme, when shed, the large seed of Barringtonia racemosa (a species of tree common upstream of the estuary along the eastern coast of sub-tropical and tropical Africa) consists solely of an hypertrophied axis, in which root and shoot meristems are not yet differentiated (Berjak et al. 1996). Further, although less extreme examples of recalcitrant axes which are too large to be flash-dried and rapidly frozen, include those of cocoa (Theobroma cacao) and the valuable yellow-wood, Podocarpus henkelii (personal obser-
vations). In such cases — and in others where although small, the zygotic embryonic axis proves to be impossible to manipulate for cryostorage — alternative explants must be developed. While these might be shoot meristems or apices, axillary buds, somatic embryos or embryogenic callus, all require additional, preliminary steps before cryopreservation can be attempted — i.e. the successful generation of growing plants from the explants. As observed above, even when excised zygotic axes provide an ideal means for germplasm regeneration, the first step in establishing the protocol for an individual species, is the development of in vitro conditions facilitating normal plant production.

Although the general guidelines for cryopreservation of recalcitrant seed material (partial drying followed by ultra-rapid cooling) have been established, the details of the protocol to be determined on a species-by-species basis. The ultimate objective of our studies is to increase our understanding of the processes involved, so that cryopreservation protocols become more predictive and less empirical in nature. Considering the increasing number of tropical and sub-tropical species that are being revealed to have non-recalcitrant seeds, development of such cryopreservation protocols is of great practical importance in Africa.

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