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Translating theory into practice for conservation of germplasm of recalcitrant-seeded species

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ABSTRACT
Recalcitrant seeds, which are metabolically-active and desiccation-sensitive, can be stored in the short-term at non-injurious temperatures under conditions precluding dehydration, but associated problems include fungal proliferation and initiation of germination. The only means for long-term conservation of the germplasm of recalcitrant-seeded species is by cryopreservation in liquid nitrogen (LN), which is not possible for intact seeds. The means to achieve this – along with attendant problems which are gradually being overcome – constitute the main subject matter of this paper. Hence, excised embryonic axes (or occasionally, embryos) are the ideal explants to be cryopreserved. As *a priori* requirements, however, a non-injurious decontamination procedure, and a suitable medium for *in vitro* germination of the axes must be developed. Thereafter, preparative procedures to condition axes for LN immersion must be refined, implemented and their possible impact on axis viability ascertained at each stage. These include: assessing the effects of cryoprotectants, to which axes of some species are intolerant and determining the responses of axes (whether cryoprotected or not) to flash drying, which involves ascertaining the least amount of water to be very rapidly removed to facilitate cooling to cryogenic temperatures without lethal ice formation. Further pivotal parameters to be optimised are the rate of cooling to cryogenic temperatures and the nature of the medium used for re-warming (thawing) and rehydration of explants after cryopreservation. Each step in the cryopreservation protocol, including excision, is accompanied by generation of free radicals/reactive oxygen species (ROS), the effects of which can be lethal – and particularly because the consequent damage is cumulative. Hence, for successful cryopreservation, it is imperative that means are developed – as discussed – to counteract ROS-mediated damage.

Keywords: Cathodic water, cryopreservation, desiccation-sensitive seeds, embryonic axes, reactive oxygen species, ROS-mediated damage.

De la teoría a la práctica en la conservación de germoplasma de especies con semillas recalcitrantes

RESUMEN
Las semillas recalcitrantes, que son metabólicamente activas y sensibles a la desecación, se pueden almacenar a corto plazo a temperaturas no perjudiciales en condiciones que impidan la deshidratación, pero tiene como problemas asociados la proliferación de hongos y la iniciación de la germinación. El único medio para la conservación a largo plazo del germoplasma de especies recalcitrantes es mediante la crioconservación en nitrógeno líquido (NL), lo cual no es posible con semillas intactas. Por lo tanto, los ejes embrionarios (o en ocasiones los embriones) son los explantes ideales para ser crioconservados. Como requisitos *a priori*, se requiere de un procedimiento de descontaminación no perjudicial, y un medio de cultivo adecuado para la germinación *in vitro* que deben ser desarrollados. A partir de entonces, los procedimientos de preparación para acondicionar ejes para inmersión en NL deben ser refinados, implementan y su posible impacto en la viabilidad eje determinarse en cada etapa. Estos incluyen: la evaluación de los efectos de los crioprotectores, a la que los ejes de algunas especies son intolerantes y la determinación de las respuestas de los ejes (si cryoprotected o no) de flash de secado, lo que implica la determinación de la menor cantidad de agua a ser muy retirado rápidamente para facilitar la refrigeración de temperaturas criogénicas sin formación de hielo letal. Parámetros más fundamentales para ser optimizados son la velocidad de enfriamiento a temperaturas criogénicas y la naturaleza del medio utilizado para re- calentamiento (descongelación) y la rehidratación de los explantes después de la crioconservación. Cada paso en el protocolo de crioconservación, incluyendo escisión, está acompañada por la generación de radicales libres / especies reactivas de oxígeno (ROS), los efectos de que puede ser letal - y en particular debido a que el daño consiguiente es acumulativo. Por lo tanto, para la crioconservación con éxito, es imperativo que se desarrollen medios - como se discute - para contrarrestar el daño mediado por ROS.

Palabras clave: agua catódica, crioconservación, daño mediado por ROS, ejes embrionarios, especies reactivas del oxígeno, semillas sensibles a desecación.

CONTENT

INTRODUCTION

Although orthodox (desiccation tolerant) seeds appear to be produced by the great majority of species for which categorisation has been done, recalcitrant seeds are produced by a significant number – particularly among tropical and sub-tropical forest trees (Tweddle *et al.*, 2003; Sacandé *et al.*, 2004). Unlike the orthodox condition which is characterised by virtual cessation of metabolism accompanied by intracellular de-differentiation, recalcitrant seeds are always metabolically active, with the cells of the embryos/embryonic axes remaining differentiated (Farrant *et al.*, 1997; Pammenter and Berjak, 1999; Berjak and Pammenter, 2004; Berjak and Pammenter, 2008). As a consequence of their metabolically-active state, and because they either do not have – or do not express – the protective mechanisms which enable the acquisition of desiccation tolerance, recalcitrant seeds remain sensitive to desiccation during development and after they are shed or harvested (Pammenter and Berjak, 2013). Furthermore, as long as they remain as hydrated as when they are shed, germination is initiated without the requirement of an external source of water, and is accompanied by an increasing degree of desiccation sensitivity (Berjak *et al.*, 1989).

Under natural conditions in humid mesic environments, recalcitrant seeds should initiate germination and establish seedlings rapidly. However, under less favourable conditions, unless the seeds are characterised by coverings that significantly retard water loss (e.g. the South African cycad, *Encephalartos natalensis* [Woodenberg, 2009]) dehydration will ensue. As a consequence of relatively slow water loss, and, extrapolating from our laboratory experiments (e.g. Pammenter *et al.*, 1998), recalcitrant seeds of most species will have lost viability by ~0.8 g g^{-1} (g water g^{-1} dry mass of seed tissues). Alternatively, as recalcitrant seeds are hydrated and consequently succulent, after shedding they have been found to harbour a wide spectrum of fungi (Sutherland *et al.*, 2002) and are also extensively predated. Additionally, as is the case in South Africa, many recalcitrant-seeded species are among those extensively harvested for traditional medicinal purposes, and are further threatened by habitat loss (Berjak, 2005). All-in-all therefore, there can be no argument against the compelling case for *ex situ* conservation of the germplasm of recalcitrant-seeded species.

Long-term germplasm conservation of the germplasm of such species can be achieved only by cryopreservation (Engelmann 2011a; Walters *et al.*, 2008; 2013), and the means to achieve this – along with attendant problems which are gradually being overcome – constitute the main subject matter of this paper. However, for practical purposes, short-term storage of recalcitrant seeds is a co-requirement, and is initially considered.

HYDRATED STORAGE OF RECALCITRANT SEEDS IN THE SHORT- TO MEDIUM-TERM

It is necessary to consider the purpose for which recalcitrant germplasm is to be stored. If planting material is required for use soon after the seeds have been harvested, then every attempt needs to be made to optimise the

means to store them intact (or with only the pericarp and/or testa removed). A second reason, for which the seeds need to be stored, is for their use in experiments to determine their characteristics and post-harvest responses, which often show marked variability – not only across species – but also inter- and intra-seasonally (Berjak and Pammenter, 2004). Thirdly, short-term seed storage is important for the preliminary work which is necessary to establish the parameters applicable to development of a cryostorage protocol for the embryonic axes or embryos. However, consideration of several factors is necessary to optimise short-term storage: these (and detailed information about explant cryopreservation) were commissioned by, and are now embodied in, a draft document on genebank standards (FAO, 2013).

Seeds should ideally be harvested directly from the mother plant, as if they have been shed, they (or the fruits) will have almost invariably become contaminated by soil-borne micro-organisms (Sutherland *et al.*, 2002). Also, it is often difficult to ascertain for how long material has been on the ground – and in this context seeds to be stored must be in the best possible condition: after shedding, some drying may have occurred which probably will compromise storage life span (once collectors become familiar with a species, it is likely that they would be able to identify newly-shed fruits or seeds from any that fell earlier). Whether or not the material was harvested directly or after being shed, it is imperative that fruits be decontaminated and, as soon as the seeds are extracted and cleaned of any adherent fruit pulp, they too need to be surface-decontaminated. While the most widely-used surface sterilants are 1% solutions of sodium hypochlorite (NaOCl), calcium hypochlorite ($Ca[OCl]_2$), or 0.1% mercuric chloride ($HgCl_2$), we have found 0.03% sodium dichloroisocyanurate (NaDCC), which has low phyto-toxicity, to be superior, as reviewed by Barnicoat *et al.* (2011). As a caveat, all physically damaged seeds (the damage often having been caused by insects or other animals), or any showing discoloration – which could be indicative of fungal activity – must be discarded.

It is imperative to maintain saturated relative humidity (RH) in the storage containers to prevent even slight dehydration of the seeds – which is known to stimulate germination thus shortening storage life span – while more severe dehydration will rapidly impact on vigour and viability of the desiccation-sensitive seeds (Pammenter and Berjak, 2013). To facilitate hydrated storage, we generally use sealing plastic buckets with sterile paper towel saturated with a 1% NaOCl solution in the base, suspended ~100 mm above which is a pre-sterilised plastic grid on which the seeds are to be placed – preferably in a monolayer. After decontamination for brief period surface moisture must be removed, the seeds dusted with a broad-spectrum fungicide and then placed in storage. Before sealing the containers, absorptive material – such as a few sheets of dry, sterile paper towel – should be inserted inside the lid to absorb condensate. Because recalcitrant seeds of tropical/sub-tropical species may be chilling-sensitive, the storage containers should then be maintained at the lowest temperature seeds of individual species will tolerate, which needs to have been previously ascertained.

However effectively surface contaminants may have been removed, there remains the serious problem of internally-borne fungal inoculum, which is prevalent particularly in the tropics and sub-tropics (Sutherland *et al.*, 2002). If such contamination is confined immediately below the pericarp or testa, then removal of these coverings and 'sub-surface' decontamination will be effective, after which the seeds could be encapsulated with alginate gel incorporating a fungicide (Motete *et al.*, 1997). Deeper-seated fungal inoculum must be effectively removed or its proliferation will rapidly degrade the hydrated-stored seeds. For this, pre-storage treatment with appropriate systemic fungicides could be effective – as long as these have no adverse effects on the seeds (FAO, 2013). Alternatively, a mixture of fungicides as appropriate to the contaminating species could be periodically applied as an aerosol spray to the seeds during hydrated storage (Calistru *et al.*, 2000). As a caveat, if contaminating fungi, which elaborate antibiotics, are able to be eliminated, then any seed-associated bacteria are likely to proliferate. This becomes a problem particularly when embryonic axes/embryos, which need to be cultured *in vitro*, are to be cryopreserved (see later). Therefore, bactericidal treatment may be necessary: we have found exposure of the seeds to Hibitane™

(available as a 5% w/v solution of chlorohexidine gluconate) and/or dusting with neomycin sulphate to be effective.

It is imperative that the seeds be periodically inspected during hydrated storage, and any showing fungal proliferation be removed and destroyed, while those remaining is again decontaminated (FAO, 2013). However, where widespread contamination of the seeds becomes apparent, the whole accession should be destroyed (by incineration or autoclaving) even if re-collection means waiting for the next season. In concluding this section, it needs to be emphasised that the seed-associated microflora pose one of the most severe problems for recalcitrant germplasm conservation – be this as seeds, or for cryopreservation as embryonic axes/embryos.

SEED-DERIVED EXPLANTS AND CRYOSTORAGE

Preliminary requirements

Cryostorage is generally carried out in liquid nitrogen (LN), which requires that specimens be very small, and able to be rapidly dehydrated and cooled to cryogenic temperatures (Pammenter and Berjak, 2013). However, almost invariably intact recalcitrant seeds are far too large and too hydrated to be cryostored. Hence, embryos excised with part or all of the cotyledon from endospermous seeds may be used (e.g. seeds of *Landolphia kirkii*, a dicot [Kistnasamy *et al.*, 2011], or of the monocotyledonous Amaryllidaceae [Sershen *et al.*, 2012a; b]), but in all cases where the cotyledons are fleshy they must be removed, leaving only the embryonic axis as the explant (see below). In developing a cryopreservation protocol, it is imperative that the effect of each step is assessed in terms of retention of vigour and germinability – and the ability for seedling establishment (Fig. 1). As excision of either embryos or embryonic axes removes them from the nutrient supply which would normally be mobilised for germination and seedling establishment, it is imperative that a medium for *in vitro* culture of explants is developed in advance of all procedural trials. In general, the first medium to be assessed is MS-based (Murashige and Skoog, 1962), but whether the MS components are incorporated in the agar at full-strength or half-strength (or less) for optimal development of the explants often needs to be established. In cases of woody species, a better medium may be WPM (Woody Plant Medium [Lloyd and McCown, 1980]). In some cases, additives to media, such as activated charcoal which adsorbs phenolics if exuded by the explants, may improve their development *in vitro*. The best medium formulation – and the optimal conditions of lighting and photoperiod (following initial culturing of explants in the dark to avoid photo-oxidative damage [Touchell and Walters, 2000]), and of temperature – all need to be established before any objective assessment of the effects of subsequent manipulations for, and following, cryopreservation is possible.

Successful explant culture demands that fungal (and bacterial) inoculum has been eliminated. In parallel with the trials to optimise the culture medium and conditions, therefore, the effects of various sterilants on the explants must be ascertained. As described for decontamination of the seeds themselves, the impact of NaOCl, $Ca(OCl)_2$, $HgCl_2$ and NaDCC should be assessed in terms of both the elimination of contaminants and, importantly, of non-injury of the explants. While NaOCl has generally been the sterilant most commonly used (with $Ca(OCl)_2$ and $HgCl_2$ being tested when NaOCl has proved injurious), we would now advocate that the efficacy of NaDCC be tested in the first instance.

It must be emphasised, however, that medium formulation, decontamination and culture conditions frequently need to be modified for explants of different species.

Selection of the optimal stage of seed development

The development of recalcitrant seeds is on-going, and germinative metabolism is imperceptibly initiated without the 'punctuation' of the dry state which occurs in orthodox seeds. Just as the sensitivity of recalcitrant seeds to desiccation changes with physiological status (Pammenter and Berjak, 2013), so do the responses of the embryos/embryonic axes to the procedures involved in cryopreservation. For example, a study on a spectrum of amaryllid seeds showed that initiation of germination is accompanied by both an increase in desiccation sensitivity of the embryos and a

diminishing ability to survive cryogen exposure (Sershen *et al.*, 2008). In contrast, a far better ability for seedling production by axes of *Strychnos gerrardii* was obtained when they were excised from seeds which had been in hydrated storage for 35 d, compared with those which had not been stored (Berjak *et al.*, 2011). Recalcitrant seeds across species may be shed at a stage when considerable development is still necessary for the natural initiation of germination, or, at the other extreme, when germination follows immediately (Pammenter and Berjak, 2013). While it is not possible to predict the developmental stage at which embryos/embryonic axes should be excised on a species basis, it is conjectured that this would be at the point just prior to incipient germination, at which they would be relatively least sensitive to desiccation. However, it is vital to understand the physiology in order to establish the optimal developmental stage of seeds of individual species in the context of embryo or embryonic axis responses to cryo-procedures, and this can be accomplished only by experimentation.

Explant excision – understanding the nature of, and minimising, associated injury

Explant excision from surface-decontaminated seeds – which is always carried out under sterile conditions in a laminar air-flow – must be done with the utmost care to minimise physical injury. Excision of entire embryos – as in the case of the amaryllids (Sershen *et al.*, 2012a; b) – does not appear to be particularly injurious, possibly because no physical continuity exists between the single cotyledon and the endosperm. However, in the absence of ameliorative treatment, similar separation of embryos from the endosperm of *Strychnos gerrardii* – precluded subsequent shoot development (Goveia, 2007) unless ameliorative treatment was applied (Berjak *et al.*, 2011). This provides a further example of how difficult predicting – or explaining – the responses of explants to particular manipulations can be.

In the case of embryos with fleshy cotyledons, it is necessary ultimately to remove all the latter tissue, leaving just the axis as the explant (Fig. 1). This is done to minimise explant size, in the interest of achieving the most rapid drying and cooling rates (see below). Despite use of appropriate instruments – e.g. very fine, pointed scalpel blades or hypodermic needles (Benson *et al.*, 2007) – an enduring problem with tropical/sub-tropical species has been necrosis of the axis shoot apical meristem (Goveia *et al.*, 2004; Perán *et al.*, 2006; Hajari *et al.*, 2011) or callus formation (Engelmann, 2011b). While much of the earlier work simply recorded the lack of ability for shoot development after explants were retrieved from cryopreservation, more recently it has been shown that potentially lethal damage of the shoot apical meristem accompanies virtually all the cryo-procedures, starting with explant excision. Goveia *et al.* (2004) were the first to suggest that necrosis of the shoot apical meristem could be the result of an injury-related burst of ROS initiated by severing the cotyledonary attachments, which has since been shown to be the case (Whitaker *et al.*, 2010; Berjak *et al.*, 2011). Thus a concerted effort has been made to implement anti-oxidant treatments in conjunction with axis excision (Fig. 1).

In this regard, Naidoo *et al.* (2011) developed a procedure involving primary excision of the axes with small cotyledonary segments attached, and immediately transferring these explants to a semi-solid MS-based culture medium incorporating 1 ml l^{-1} of the powerful anti-oxidant, dimethyl sulphoxide (DMSO/Me_2SO) with the shoot apices immersed in the medium. After 6 h incubation in the dark, explants were individually immersed in a 1% (v/v) aqueous solution of DMSO for complete removal of the cotyledonary tissue, after which the axes were submerged in the DMSO solution for a further 30 min. These studies were conducted on explants of three recalcitrant-seeded species, for which it was found that the post-excision soaking was efficacious for two, but not for the third (Naidoo *et al.*, 2011): however, in all three cases, a significant proportion of the axes retained the ability for shoot production – which had never previously been achieved.

Injury- (stress-related) bursts of ROS are, however, not confined to the excision step. In fact, evolution of ROS has been shown to accompany each of the procedures necessary for explant cryopreservation – including the final step itself, *viz.* retrieval from LN followed by re-warming and rehydration (Whitaker *et al.*, 2010; Berjak *et al.*, 2011; Naidoo, 2012). As we were reluctant to use DMSO in subsequent procedures in view of the potential cyto-toxicity

of its accumulation (despite its being a component of plant vitrification solution 2, PVS2 [Sakai *et al.*, 1990]), we developed a novel procedure using the powerful reducing capacity of cathodic water to ameliorate ROS-mediated damage (Berjak *et al.*, 2011). We prepare cathodic/reducing water by electrolysis of a solution of 0.5 µM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$ (Ca/Mg) in distilled water. (This is the non-electrolysed Ca/Mg solution that we have previously used in most of our cryo-procedures, which was first developed by Mycock [1999] as a medium for cryoprotectants which enhanced the quality of the explants retrieved from cryostorage.)

In developing the cryo-protocol for axes of a recalcitrant-seeded species, *Strychnos gerrardii*, which were not cryo-protected, we tested the effects of cathodic water after excision, following flash drying (Fig. 1), and for thawing and rehydration following retrieval from LN (Berjak *et al.*, 2011). At all these procedural stages, use of cathodic water strongly promoted shoot development as evidenced by seedling establishment. Note that once a protocol for cryopreservation of axes of a particular species has been successfully established, rehydration after flash drying would not be carried out. However, when developing and optimising the protocol, it is necessary to assess the potential of each step in terms of damage or lack thereof by *in vitro* germination and the potential for seedling establishment, as indicated in figure 1.

Decontamination of explants

Once a cryopreservation protocol has been established, explants would not normally be decontaminated after excision – but only when they have been rehydrated after retrieval from LN (see later). However, *in vitro* culture of explants – be they embryos or axes – is necessary after each procedural stage during *development* of a cryo-protocol (Fig. 1), as it is imperative to be able to identify manipulations having deleterious effects in order to introduce modifications to counteract resultant damage. As any fungal contaminants will rapidly proliferate when the axes are incubated *in vitro*, it is necessary to apply the most effective, non-injurious decontamination regime for the shortest time possible, and the recommendation is in the first instance to use the procedure found to be best for decontamination of the seeds (see Preliminary requirements, above). In certain cases, fungicide incorporation in the culture medium might also be necessary. It should be noted, however, that once fungal contaminants and their antibiotic effects are eliminated if there is any bacterial inoculum associated with the explants then bacteria will proliferate. In such cases as discussed above, bactericidal treatments need also to be developed. Nevertheless, there are instances where the problems posed by explant-associated microbial inoculum are not able to be overcome within any one season, which will necessitate postponing trials. In this regard, the quality of recalcitrant seeds – and hence their embryos/embryonic axes – is variable both intra- and inter-seasonally. After a 'bad year', seeds harvested in a following season may well be of excellent quality. As a *caveat* in all cases, however, seeds collected towards the end of a season have often been found to be of far poorer quality – and considerably more contaminated – than those harvested earlier (Berjak and Pammenter, 2004).

Provision of cryoprotectants – a procedure which can be efficacious – or deleterious

The aim of exposure of explants to chemical cryoprotective substances, some of which penetrate the tissues while others do not, is to concentrate cellular contents to the extent that on (or even prior to) cooling to cryogenic temperatures there is the potential for homogeneous intracellular solidification without the formation of crystalline ice. This is known as vitrification or formation of the glassy state. Non-penetrating cryoprotectants act by osmotic removal of water, thereby increasing intracellular solute concentrations (Panis and Lambardi, 2005). This category of cryoprotectants, none of which penetrates the tissues, includes sucrose and polyethylene glycol: in contrast, penetrating cryoprotectants, e.g. DMSO and glycerol, not only enter the explant tissues, but cross the plasmalemma (plasma membrane/cell membrane) thus acting intracellularly (Benson, 2008a). Penetrating cryoprotectants can act as solutes, increasing the concentration of intracellular particles which

depresses (lowers) the freezing point of water, but also act as solvents for small solutes such as electrolytes, preventing high, potentially toxic, concentrations building up as water is lost (Mazur, 2004). There are also cryoprotective substances which are taken up by explants, but penetrate the cell walls only, e.g. proline, mannitol, oligosaccharides and low molecular weight polymers (Tao and Li, 1986; Benson, 2008b): in effect, these would act as non-penetrating cryoprotectants. Generally, cryoprotectant solutions include both non-penetrating and penetrating components (Panis and Lambardi, 2005).

While the effects of cryoprotectants should dehydrate explant cells and increase intracellular viscosity non-injuriously, we have documented several cases where cryoprotection has proved to be deleterious. Axes of *Landolphia kirkii*, were excised with segments of both thin cotyledons which circumvented excision-related injury. However, after exposure to cryoprotectants (sucrose, glycerol and DMSO singly and in all combinations) none survived the further evaporative dehydration which was necessary to achieve water contents facilitating cryogen exposure, however without cryoprotectant treatment, 70% of axes remained viable after flash drying to a mean water content of 0.28 g g^{-1} (Kistnasamy *et al.*, 2011). Work on axes of various other tropical/sub-tropical recalcitrant-seeded species has also revealed problems. For example, while axes of *Lansium domesticum* survived cryoprotectant pre-culture on an MS-based medium incorporating 0.3 M sucrose followed by vitrification with PVS2 (see below), subsequent exposure to LN was lethal (Normah *et al.*, 2011). Those authors also reported lack of seedling production after cryopreservation of another Malaysian species, *Baccaurea motleyana*, where axes were pre-cultured on a medium incorporating DMSO and sucrose and then treated with 60% PVS2. In work with a spectrum of amaryllid species, cryoprotection (using glycerol and sucrose alone or in combination) improved axis survival after cryogen exposure for 10 of the 15, species, but was deleterious to four others, while making no difference to survival in the case of one species (Sershen *et al.*, 2007). Such observations are presently difficult to explain, but require further investigation as it is important to understand the conflicting advantages and disadvantages of similar treatment of axes of different recalcitrant-seeded species, even of the same family. What may provide a valuable clue in cases where cryoprotection is actually damaging, comes from the work of Naidoo (2012) on axes of *Trichilia dregeana* where none survived cryoprotection when the solvent was distilled water. However, when the mixture of DMSO and glycerol was made up in cathodic water, both a root and a shoot were produced by 40% of the axes. This argues that, at least for axes of the tropical/sub-tropical species, *T. dregeana*, application of the cryoprotectants when used conventionally was accompanied by lethal ROS generation – a deduction supported by a significant reduction in accumulation of the ROS, hydrogen peroxide, recorded when the solvent was the highly reducing cathodic water (Naidoo, 2012).

Cryoprotectants act to lower explant water content. However, further dehydration by physical evaporative means (see below) may well be necessary to facilitate survival of cryogen exposure (Fig. 1).

Vitrification-based methods – extensions of the principles of cryoprotection

'Classical' cryopreservation techniques rely on slow cooling to a pre-defined temperature, during which ice forms in the external medium, with the cells becoming increasingly dehydrated as water migrates from the cells and freezes extracellularly: thus dehydration is freeze-induced (Engelmann, 2011a). In contrast, in the newer protocols, dehydration is carried out by the use of cryoprotectants as vitrification media and/or physical evaporative drying prior to cooling (Fig. 1). These procedures are aimed at achieving the glassy state and have been developed primarily for cryopreservation of shoot apices and other vegetative explants (Sakai and Engelmann, 2007; Engelmann, 2011a). As discussed above, problems have been encountered when cryoprotectants or vitrification-based procedures have been applied to recalcitrant embryonic axes, but some successes have also been reported, as documented below in the context of the procedure used.

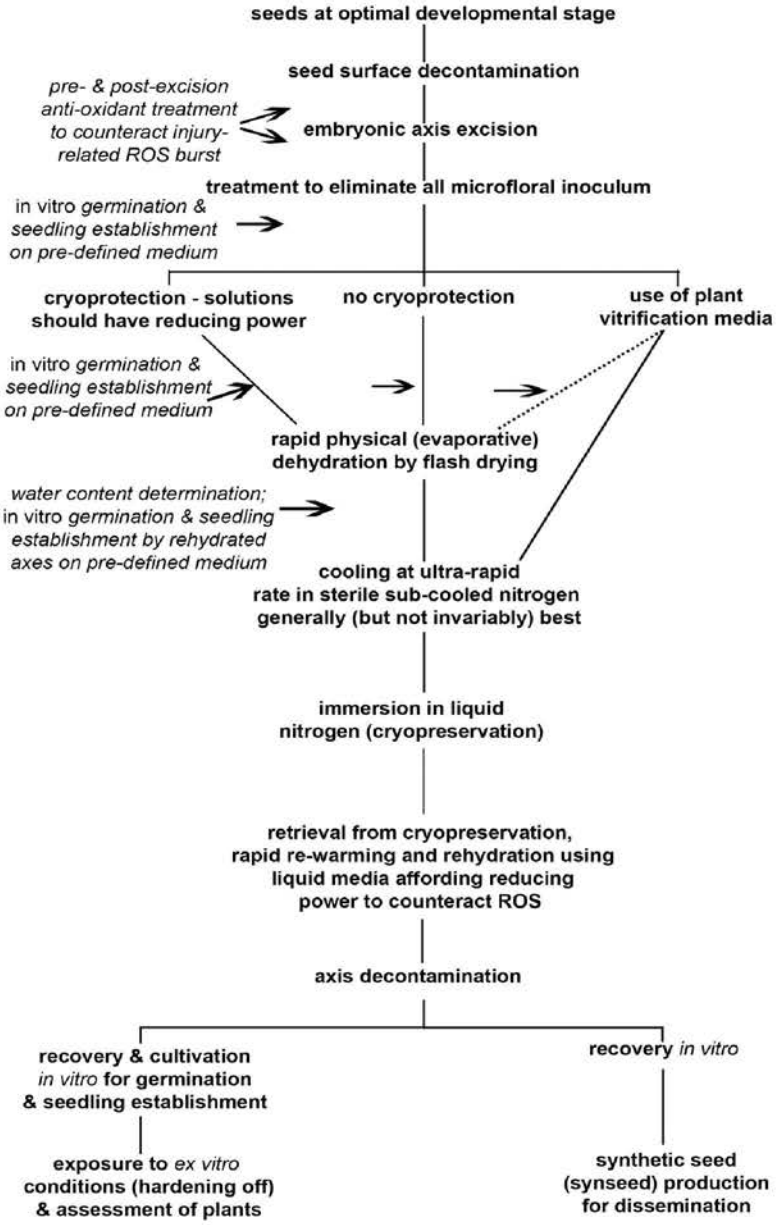

Figure 1. The successive steps which are involved in developing a protocol for cryopreservation of embryonic axes are documented. The italicized text on the left indicates assessements which must be carried out at each stage (arrowed) to ascertain whether or not the manipulation has had adverse effects and therefore requiring to be amended. Once a protocol has been successfully established, then assessment of individual steps falls away.

Dehydration by flash drying

Besides the chemi-osmotic procedures involving cryoprotectants to enable vitrification as outlined below, one means to lower axis water contents to levels which should facilitate survival of cryogenic cooling, is physical (evaporative) dehydration by flash drying (Fig. 1). Early in our work on the effects of dehydration on recalcitrant seeds, embryos and axes, we discovered that the more rapidly dehydration could be achieved, the greater was the

proportion of water that could be removed without lethal effects – at least in the short term (Berjak *et al.*, 1990). This led to our developing the flash drying technique (reviewed by Pammenter *et al.*, 2002) which enables rapid dehydration of explants to water contents that could facilitate intracellular vitrification upon cooling to cryogenic temperatures. In minimising the time taken for dehydration, what is effectively achieved is minimising the *time* during which potentially lethal metabolism-linked damage occurs and accumulates (Pammenter *et al.*, 1998; Pammenter and Berjak, 1999; 2013; Walters *et al.*, 2001). Followed immediately by cryogenic cooling, flash drying even in the absence of cryoprotection has enabled successful cryopreservation of axes of a spectrum of recalcitrant-seeded species: however, an *a priori* requirement is that the extent of flash drying tolerated (in conjunction with the cooling rate used) be ascertained per species (Berjak and Pammenter, 2008). Ideally, the least amount of water to be removed which will enable explant survival of cryogen exposure needs to be established. As this is likely to vary if cryoprotectants have been used, in trials aimed at developing cryo-protocols the flash-drying time course in the context of viability retention needs to be established both before and after cryoprotection. While recourse to flash drying alone as a means of dehydration is imperative in cases where explants are lethally damaged by cryoprotectants, further water loss by this means can be used in conjunction with other vitrification techniques, e.g. encapsulation-dehydration and pregrowth-dehydration (see below).

Encapsulation-dehydration

This procedure involves encapsulating explants in alginate 'beads' which are then pre-grown in a sucrose-enriched liquid medium for periods up to 7 d, after which they are dried in a laminar air-flow – or by flash drying – to the required water content and then cooled very rapidly (Engelmann, 2011a). This technique has proved successful for embryos of peach palm, *Bactris gasipaes* (Steinmacher *et al.*, 2007; and those of tropical *Ilex* species, Mroginski *et al.*, 2008).

Vitrification

This approach, which ultimately exposes the explants to a highly concentrated vitrification solution, e.g. PVS2 (Sakai *et al.*, 1990), requires that they first be pre-cultured on a medium incorporating cryoprotectants. This is followed by a loading step involving explant exposure to a solution which is less concentrated than PVS2, to pre-condition them osmotically. While the loading solution comprises 2 M glycerol and 0.4 M sucrose, PVS2, which is 7.8 M, is made up of 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (Sakai and Engelmann, 2007). There has been some success with the use of PVS2 for cryopreservation of embryos of *Castanea sativa, a* temperate recalcitrant-seeded species (San José *et al.*, 2005), and those of *Sterculia cordata*, described as being intermediate/recalcitrant-seeded (Nadarajan *et al.*, 2007). In this regard, however, it needs to be emphasised that successful cryopreservation of explants from temperate recalcitrant and intermediate seeds generally appears to be easier to achieve, than when similar procedures are applied to embryos/axes from seeds of tropical/sub-tropical origin which have been unequivocally established as being recalcitrant. While vitrification as described here has not generally been successful for zygotic embryos/axes of tropical/sub-tropical species established as being recalcitrant-seeded (Normah *et al.*, 2011), an exception is provided by Thammasiri (1999), who reported that ~50% of axes of jackfruit (*Artocarpus heterophyllus*) treated with PVS2 produced seedlings after cryopreservation. Variations of plant vitrification solutions have been developed in cases where use of PVS2 was deleterious: for example, Nishizawa *et al.* (1993) developed PVS3, which does not contain DMSO.

Encapsulation-vitrification

This combines the benefits of encapsulation-dehydration and vitrification (Sakai and Engelmann, 2007). However, zygotic embryos/ embryonic axes were not included among the successes using this procedure as reported by those authors.

Pre-growth

In this procedure, explants are cultured on media in which cryoprotectants are incorporated, after which they are very rapidly cooled. The technique, which was developed for banana meristems which were pre-cultured

for two weeks on a medium incorporating 0.4 M sucrose (Panis *et al.*, 2002), does not seem to have found application for recalcitrant zygotic embryos/axes, unless complemented by evaporative dehydration, as described next.

Pre-growth–dehydration

Zygotic embryos of coconut (*Cocos nucifera*), a tropical recalcitrant-seeded species, have been successfully cryopreserved using this technique (Engelmann, 2011a). Sisunandar *et al.* (2010), who, however, did not use a pre-growth step, reported successful cryopreservation of zygotic embryos of particular cultivars of *C. nucifera* following optimisation of rapid dehydration (flash drying), rapid cooling and re-warming rates and recovery *in vitro*.

Droplet-freezing

This procedure, whereby explants which have been exposed to liquid cryoprotectant solutions are rapidly cooled in tiny droplets of the cryoprotectant medium on aluminium foil, has been successfully used to cryopreserve shoot apices (Engelmann, 2011a). Whether or not the method could be applied to zygotic embryos/axes is presently not known.

Droplet-vitrification

Using this technique, explants are exposed to loading and vitrification solutions as described above, and then cooled rapidly in minute droplets (5-10 µl) of PVS2 on aluminium foil (Sakai and Engelmann, 2007). As pointed out above, PVS2 may have deleterious effects on explants: thus Kim *et al.* (2009a; b) formulated alternative loading and vitrification solutions for droplet-vitrification procedures of explants which did not tolerate either PVS2 or PVS3. Again, however, the studies focused on vegetative explants (of garlic and chrysanthemum).

Cryopreservation using most of the chemi-osmotic vitrification-based protocols has been focused on shoot apices/meristems, with the applicability of these procedures to zygotic germplasm of recalcitrant-seeded species seemingly being limited. In comparison with apices and meristems in which most of the cells are essentially compact and not highly vacuolated, embryonic axes comprise a variety of tissues, all of which are unlikely to react in the same way to chemi-osmotic vitrification. This may well impose a major restriction in the use of the vitrification-based protocols for embryos/axes – as presently interpreted from the sparse number of reports on their successful cryopreservation by means other than rapid evaporative dehydration either following pre-growth and/or direct exposure to cryoprotectants, or not (Fig. 1). However, modification of chemi-osmotic vitrification methods to render them suitable for explants derived from recalcitrant seeds may ultimately yield positive results.

Cooling to cryogenic temperatures

The responses to, and optimisation of, cryogenic cooling cannot be confidently undertaken until one is assured that all the procedures to this point have been optimised as demonstrated by germination and seedling establishment by most of the explants (Fig. 1). In general (but not without exception [see below]) – and particularly in the context of the vitrification-based procedures – ultra-rapid (non-equilibrium) cooling is advocated. The most rapid cooling rate to be achieved based on the use of LN, is to plunge explants (whether naked or encapsulated or within droplets of cryoprotectant including PVS2) into what is called nitrogen slush. This is prepared by applying a slight vacuum to LN in, e.g. a polystyrene (styrofoam) cup, within a desiccator. The temperature of nitrogen slush, -210°C, avoids the boiling of LN which occurs at -196°C thus forming a nitrogen gas bubble around the explant which retards the cooling rate. However, a *caveat* is necessary: liquid nitrogen, which is made from atmospheric nitrogen, is generally contaminated by fungal and bacterial spores, with the result that, after retrieval from cryopreservation, explant culture *in vitro* is accompanied by fungal and/or bacterial proliferation. We recommend that the LN to be used for cooling be poured into an insulated polystyrene container and then exposed to ultra-violet irradiation for around 15 min prior to use – whether as nitrogen slush or as LN (see below).

Cooling rate has emerged as another variable which will affect the success or failure of cryopreservation. While cooling of naked

explants is most rapid in nitrogen slush, plunging them into LN (at -196°C) is more rapid than the rate achieved if explants are enclosed in aluminium foil envelopes held with pre-cooled forceps and plunged into LN, which is, in turn, more rapid that the rates achieved if they are enclosed within cryotubes. If required for experimental purposes, assessment of cooling rates can be made using a thermocouple (75 µm diameter wire and *c.* 280 µm bead size) as described by Wesley-Smith *et al.* (2004).

While explants which have been pre-treated with a vitrification solution – e.g. PVS2 – are cooled in a small volume of that solution (~0.5 ml) within cryotubes (Sakai and Engelmann, 2007), naked axes (or embryos) cooled in nitrogen slush or LN must subsequently be contained for cryopreservation. Our practice is to use a shallow polystyrene tray containing UV-sterilised LN into which the explants are introduced and then to transfer them under the LN to pre-cooled cryotubes. The tubes are then capped loosely to counteract pressure build-up while they are being clipped into cryocanes which are then rapidly immersed into LN in the cryovat (cryostorage container). At this stage, the tubes are capped more tightly.

For recalcitrant-seeded species, although we have generally found cooling in nitrogen slush to be the best in terms of promotion of the ability for seedling formation after explant retrieval from cryopreservation, this has not invariably been the case. As an isolated example, survival of the lipid-rich recalcitrant axes of *Landolphia kirkii* (which did not tolerate cryoprotection, as mentioned above) was best when cooling at 1°C min^{-1} to -70°C was employed (Kistnasamy *et al.*, 2011) – for reasons that must still be ascertained. However, after removal of the testa, good survival of intermediate seeds of *Swietenia macrophylla* after slow cooling was reported, noting that in this case seed water content was in the region of 0.05 g g^{-1} (Normah *et al.*, 2011). Parallel results were obtained for intermediate lipid-rich seeds of *Coffea arabica* which were slowly cooled before being exposed to LN (Dussert and Engelmann, 2006).

Even in the case of recalcitrant embryos or embryonic axes, there is a relationship between explant water content (which necessarily must remain considerably higher than is the case for intermediate seeds/explants) and cooling rate. Generally, if sufficiently low water contents can be attained without compromising viability, explants will tolerate a broad range of cooling rates as shown for axes of *Poncirus trifoliata* having 0.26 g water g^{-1} dry matter (Wesley-Smith *et al.*, 2004). There is an upper water content limit above which explants are unlikely to survive cryogen exposure, irrespective of the cooling rate. However, at relatively high water contents but not approaching the upper limit, better survival in terms of normal post-cryo seedling production is obtained after rapid cooling, e.g. for axes of *Aesculus hippocastanum* (Wesley-Smith *et al.*, 2001). Generally, seedling (i.e. root and shoot) production after cryogen exposure by explants from recalcitrant-seeded species of temperate origin (e.g. *A. hippocastanum* [Wesley-Smith *et al.*, 2001] and *Quercus robur* [Berjak *et al.*, 1999]) appears to be far more successful. A prevailing problem with embryos/axes excised from seeds of most tropical/sub-tropical origin is the lack of shoot production after exposure to cryogenic temperatures, irrespective of rapid cooling rates (e.g. for axes of *Ekebergia capensis* [Perán *et al.*, 2006; Hajari *et al.*, 2011]). This may be explained in terms of the magnitude of the oxidative burst (of the ROS, superoxide) after cryogen exposure reported for axes of *Trichilia dregeana*, which was described as being «approximately twice as great and [to] decline much more slowly» than the burst displayed by axes of the temperate recalcitrant-seeded species, *Castanea sativa*.

Retrieval from cryopreservation – re-warming and rehydration

When explants are retrieved from LN there is the danger of ice crystallisation during slow re-warming, irrespective of maintenance of the vitrified state while the explants are in cryostorage. Hence the general practice is to warm the explants at ~37 – 40°C, so that they rapidly traverse the temperature range in which ice crystallisation could occur. In the case of explants held in ~0.5 ml PVS2, the cryotubes are rapidly immersed in sterile distilled water at 40°C and vigorously shaken for 1.5 min, which achieves a warming rate of ~250°C min^{-1} (Sakai and Engelmann, 2007). Our practice is different in that for thawing we essentially reverse the mechanics of the cooling procedure. Cryotubes are opened under UV-sterilised LN and the explants then tipped into sterile LN in a

polystyrene cup; the LN within the cup is then decanted and immediately replaced with the re-warming solution (see below) at 37 – 40°C. This achieves warming rates which are one to two orders of magnitude more rapid (Wesley-Smith, 2002) than when cryotubes are immersed in a water bath at the same temperature.

In the case of explants vitrified using e.g. PVS2, an unloading step is implemented to facilitate the egress of the vitrification solution from the explant tissues. Hence after warming, the PVS2 is decanted from the cryotubes and replaced by 2 ml of basal culture medium containing 1.2 M sucrose, in which the explants are held for 20 min (Sakai and Engelmann, 2007). For explants which have been processed by encapsulation-dehydration, after re-warming and rehydration for ~10 min in liquid medium, the capsule is recommended to be removed prior to the explants being cultured on a recovery medium (Engelmann *et al.*, 2008).

For some years we used the Ca/Mg solution developed by Mycock (1999) as the re-warming and rehydration medium for naked embryos/axes, with some success (e.g. Sershen *et al.*, 2007). However, with the realisation of the potential for considerable ROS-mediated damage at all stages in a cryopreservation protocol – including, significantly, following explant retrieval from cryostorage (Whitaker *et al.*, 2010; Berjak *et al.*, 2011), we decided that the medium used for both re-warming and rehydration should have considerable reducing power (Fig. 1). Consequently we now use either cathodic water alone or an ascorbic acid solution made up in cathodic water. After cryogen exposure, 70% of axes of *Strychnos gerrardii* which had not previously been exposed to any cryoprotectants, established seedlings (i.e. producing both a root *and* a shoot) *in vitro* after re-warming for 2 min and rehydration for 30 min in cathodic water in the dark. In comparison, <10% were capable of seedling establishment when Ca/Mg was used as the thawing and rehydration medium (Berjak *et al.*, 2011). However, while this procedure was remarkably successful for *S. gerrardii* axes, we have yet to succeed in terms of shoot production by axes of *Trichilia dregeana after* cryogen exposure using either cathodic water alone or as the solvent for 1% ascorbic acid (Naidoo, 2012). We presently ascribe these seemingly anomalous results to the time taken for dehydration of axes of these two species under identical flash-drying conditions: whereas those of *S. gerrardii* became sufficiently dehydrated to withstand cryogen exposure within 30 min, *T. dregeana* axes required ~120 min, during which associated metabolism-linked damage would have occurred. As oxidative (ROS-mediated) damage is viewed as being cumulative, it is presently proposed that cryogen exposure caused necrosis of the already-damaged shoot apical meristems. Consequently, our focus is on accelerating dehydration rate during the process of flash drying.

Explant decontamination and recovery

Before the rehydrated explants can be cultured *in vitro*, they must be decontaminated by the means initially found not to be injurious. This is particularly important in view of contaminating inoculum present in LN. Even though the LN used for explant cooling and retrieval is routinely UV-sterilised, some seepage of LN into conventional cryotubes from the cryostorage container seems to be inevitable. It is not practical – or possible – to UV-sterilise the large volume of LN in which the cryotubes are immersed, hence precautionary decontamination of the explants *after* rehydration, but before plating, is necessary. Our observations suggest that a bactericide needs to be incorporated in the sterilant (even if not used initially) as bacterial inoculum seems to be particularly prevalent in the LN. As an aside, the explant-containing cryotubes can be stored in the vapour phase above LN (at temperatures never lower than -140°C), which will avoid LN seepage into the tubes.

As mentioned above in relation to the work of *S. gerrardii*, rehydration was carried out in the dark. Rehydrated axes plated on the optimised germination medium should similarly be maintained in the dark until signs of recovery are apparent. This is a precaution aimed at avoiding photo-oxidative stress (Touchell and Walters, 2002), noting that axes of recalcitrant seeds are green in many cases, thus indicating the potential for radiation absorption and consequent chlorophyll excitation.

The final stages – seedling establishment *in vitro* and transfer to *ex vitro* conditions

Once rooting and shoot development has occurred, it is generally necessary to sub-culture the seedlings, both because of nutrient depletion of the medium and the need to use taller containers to allow for growth. *In vitro* culture conditions should facilitate the development of functional root systems, after which the high RH within the culture vessels will need to be gradually reduced: this can be achieved by periodic transient opening of the lids for lengthening periods over about 5 – 7 d. When seedlings are ready for transfer into sterile soil, all culture medium should be gently washed off the roots before they are planted (e.g. Hartmann *et al.*, 2002). As they have been removed from the carbon source in the culture medium, the seedlings will gradually become autotrophic. Because the leaves require some time to become cuticularised, initially it will be necessary to maintain the transplanted seedlings under high RH conditions. This is easily achieved by enclosing individual pots in plastic bags, which are then gradually perforated. Alternatively, if the facilities are available, seedlings can be maintained in a mist-tent. Once gradually acclimatised, the young plants should be maintained in a greenhouse/glass-house or shade-house, until ready for nursery or field planting.

It is important that plants produced from cryopreserved embryos or axes be monitored, to ascertain whether or not they develop normally. In this regard, Konan *et al.* (2007) monitored the field performance for up to 12 y of 440 oil palms (*Elaeis guineensis*) produced from polyembryonic cultures which had been cryopreserved, of six elite clones. Those authors reported that some abnormal plants were produced by only one clone. In terms of genomic stability, conventional molecular analyses sample only 0.001% of the DNA (Harding, 2004). That author points out that over and above molecular biological analyses, phenotypic, histological, cytological and biochemical assessments all can contribute valuable information about possible effects of cryopreservation on subsequent plant growth and development. It is also important to note that epigenetic variations such as DNA methylation could occur as a consequence of cryopreservation, but may be reversible with on-going plant development (Harding, 2004).

Provenance effects

Besides marked inter-species differences in the responses of embryos or axes to a cryo-procedure, there is also provenance-related variation within individual species. For example, In trying to resolve the problems with cryopreservation of axes of *Ekebergia capensis*, Hajari *et al.* (2011) developed a novel method of producing plantlets from adventitious buds induced to form from the roots of 'shootless seedlings' after retrieval from the cryogen. Using seeds from a warm-temperate provenance, roots of 40% of the 'shootless seedlings' produced an average of five shoots each, compared with seeds from a sub-tropical provenance where 95% of 'shootless seedlings' produced an average of 17 shoots per individual. In terms of 'degrees of recalcitrance' of seeds of individual species, Daws *et al.* (2004; 2006) have recorded marked provenance-related differences in degree of development and responses to dehydration for two Northern Hemisphere species, *Aesculus hippocastanum* and *Acer pseudoplatanus*, respectively. In the context of axis cryopreservation, such provenance-related differences emphasise the unpredictability of being able to apply a cryo-protocol broadly, even within individual species.

Alternative explants

Development of adventitious buds on the roots of the 'shootless seedlings' of *E. capensis (*Hajari *et al.,* 2011) provides an example of an alternative explant which maintains the genetic traits of the seed from which the axis was derived. That approach could have applicability for other species where shoot apical meristem necrosis appears to be unavoidable. As an example of a more conventional approach, shoot apices from seedlings of another recalcitrant-seeded species, *Trichilia emetica*, were multiplied *in vitro* and their capacity for plantlet production ascertained. Shoot apices were then cryopreserved (using slow cooling) following pre-culture and exposure to PVS2, with 71% producing plantlets when retrieved from the

cryogen (Varghese *et al.*, 2009). Working with shoot apices of *Garcinia mangostana*, Ibrahim and Normah (2013) assessed the effects of various pre-culture parameters followed by exposure of shoot apices to PVS2. After cryopreservation and unloading using MS with 0.4 M sucrose and 2 M glycerol, and optimisation of the photoperiod during recovery, those authors reported survival (greening and initiation of growth) of 50% of the explants. It is interesting that PVS2 is able to be used to advantage for seedling shoot apices of recalcitrant-seeded species, whereas it may not be tolerated by the newly-excised embryonic axes, as discussed above. Generally, embryonic axes of recalcitrant-seeded species are small structures (relative to the total seed mass and volume), and so provide usable explants for cryopreservation. However, there are those species where the axes are simply too large to be rapidly dried or cooled. In all such cases, explants alternative to the zygotic axes need to be developed.

Dissemination of material retrieved from cryopreservation

There may be instances where explants that have been cryopreserved are required to be transported to a distant site. In such cases, noting that cryostorage vats cannot be transported, one option is to use what is called a dry-shipper in which an absorbent material charged with LN facilitates storage of cryopreserved samples in the vapour phase (Benson, 2008a). However, receipt of material in a dry-shipper assumes that the destination laboratory/institute is equipped with facilities for onwards *in vitro* cultivation of the explants. Furthermore, dry-shippers are expensive and need to be returned.

In order to overcome such problems, we are experimenting with the production of synthetic seeds (synseeds) also called artificial seeds, as developed for somatic embryos (e.g. Ravi and Arnand, 2012). After re-warming, rehydration and decontamination (Fig. 1) our procedure involves a short recovery period in the dark, with subsequent encapsulation of axes individually within calcium alginate beads. In preliminary (unpublished) experiments we found that encapsulated axes of *E. capensis* survived for at least a month at 16°C, during which time there was no root protrusion from the beads. In the production of such synseeds, a variety of additives – e.g. nutrients, growth regulators and fungicides – could be included in the alginate encapsulation. Although this research is in its infancy for recalcitrant embryonic axes, it promises to provide a means for dissemination of explants after cryopreservation to any distant location. It is also possible that the encapsulated explants could be germinated directly on/in sterile soil, which would avoid the necessity for further *in vitro* cultivation on medium at the destination.

CONCLUDING COMMENTS

While the procedures as outlined in figure 1 are complex and require to be optimised presently for explants of individual species – there are no options other than cryopreservation for *ex situ* long-term germplasm conservation of recalcitrant-seeded species. The procedures involved in developing and implementing cryo-protocols are labour-intensive and expensive (Pence, 2011) but cannot be circumvented: there are no short-cuts. Although *in situ* growth of plants in their natural environment affords the best means of conservation, given the global threats to biodiversity, complementary *ex situ* practices are imperative. However, considerable research is still necessary to overcome problems, particularly when the aim is to to cryopreserve embryos/embryonic axes of tropical/sub-tropical species. Nevertheless, gradual progress is being made based by assessment of, and elucidating, the effects of each of the steps in a cryo-procedure, the most recent being the indication that effects of ROS bursts need to be counteracted at each procedural stage.

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