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Development of the understanding of seed recalcitrant and implications for *ex situ* conservation

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ABSTRACT
Recalcitrant seeds are shed at high water content, are desiccation sensitive and cannot be stored under conditions conventionally employed for desiccation-tolerant orthodox seeds. Recalcitrant seeds are metabolically active when shed, with high rates of respiration and a high degree of intracellular differentiation. Development grades gradually into germination, with no punctuating quiescent period, and many recalcitrant seeds will germinate if maintained at their shedding water content. Consequently, storage in the hydrated condition is strictly a short-term option only. Although recalcitrant seeds are desiccation sensitive, if excised embryonic axes are dried very rapidly they will survive to lower water contents than those dried slowly. When axes are dried they are initially subject to aqueous-based oxidative degradative processes. Axes dried slowly are exposed to these conditions for an extended period of time, accumulate considerable damage and die at high water contents. Rapidly dried axes accumulate less damage over a shorter period and so survive to lower water contents. The response of excised axes to rapid drying opens up the possibility of cryo-storage. Partial drying reduces the chances of ice formation on subsequent exposure to cryogenic temperatures, and if the drying is rapid, will reduce oxidative damage. Rapid cooling can induce the remaining intracellular water to vitrify, permitting cryo-storage without damaging ice crystal formation. However, because of the highly variable physiology of recalcitrant seeds and axes, detailed protocols have to be established on an individual species basis, guided by these concepts.

Key words: Cryopreservation, damage, desiccation, recalcitrant seeds, storage

Desarrollo de la comprensión de las semillas recalcitrantes y las implicaciones para la conservación *ex situ*

RESUMEN
Las semillas recalcitrantes se desprenden cuando el contenido de agua es alto, son sensibles a la desecación y no se pueden almacenar en condiciones convencionalmente empleadas para las semillas ortodoxas-tolerantes a la desecación. Las semillas recalcitrantes son metabólicamente activas cuando se desprenden, tienen altas tasas de respiración y un alto grado de diferenciación intracelular. Si se mantiene elevado el contenido de agua de las semillas, hay un desarrollo gradual en la germinación y no se interrumpe la dormancia, muchas semillas recalcitrantes germinarán. En consecuencia, el almacenamiento en el estado hidratado es estrictamente una opción a corto plazo. Aunque las semillas recalcitrantes son sensibles a la desecación, si los ejes embrionarios se separan y se secan muy rápidamente van a sobrevivir a contenidos de agua bajos más que si se secan lentamente. Cuando los ejes se secan se someten inicialmente a un proceso de degradación oxidativa de base acuosa. Los ejes que se secan lentamente están expuestos a estas condiciones durante un período prolongado de tiempo, se acumulan daños considerables y mueren en presencia de altos contenidos de agua. Los ejes que se secan rápidamente acumulan menos daño en un período de tiempo más corto y así pueden sobrevivir a bajos contenidos de agua. La respuesta de los ejes embrionarios al secado rápido abre la posibilidad de crio-almacenamiento. El secado parcial reduce las posibilidades de formación de hielo en la posterior exposición a temperaturas criogénicas, y si el secado es rápido, reducirá el daño oxidativo. El enfriamiento rápido puede inducir el agua intracelular restante para vitrificar, lo que permite el crio-almacenamiento sin dañar la formación de cristales de hielo. Sin embargo, debido a la fisiología altamente variable de las semillas recalcitrantes y los ejes, no se han establecido protocolos detallados sobre la base de especies individuales, guiados por estos conceptos.

Palabras clave: almacenamiento, crioconservación, daño, desecación, semillas recalcitrantes

CONTENT

INTRODUCTION

Recalcitrant seeds are so-called because they are just that - difficult to store and difficult to handle. They cannot be stored using the conventional approaches of low seed water content and low (sub-zero) temperatures used to store desiccation-tolerant orthodox seeds. Recalcitrant seeds are shed at high water content, are desiccation sensitive and will die if dehydrated, but if maintained at the shedding water content they will germinate in storage or will succumb to fungal proliferation, both of which also lead to the death of the seeds. The only feasible method of storing the germplasm of recalcitrant-seeded species would appear to be cryostorage in liquid nitrogen, but the hydrated state and the desiccation sensitivity of the seeds pose considerable difficulties. The development of protocols for the successful *ex situ* cryoconservation of the germplasm of recalcitrant-seeded species requires an understanding of the physiology of recalcitrant seeds.

The phenomenon of seed recalcitrance was formally described and named by Roberts (1973), based on the storage behaviour of these seeds. Much of the early work concentrated on empirical studies aimed at enhancing the storage life span of these seeds, but the observed responses were complex and often generated confusion rather than an understanding (Chin and Roberts, 1980; King and Roberts, 1980). However, since those early days there have been some significant advances that have opened the door to possible cryopreservation of germplasm of recalcitrant-seeded species.

This paper presents an historical overview of the development of such an understanding, and implications for long-term storage.

CHARACTERISTICS OF SEED RECALCITRANCE

Germination in storage

Although germination in storage of several species was documented (King and Roberts, 1980), this was viewed as a 'nuisance', an impediment to successful storage. Numerous attempts were made to reduce germination in storage (including partial drying [sub-imbibed storage] and reduced temperatures), but the significance of the phenomenon in terms of explaining recalcitrant seed behaviour was not appreciated. If germination in storage occurs it implies that the seeds must be metabolically active and this metabolic activity is one of the underlying reasons as to why recalcitrant seeds are desiccation sensitive (Berjak *et al.,* 1984; Leprince *et al.*, 1999; Walters *et al.*, 2001; Berjak and Pammenter 2004; 2008). Unlike the situation in orthodox seeds, which undergo a period of maturation drying at the end of their development, in recalcitrant seeds there appears to be no clearly discernable switch from developmental metabolism to germinative metabolism and germination is entrained on (or even before) shedding (Farrant *et al.*, 1993).

Not all species show germination in the sense of radicle protrusion but all of them will show ultrastructural changes indicative of enhanced subcellular organisation and metabolism, particularly the development and increasing complexity of membranous structures such as mitochondria, endoplasmic reticulum and plastids (Farrant *et al.*, 1985; 1986; 1989). This ultrastructural and biochemical development leads to an increase in desiccation sensitivity, which itself can lead to death of the seeds unless additional water is provided (Pammenter *et al.*, 1994).

The significance of germination in storage and the associated increase in sensitivity depends upon the rate at which germinative metabolism proceeds. For species where germination is slow, metabolic rates are low, desiccation sensitivity is less and storage life span is extended (Berjak *et al.*, 1989; Farrant *et al.*, 1989). The rate of germinative metabolism can explain much of the confounding data about recalcitrant seed storage.

Developmental status

Desiccation-tolerant orthodox seeds undergo a final development stage of maturation drying, during which metabolism effectively ceases, ultrastructural de-differentiation occurs, desiccation tolerance is acquired and most of the seed water is lost. Recalcitrant seeds do not undergo these developmental phases and remain fully hydrated, metabolically active and desiccation sensitive (Finch-Savage, 1996). It is this high degree of subcellular development and metabolic activity that underlies the desiccation sensitivity of recalcitrant seeds: changes in developmental status will influence storage life span (Farrant *et al.*, 1989) and desiccation sensitivity (Pammenter and Berjak, 1999). It has been suggested that mild drying prior to storage (so-called sub-imbibed storage) could reduce germination and so extend storage life span (King and Roberts, 1980). However, mild dehydration appears to enhance the rate of germination (e.g. Fu *et al.*, 1994; Tompsett and Pritchard, 1998, Drew *et al.*, 2000). The reason for this is unknown, although it is conjectured that mild dehydration could be a signal of an imminent dry period, and in the field a rooted seedling being able to take up water from the soil, would be more resilient than would be a seed. However, the consequence for storage is enhanced metabolism leading to increased desiccation sensitivity and shorter seed storage life span (Drew *et al.*, 2000; Eggers *et al.*, 2007).

Effects of rate of drying

A recognition of the significance of the rate of drying was perhaps the major breakthrough in understanding aspects of recalcitrant seed behaviour. For some species it was reported that seeds that had been dried rapidly survived to lower water contents than those dried slowly (King and Roberts, 1980; Berjak *et al.*, 1984; Farrant *et al.*, 1985; Pritchard, 1991), although this was not observed in all cases (e.g. Tompsett, 1982; Probert and Longley, 1989). We initially attributed the effect of drying rate to the fact that during slow drying the seeds were exposed to high water contents for extended periods of time during which they experienced continued germinative development. This continued development increased the desiccation sensitivity and so the seeds died at high water contents (Farrant *et al.*, 1986). This hypothesis is similar to that provided by King and Roberts (1980). Whether or not this phenomenon is apparent depends upon the rate of germination in storage (Berjak *et al.*, 1989) which could explain the conflicting results of Tompsett (1982) and Probert and Longley (1989). Whatever the cause of the influence of drying rate on the response to dehydration, it does mean that there is no such thing as a species-specific 'critical water content' below which seeds lose viability; the water content corresponding to death depends upon the rate at which the seeds are dried. The concept of a critical water content ignores the importance of the time factor in the response of biological systems to stress (Pammenter *et al.*, 2003).

The hypothesis that the effect of drying rate is a consequence of continued development in slowly dried seeds would hold only if the drying rate was of the same order as the rate of germination in storage. This may not be the case and there must be another explanation of the phenomenon.

The alternative hypothesis considers the accumulation of damage during drying. Material that is dried very rapidly – in a matter of minutes to a few hours – can survive (in the short-term) to lower water contents than that dried slowly (several hours to days). The effect is more pronounced the more rapid is the drying, but most recalcitrant seeds are too large to achieve the drying rates at which this phenomenon is marked. However, excision of the embryo, or embryonic axis if the cotyledons are fleshy, yields an explant that is of a size permitting rapid drying, and partially dried excised axes can regenerate seedlings (Normah *et al.*, 1986). This led to the development of the technique of 'flash drying' where excised axes are dehydrated very rapidly in a dry air stream (Berjak *et al.*, 1990). Recalcitrant seeds, and particularly their embryonic axes, are metabolically active. As dehydration proceeds, at high to intermediate water contents aqueous-based degradative processes occur, probably mediated by reactive oxygen species (ROS), as a consequence of the loss of metabolic control or failure of antioxidant systems (Côme and Corbineau, 1996; Varghese *et al.*, 2011). Such damage is termed 'metabolism-linked' damage (Walters *et al.*, 2001) and is characterised by being aqueous-based. Material dried slowly spends a long period of time in the water content

range at which aqueous-based deleterious processes can occur, so accumulates considerable metabolism-linked damage and dies at a high water content. On the other hand, material that is dried rapidly spends a short period in the water content range where metabolism-liked aqueous-based processes can occur, damage accumulation is limited and death occurs at low water contents. It must be emphasised that rapid drying does not induce desiccation tolerance in recalcitrant seeds or embryonic axes: material that has been rapidly dried will not survive for more than a day or two at most (Walters *et al.*, 2001).

No matter how fast the drying there is a lower limit below which recalcitrant axes will not survive, and this appears to coincide with, or be slightly higher than, the water content at which the remaining tissue water is non-freezable (Pammenter *et al.*, 1991, 1993; Pritchard, 1991 [expressed as minimum water potential survived]; Finch-Savage, 1992 [expressed as matric bound water]; Berjak *et al.*, 1993; Pritchard and Manger, 1998). Presumably this non-freezable water is bound to the surfaces of intracellular structures and macromolecules. Unlike orthodox seeds, removal of the structure-associated water is lethal for recalcitrant types, and this is referred to as desiccation damage *sensu stricto* (Walters *et al.*, 2001).

We can conceive of three 'types' of damage occurring on drying of recalcitrant seeds or axes:

1. Mechanical damage: this is associated with volume reduction on loss of water (Iljin, 1957). This has not received much attention but presumably would be important in tissues with large, highly vacuolated cells.
2. Metabolism-linked damage: this occurs at high to intermediate water contents and is a consequence of failure of co-ordination of metabolism, leading to aqueous-based oxidative degradation, probably mediated by ROS. Damage accumulation, and the water content at which death occurs through this process, is strongly influenced by drying rate.
3. Desiccation damage *sensu stricto*: this occurs when structure-associated water is removed, leading to a loss of subcellular integrity. At present it is not understood why orthodox seeds can tolerate the loss of most of the structure associated water but recalcitrant seeds and axes cannot, but it could be a consequence of the highly differentiated intracellular condition of the recalcitrant tissue relative to the orthodox material.

When assessing the impact of drying of recalcitrant seeds or axes these different processes leading to viability loss must be borne in mind. Although we tend to consider them separately Liang and Sun (2000) have suggested that (for axes of *Theobroma cacao* at least), there is an optimum drying rate where the combination of mechanical and metabolism-linked damage is at a minimum.

There is one possible phenomenon that should not be overlooked when considering drying rate and that is uneven drying. During rapid drying of an embryonic axis it is likely that water will be lost initially from the apoplasm and outer cell layers before being lost from the inner cells: indeed, the rate of diffusion of water from the interior cells to the surface of the axis may be the rate limiting step in drying. When water content is measured gravimetrically it is the bulk average of all the tissues in the axis that is being measured, and this technique would mask any differences between tissues. It is possible (even likely) that the inner (and most metabolically active) cells in the meristem would actually be at a higher water content than that measured gravimetrically for the bulk axis. These cells may apparently suffer less damage simply because they are not as dry as bulk water content measurements would indicate. This phenomenon of uneven drying could be important in the response of axes to rapid drying, but it has not been investigated in detail as yet (see Pammenter *et al.*,1998 for a more detailed discussion).

Another complicating factor is that most of the investigations into the effects of drying rate have compared excised axes with axes dried in whole seeds. The reason for this is that it is technically almost impossible to dry excised axes slowly (because of their small size), and to dry whole seeds very rapidly (because of their large size). However, it is possible to dry whole seeds of *Ekebergia capensis* at sufficiently different rates to observe the effect of drying rate (Pammenter *et al.*, 1998), and the results are essentially the same: seeds dried rapidly survive to lower water contents than seeds dried slowly. We are confident that the effect of

drying rate is just that, and not a consequence of separating the axis from the rest of the seed.

Oxidative damage

A free radical is a chemical species with an unpaired electron in an outer orbital. As such it is highly reactive, and when it extracts an electron from a substrate it is a very powerful oxidising agent. Compounds that are powerful oxidising agents and contain oxygen are termed reactive oxygen species (ROS); many (but not all) are free radicals. Aerobic organisms undergo respiration where electrons pass down a chain of acceptors with oxygen as the terminal electron acceptor. During this process electrons may 'leak' from the electron transport chain and directly reduce molecular oxygen to superoxide ($^{\bullet}O_2^-$), a free radical ROS. Similarly, photosynthetic organisms can generate ROS (superoxide and singlet oxygen) during photosynthetic electron transport (reviewed by Halliwell, 2006; Gill and Tuteja, 2010).

Superoxide can be dismutated by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) and O_2. Hydrogen peroxide is a non-free-radical ROS which has the ability to form the hydroxyl radical ($^{\bullet}OH$) via so-called Fenton chemistry involving ferrous iron. The reactivity of the different ROS varies; $^{\bullet}OH$ is highly reactive and can directly oxidise unsaturated bonds in the fatty acid components of lipids, and proteins and nucleic acids. The products of these reactions are often free radicals themselves and can promote further oxidative processes via chain reactions, or can produce toxic by-products.

Reactive oxygen species are normal components of aerobic metabolism but there is an increase in production under conditions of stress, *inter alia* physical damage, desiccation and chilling/freezing stress (reviewed by Smirnoff 1993; Walters *et al.*, 2002; Gill and Tuteja, 2010). Coordination of metabolism fails because different metabolic processes can react to stress at different rates, resulting in an increase in ROS production. Under non-stressful conditions the production and activity of ROS are tightly controlled by a spectrum of anti-oxidants. These include non-enzymic (glutathione [GSH], ascorbic acid [AsA], and á-tocopherol, which is lipid soluble) and enzymic (SOD, a range of catalases and peroxidases, and glutathione reductase [GR], amongst others) anti-oxidants (reviewed by Gill and Tuteja 2010). When a plant is placed under stress there could be an increase in the production of ROS or the activity of the anti-oxidant systems could decline such that the oxidative activity of the ROS overwhelms the anti-oxidant capacity, and degradative oxidative processes predominate.

Excision induced damage

A common problem with many species, particularly those with fleshy cotyledons and of tropical origin, is that excised axes will produce a root in culture but not shoots, even without the additional stresses of drying and subsequent cryopreservation. This has been attributed to a wounding-induced oxidative burst of superoxide at the site from where the cotyledons were excised. (Goveia *et al.*, 2004; Roach *et al.*, 2008; Whitaker *et al.*, 2010; Berjak *et al.*, 2011), and if the excision site is close to the apical meristem this oxidative burst could damage the meristem, inhibiting shoot formation (Pammenter *et al.*, 2011). Interestingly, the magnitude of the burst from *T. dregeana* (Whitaker *et al.*, 2010), which is tropical, was double that from *Castanea sativa* (Roach *et al.*, 2008), which is of temperate origin, indicating the generally higher sensitivity (to all the steps of cryopreservation) of recalcitrant seeds from tropical regions relative to those of temperate origin

Dehydration-induced damage

However, it is not only excision, but all the subsequent steps involved in cryopreservation, that can induce ROS, and an oxidative burst has been shown to occur on excision, drying and retrieval from cryopreservation of axes of *T. dregeana* (Whitaker *et al.*, 2010) and of *Strychnos gerrardii* (Berjak *et al.*, 2011).

There has been considerable interest in the involvement of oxidative metabolism in the response of tissues to drying,. Certainly, in the case of desiccation tolerant vegetative tissue ('resurrection plants'), the importance of antioxidant systems during drying has been clearly demonstrated (reviewed by Farrant *et al.*, 2012) but little attention seems to have been paid to the maturation drying phase of orthodox seeds. It may be that, because of the de-differentiation and metabolic 'switch off' that

occurs prior to, or commensurate with, maturation drying, the production of ROS is reduced relative to that in recalcitrant seeds and there is less need for highly active antioxidant systems. Alternatively, it could be the possession of highly efficient antioxidant systems that permits orthodox seeds to survive the process of maturation drying.

Under non-stressful conditions ROS are part of normal metabolism with an important role in cell signalling, and the pro-oxidant and antioxidant status is in balance, preventing excessive pro-oxidants causing damage but permitting ROS signalling. Under stress conditions (with dehydration being of interest here) metabolism can become unregulated leading to an increase in damaging ROS which, in turn, can induce the production of defence compounds, e.g. the antioxidants mentioned above.

However, when assessing oxidative metabolism in response to drying, some of the data are rather equivocal, and there appears to be no consistent pattern in responses. Different responses occur in different tissues of the same seed (Hendry *et al.*, 1992; Pukacka and Ratajczak, 2006, suggesting that different organs have different protective mechanisms), and individual antioxidants respond differently from each other, and at different stages during the drying process.

A sign of lipid peroxidative damage (probably initiated by the hydroxyl radical) is the accumulation of thiobarbituric acid reactive substances (TBARS) in recalcitrant material; in some instances TBARS have been shown to increase on drying (Hendry *et al.*, 1992 [in axes but not cotyledons]; Chaitanya and Naitani, 1994; Varghese and Naitani, 2002; Francini *et al.*, 2006; Cheng and Song, 2008), whereas in other studies no marked increase was observed (Niedzwiedz-Siegien *et al.*, 2004; Xin *et al.*, 2010). In the studies by Varghese *et al.* (2011), although there was no accumulation of TBARS with drying time, material dried rapidly consistently showed lower levels than that dried slowly. The ratio of reduced to oxidised glutathione (GSH/GSSG), which is a good indicator of the oxidation state of a tissue, does show more consistent behaviour, with a general decline (more oxidised) with drying (Tommasi *et al.*, 2006; Pukacka and Ratajczak, 2006; Varghese *et al.*, 2011).

If ROS-mediated degradation is a major cause of death of dried desiccation-sensitive material, an increase in ROS activity might be expected. Increases in levels of ROS on drying have been reported by Hendry *et al.* (1992), although this was confined to embryonic axes, and by Pukacka and Ratajczak (2006) and Cheng and Song (2008), whereas some reports indicate no increase in ROS accumulation during drying (e.g. Xin *et al.*, 2010; Varghese *et al.*, 2011) suggesting either no increase in ROS production, or efficient quenching by antioxidants. However, a *caveat* must be noted here: the conventional assays for superoxide (Misra and Fridovich, 1972) and hydroxyl radical (Schopfer *et al.*, 2001) measure extracellular production of these ROS, and the values obtained may not reflect intracellular levels where presumably most of the damage occurs.

Antioxidant activity can also change with drying. A not unexpected response is an initial increase in activity, followed by a decline, indicating failure of the antioxidant systems. A variant on this pattern was observed by Hendry *et al.* (1992) in seeds of *Quercus robur* Antioxidants in the cotyledons were predominantly enzymic and retained activity during drying, whereas in axes the antioxidants were predominantly non-enzymic. Death of the seeds was suggested to be a consequence of failing antioxidant activity in the axes. A similar failure of the antioxidant system in neem (*Azadirachta indica*) was reported by Varghese and Naithani (2002). In cotyledons of *Acer saccharinum* (Pukacka and Ratajczak, 2006) there was an initial increase in non-enzymic antioxidants, followed by a decline as drying progressed, whereas in the axes it was enzymes of the ascorbate-glutathione pathway that increased on drying (this is the reverse of the location of enzymic and non-enzymic antioxidants reported for *Quercus robur* (Hendry *et al.*, 1992).

The lack of a clear theme in the response of the redox system in recalcitrant seeds or axes to drying is perhaps not unexpected. There is no taxonomic relationship between species producing recalcitrant seeds, and so they have different evolutionary histories, occur in a range of habitats and could have different strategies for protecting against oxidative damage. It

should also be remembered that ROS are extremely reactive and there may be considerable reactivity and turn-over with little accumulation (increase in pool size). Perhaps more important is that the responses obtained can (will) depend on the drying conditions, and this varies among investigators as well as between species which have different drying characteristics.

As an example, embryonic axes of *Trichilia dregeana* were dried rapidly or slowly and viability and ROS (using extracellular superoxide and hydroxyl radicals as markers) and anti-oxidant activity monitored (Varghese *et al.*, 2011). With rapid drying there was no relationship between viability, ROS or anti-oxidant activity, suggesting that processes other than oxidative degradation were involved in viability loss. On the other hand, with slow drying there was no relationship between viability and ROS, but a clear relationship between viability decline and reduced anti-oxidant activity. These data indicate that a decline in anti-oxidant activity rather than an increase in ROS may lead to damage and viability loss, under slow drying conditions, and that death under rapid drying was not a result of metabolism-linked oxidative damage. Xin *et al.* (2010) have also demonstrated a lack of relationship between accumulation of ROS and viability loss under rapid drying conditions of axes of *Antiaris toxicaria* and the authors suggested that under these conditions death was triggered by mechanical and physical damage, rather than metabolic damage. It has also been suggested that, in two amaryllid species at least, one of the roles of glycerol as a cryoprotectant is to protect the activity of certain anti-oxidant enzymes (Sershen *et al.*, 2012).

There have been attempts to reduce dehydration-induced oxidative damage by supplying exogenous antioxidants or enhancing the activity of endogenous antioxidants (e.g. Bai *et al.*, 2011; Pukacka *et al.*, 2011). Of these the use of cathodic water (the water in the cathode chamber when water is electrolysed with the anode and cathode in separate chambers connected via a salt bridge) holds the most promise (Berjak *et al.*, 2011).

Although there are variations on the oxidative defence theme, failure of the antioxidant systems, rather than excessive ROS production seems to be common. But whatever the mechanisms to regulate the redox status of the tissue, they do not function efficiently – recalcitrant seeds and axes die when dried.

Desiccation damage *sensu stricto*

Rapidly drying of excised axes permits survival to lower water contents than does slow drying. However, no matter how fast the drying, there is a lower limit in water content, below which the axes will die, and this lower limit corresponds with, or is slightly above, the water content at which the remaining water does not freeze (Pammenter *et al.*, 1993). This non-freezable water is presumably firmly associated with macromolecular structures and is sometimes referred to as 'bound water' or 'structure-associated' water. Unlike orthodox seeds, recalcitrant seeds or axes cannot survive the removal of this water.

There are a number of putative mechanisms and molecules thought to confer desiccation tolerance. Metabolic switch-off and organelle de-differentiation, together with efficient antioxidant systems predominantly protect against damage caused by the drying processes itself (i.e. metabolism-linked damage). Viability in the dry state is thought to be maintained by compounds such as late embryogenic proteins (LEAs) and sucrose and raffinose- series oligosachharides, all of which accumulate during maturation drying (reviewed by Berjak, 2006). The mechanisms by which these solutes confer desiccation tolerance are not yet clear, but they may be involved in the conversion of cellular water to the glass phase (see below).

While these putative protectants appear to be present in orthodox seeds, the situation in recalcitrant seeds is equivocal, as they have been found to be present in the seeds of a range of species from different habitats, but appearing to be absent from others. Group 2 LEAs (dehydrins) have been identified in the recalcitrant seeds of some temperate tree species (Finch-Savage *et al.*, 1994; Gee *et al.*, 1994) some temperate grasses (Gee *et al.*, 1994) and some tropical/subtropical tree species (Farrant *et al.*, 1996). On the other hand, no dehydrin-type LEAs could be demonstrated in the recalcitrant seeds of ten tropical wetland species (Farrant *et al.*, 1996).

Kalemba and Pukacka (2012) showed little difference in dehydrins and small heat shock proteins in the seeds of recalcitrant and orthodox seeds of three *Acer* species and suggested that the environmental conditions during seed development could be important modulators of dehydrin expression. (However, dehydrins have received disproportionate attention simply because they are the only type of LEAs to which an antibody is readily available.) Sucrose and oligosaccharides have been found in most recalcitrant seeds studied, although in some instances at lower concentrations than in orthodox seeds from similar habitats (Steadman *et al.*, 1996).

Whatever the situation may be with respect to the presence or absence of these putative protectants in recalcitrant seeds, if present they are obviously not functional – if water in recalcitrant seeds or axes is reduced below the non-freezable level (at which glasses may have formed), viability is lost.

Cryopreservation

Given that recalcitrant seeds cannot be stored under the conditions conventionally used for orthodox seeds, alternate methods for the long-term *ex situ* conservation of the germplasm of recalcitrant-seeded species must be sought. Currently, the most promising approach is cryoconservation in liquid nitrogen at -196°C; at this temperature chemical and metabolic activity is reduced to very low levels and theoretically material can be stored indefinitely. However, cryopreservation is not a simple matter and there are a number of factors that place constraints on our ability to successfully cryopreserve desiccation-sensitive material, the most important being the formation of large (and generally lethal) ice crystals in the tissue as the temperature is reduced below 0°C.

Although water (ice) melts at 0°C liquid water will often supercool to temperatures well below this before ice crystals form. In liquid water, hydrogen bonds between neighbouring water molecules will form and break; as the temperature decreases the kinetic energy of the water molecules decreases, reducing the rate at which hydrogen bonds are broken. At sufficiently low temperatures and low kinetic energy there is the formation of 'embryo' ice crystals where the water molecules bond together in a lattice-like symmetry, leaving open spaces, which gives rise to the lower density of ice, and the (often lethal) expansion of water on freezing (Benson, 2008). At the homogeneous nucleation point (-40°C for pure water) the temperature is sufficiently low to reduce molecular mobility to the point that permits the formation of an embryo ice crystal that is thermodynamically capable of growth. Heterogeneous nucleation occurs at temperatures higher than -40°C when impurities or irregularities provide nucleation sites on which ice crystals will can form and grow, a phenomenon known as 'seeding'. During cryopreservation it is important to prevent intracellular nucleation events as these lead to ice crystal formation.

In conventional cryopreservation of single cells (reviewed by Mazur, 2004) the cell suspension is cooled slowly. Under these conditions extracellular ice forms and the intracellular water becomes supercooled. This generates a chemical potential gradient between the interior and exterior of the cell, driving the movement of intracellular water from the cell to the exterior where it freezes. As water is lost from the cells, the intracellular contents become more concentrated, the freezing point is depressed, cellular water remains supercooled and the cells become increasingly dehydrated with decreasing temperature. As there is almost equilibrium of chemical potential across the membrane during slow cooling, this approach is often termed 'equilibrium cooling'. The success of this protocol depends on the cooling rate: if the cooling rate is too slow, osmotic effects and high electrolyte concentrations associated with dehydration may kill the cells; if the rate is too high water may not be able to leave the cells fast enough to concentrate the solution, the cells become further supercooled and intracellular ice formation is likely.

Embryonic axes are, of course, much more complex than a suspension of single cells. They have a diversity of cell types and tissues, which may respond differently from each other and the axes are seldom subjected to cryogenic procedures while suspended in a medium. Nonetheless, some of the concepts derived from work on single cells are applicable to the structurally much more complex axes, in particular, the need to reduce the water content to avoid ice formation.

Initial attempts at cryopreserving excised embryonic axes involved partial drying of the axes (generally to water contents in the region of 0.4 to 0.3 g water per g dry mater) followed by enclosure in cryo-vials which were immersed in liquid nitrogen (e.g. Pritchard and Prendergast, 1986; Chaudhury *et al.*, 1991; Pence, 1992; Chandel *et al.*, 1995), a procedure that yields a cooling rate of about 3°C s^{-1} (Vertucci, 1989). Partial drying was required to reduce intracellular ice formation during cryogen exposure, but the drying itself is injurious. Cooling axes at higher water contents would ameliorate the desiccation damage, but it would increase the likelihood of intracellular ice formation. There appears to be a window of optimal hydration for cryopreservation, limited by desiccation damage at the lower end and lethal freezing damage at the upper. It appears that the extent of drying required by conventional cooling protocols is detrimental and cooling methods that permit axes to be cryopreserved at higher water contents would be desirable. As an aside, it is difficult to interpret some of the published data in that 'success' has not always been clearly defined; it could indicate a post-thaw recovery of an axis producing a functioning seedling with both roots and shoots, an axis that produces only a root, an axis that swells and greens but never produces a root, or merely 'non-dead' disorganised callus tissue.

It is almost a central tenet of cryobiology that large intracellular ice crystals are invariably lethal, and cryopreservation protocols should be designed to avoid the formation of such crystals. One way of doing this is to induce vitrification – to induce intracellular water to form a glass. A glass is a 'solution' so concentrated and of such reduced molecular mobility that it acts as an amorphous solid. Because of the low molecular mobility water molecules cannot come together in the appropriate orientation to form ice and so tissue with the water in the glassy state will not form intracellular ice crystals, and will not suffer the damage consequent upon this. Another consequence of the extremely low molecular mobility in a glass is that all metabolism is virtually halted and so damaging oxidative processes should not proceed.

There are three ways that intracellular vitrification can be induced: (I) increase the amount of solute, (II) reduce the amount of solvent, i.e. partially dry the tissue, and (III) reduce the temperature to below the glass transition temperature (T_g). Very often all three of these processes are used in the same crypreservation protocol.

Increasing the amount of solvent can be achieved by the addition of cryoprotectants; these are substances that act to reduce damage to tissue on exposure to cryogenic temperatures. Cryoprotectants that do not penetrate the cells (e.g. sucrose, polyethylene glycol) remain in the surrounding medium and dehydrate the cells by osmotic effects. Penetrating cryoprotectants (e.g. glycerol, dimethylsulphoxide [DMSO]) increase intracellular solute concentrations which can lead to vitrification on cooling.

Partial drying will, of course, induce metabolism-linked oxidative damage, but this can be reduced by very rapid drying. Partial drying will also increase cellular viscosity and concomitantly decrease molecular mobility (Buitink *et al.*, 1998), which can have two important effects: it will reduce the rate of aqueous-based damaging oxidative processes, and the reduction in the amount of water will reduce the likelihood of ice formation and affect the readiness with which ice crystals are formed (Luyet *et al.*, 1962). Additionally, partial drying will reduce the heat capacity of the sample which will increase cooling rates on subsequent exposure to cryogenic temperatures.

Inducing vitrification by reducing temperature requires very rapid cooling to prevent intracellular ice crystal formation. Fully hydrated cells freeze at about -2°C and ice crystal growth is arrested at -80°C (Moor, 1973); if cells can traverse this range extremely rapidly the growth of ice crystals to lethal sizes may be prevented. Less than the maximum amount of ice is formed if a solution is prevented from reaching thermodynamic equilibrium within the temperature range where crystal growth is possible (Luyet, 1960). As with rapid drying this is a case of the stress (cooling) being applied faster than the damage (intracellular ice formation) can accumulate. Just as maximising the rate of drying requires small tissue pieces, so maximising the rate of cooling also requires small sample sizes to minimise heat capacity. The major way of reducing heat capacity and so increasing cooling rate is by partially drying

the sample. Other factors increasing drying rate include a large surface area to volume ratio to maximise energy exchange between the sample and cryogen, and maximum convective heat dissipation during cooling. Rapid cooling to temperatures below the glass transition temperature, T_g, will permit vitrification with minimal ice formation. An advantage of this approach is that is does not require precisely controlled cooling rates using a programmable freezer, just that cooling is sufficiently rapid.

CONCLUSIONS

Recalcitrant seeds are desiccation-sensitive and metabolically active. To achieve successful cryopreservation there are a number of conditions that must be met.

(I) Lethal intracellular ice formation must be avoided: the best way of achieving this is to induce 'freezable' water in the cells to form a glass during cooling. This requires very rapid cooling and so small specimens of low heat capacity, such as excised embryonic axes are the explants of choice.

(II) To achieve the cooling rates required for vitrification the thermal mass must be reduced as much as possible. The use of non-penetrating cryoprotectants to bring about osmotic dehydration may be helpful here, but the most efficient way is to rapidly dry the tissue in a dry air stream, as removal of some of the tissue water will reduce the heat capacity of the sample. Partial drying has other effects, particularly the increase in viscosity and reduction in molecular mobility, which will reduce the likelihood of ice formation and reduce aqueous-based degradative reactions. Drying must be rapid to reduce the accumulation of damage brought about by the metabolism-linked oxidative damage invariably associated with drying. However, samples should not be over-dried so that desiccation damage *sensu stricto* does not occur, but adequate to prevent intracellular ice formation on cooling.

(III) Although drying is essential it is our experience that it may be the most damaging step in a cryopreservation protocol. It is important to reduce oxidative damage on excision of the axis and during drying. Dehydration-induced ROS generation is generally reduced (but not eliminated) by rapid drying, Stimulation of antioxidant activity by exogenous antioxidants has been attempted and cathodic water shows considerable promise in this regard (Berjak *et al.*, 2011).

Although these requirements are understood conceptually there are still a number of remaining practical difficulties. There is considerable variation among recalcitrant seeds and so the specifics of cryopreservation protocols will have to be elucidated on a species by species basis (and possibly also in relation to provenance Berjak and Pammenter, 2013). When tissue is rapidly dried there will be a heterogeneous distribution of water across the tissue, and so different cells in the same axis will respond differently to treatments. Cryoprotectants are not universally effective and, in fact, they are toxic to the axes of some species (Berjak and Pammenter, 2013). Some species are just not amenable to cryopreservation of the axes. This is often simply because the axes are too large to permit the rates of drying and cooling required bringing about vitrification. In cases like this it will be necessary to investigate the potential of alternative explants (meristems or buds or other explants) for cryopreservation.

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