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Different Assessments of the Effect of Drying Rates on Recalcitrant Seed Material

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Abstract

The results of the germination and tetrazolium (TZ) tests of axes of *Pisum sativum*, *Quercus robur* and *Trichilia dregeana* were in agreement during both drying and wet storage. The TZ test overestimated viability of *Avicennia marina*, *Trichilia emetica* and *Strychnos madagascariensis* axes in comparison to the germination test during dehydration. Thus, the germination test is a method of choice during desiccation and the TZ test may be a better indicator of viability during hydrated storage than drying. The survival of axes of *Q. robur* during slow dehydration and moist storage was unexpectedly poor possibly as a result of an unfavourable temperature during treatments. Hence, rapid desiccation is recommended for determinations of minimum 'critical water contents'. The relationship between electrolyte leakage and water content during drying and wet storage of axes of *T. dregeana* harvested in 2001 and *S. madagascariensis* showed a 'classic pattern' as dehydration and hydrated storage proceeded. Consequently, the conductivity test is a poor measure of the 'critical water contents' but may be an indicator of differences in vigour among harvests of the same species along with the germination and TZ tests during slow desiccation. Less leakage occurred during rapid than slow drying in all species. Desiccation-sensitive seeds can be divided into three categories on the basis of the predominant mechanism of loss of viability during dehydration: minimally, moderately and highly desiccation-sensitive in which desiccation, metabolic and physical damage predominate, respectively. Irrespective of the mode of dying, the effect of rate drying of was always apparent.

Keywords

Conductivity, Desiccation, Drying Rate, Recalcitrant, Storage, Viability

1. Introduction

Recalcitrant seeds are shed at high water contents, are desiccation-sensitive and are metabolically active (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2008; Ntuli et al., 1997, 2007, 2009, 2013, 2014). Because of these characteristics recalcitrant seeds cannot be stored under the conventional conditions of low (sub-zero) temperatures (the high water content leads to lethal ice crystal formation), and low water content (because they are desiccation-sensitive). Currently, such seeds can be stored only in the hydrated condition and that for only a short while, and the only potential route for the long-term storage of recalcitrant seeds is cryopreservation in liquid nitrogen, or the vapour above the liquid. However, most recalcitrant seeds are too large to permit the very rapid cooling required to prevent ice crystal formation during immersion in liquid nitrogen.

To overcome this, an approach that has been adopted is to excise the embryo or embryonic axis from the seed, to yield an explant that is a fraction of the size of the seed and so more amenable to rapid cooling (Wesley-Smith *et al*., 1992). Additionally, the excised tissue can be partially dried, which reduces the thermal mass and further enhances the rate of cooling, and also reduces the water present available for ice crystal formation (Wesley-Smith *et al.*, 2001). The partial drying step is crucial: if the tissue is not dried enough then lethal ice crystal formation can occur; if drying is excessive, then desiccation damage can ensue. The rate of drying is an important factor: excised embryos or embryonic axes that are dried rapidly can survive, transiently to lower water contents than those dried slowly (Pammenter *et al*., 1991), providing

material more suitable for cryopreservation.

The preparative processes for cryopreservation are laborious and so it is essential to know the effects of these individual processes on viability before proceeding to the next step. Because the embryos or axes have been removed from the storage reserves of the seed, viability is assessed by growth in tissue culture, a technique that can take several weeks to yield results. It would be advantageous to have a method to assess viability that is more rapid and less laborious than growth in tissue culture.

There are a number of techniques that have been developed to assess the viability and vigour of (mostly desiccation tolerant, orthodox) seeds. Germination is the ultimate measure of viability as it presents an integrated response of the whole organism, but rate of germination can be indicative of the vigour of a seed lot. In addition to simple germination tests, there are other ways of assessing the 'condition' of a seed (reviewed by Pritchard, 1996), although they measure different phenomena. The tetrazolium test is used to measure seed viability (ISTA, 1996). Colourless tetrazolium chloride (2,3,5-triphenyltetrazolium chloride, TTZ) can be reduced to a red, stable and non-diffusable triphenyl formazan, and the tetrazolium (TZ) test is taken as an indicator of activity of dehydrogenases of the mitochondrial electron transport pathway, and so of respiratory metabolism and seed viability. The leakage of electrolytes from seeds or seed tissues can be used as a measure of vigour (mainly of orthodox seeds). Enhanced electrolyte leakage can be taken as an indicator of membrane integrity or of death of cells in the seed tissue and can be determined by measuring the electrical conductivity (EC) of the seed leachate.

Both the TZ and EC test are rapid and simple to conduct and may be suitable as rapid tests for excised embryos/embryonic axes. However, they were initially developed for use with orthodox seeds, and their suitability for excised recalcitrant embryo/embryonic axes has not been systematically investigated. This study measures the *in vitro* germinability, viability as assessed by the TZ test and electrolyte leakage of axes excised from a range of recalcitrant seeds. The axes were stressed by drying, either rapidly or slowly, or by storing in the hydrated state, as it has been suggested that different degradative processes may cause viability loss under different drying conditions (Walters *et al.*, 2001) and this may effect the results the different assay techniques yield.

2. Material and Methods

2.1. Material

Seeds of *Trichilia dregeana* Sond. (Meliaceae) and *Avicennia marina* (Forssk.) Vierh. (Verbenaceae) were collected in two years with an intervening year, and those of *Trichilia emetica* Vahl., and *Strychnos madagascariensis* Poir. (Loganiaceae) were collected once. All collections of these recalcitrant seeds were from several trees, in the sub-tropical coastal region of KwaZulu-Natal, South Africa. Recalcitrant seeds of the temperate species, *Quercus robur* L. (Fagaceae) were collected from two trees in Wellesbourne, U.K, where the studies on this species were conducted., while orthodox seeds of *Pisum sativum* L. var. Greenfeast (Fabaceae), were obtained from a commercial source in South Africa.

Recalcitrant seeds initiate germination immediately on (or even before) shedding Berjak *et al*., 1984) and so it is important that seeds are collected as soon after shedding as possible and stored for only a minimal period prior to experimentation. In this study all seeds of the sub-tropical species were collected within a day of shedding. The seed coverings, including the testa, were removed (to remove fungally contaminated structures), the seeds surface-sterilised and dusted with fungicidal powder (Péran *et al*., 2004). and stored in loosely-closed plastic bags at 16°C for periods not exceeding two weeks before use. Seeds of *A. marina* were used immediately after collection and the surface sterilisation of *Q. robur* was performed with 6.4% (w/v) sodium dichloroisocyanurate (Fichlor) rather than sodium hypochlorite.

Embryonic axes were excised and, in the case of those of *T. dregeana* and *T. emetica*, a block (*c*. 2mm^3) of each cotyledon was left attached to the axes to obviate injury to the shoot meristem. For *P. sativum*, seeds were set to germinate for 72 h, after which axes longer than 10 mm were excised for experimentation, each with the cotyledonary petioles attached.. In all cases, axes were accumulated on barely-moistened filter paper in closed Petri dishes until the requisite number had been excised, after which they were surface sterilised (Péran *et al*., 2004).

2.2. Treatments

For rapid dehydration silica-gel-dried air was fan propelled over axes supported on grids within sealed containers (i.e. the flash drying apparatus described by Pammenter *et al.* [2002]), while slow dehydration was carried out by suspending a third batch of axes over saturated NaCl within a sealed chamber which maintained a relative humidity of 75±1%. In all cases drying was carried out at 16°C. For hydrated (wet) storage axes were mounted on grids suspended over distilled water within sterile sealed containers, which were stored in a temperature-controlled room at 16°C. Axes that were wet-stored, dried slowly or rapidly were sampled at five intervals for water content determinations and assessment of viability. Water content of five individual axes at each sampling interval was determined gravimetrically after drying at 80°C for 48 h, and expressed on a dry mass basis (g H_2O g $^{-1}$ dm; g g^{-1}).

2.3. Assessments

Prior to tetrazolium (TZ) testing, germination assessment and measurement of leachate conductivity, axes sampled at intervals during dehydration or wet storage were maintained overnight on moistened filter paper in Petri dishes. The TZ test was performed on 20 axes at each sampling interval. Pre-

moistened axes were bisected longitudinally, with one half of each being immersed in 1% aqueous (w/v) 2,3,5-triphenyltetrazolium chloride for 24 h in the dark at 20°C, and assessed by intensity and location of staining (ISTA, 1996).

Germinability of axes of *T. dregeana*, *T. emetica*, *A. marina* and *P. sativum* was assessed *in vitro* by culturing under sterile conditions in Petri dishes on half-strength MS medium (Murushige and Skoog, 1962) containing 3 g l^{-1} sucrose. The same germination medium but supplemented with 1 mg l^{-1} benzylaminopurine (BAP) was used for axes of *Q. robur*. Cultures of 20 axes each of *T. dregeana*, T. *emetica*, *A. marina* and *Q robur* were maintained under a 16 h photoperiod at room temperature, while those of *S. madagascariensis* were maintained in the dark. Germination was scored by greening, expansion or elongation alone or in combination.

Electrolyte leakage from five individual, pre-moistened axes of all species (except for *Q. robur*) at each sampling interval was measured over 12 h using a multi-cell conductivity meter (CM 100; Reid & Associates, Durban, S.A.). For *Q. robur*, leakage from 10 replicates of individual moistened axes was measured after leaching for 12 h, using a Cardy C-172/173 compact conductivity meter (Horiba Ltd, Kyoto, Japan). Measurements were recorded at 2 V, with single axes immersed in 1 ml distilled water for all species except *A. marina*, which were immersed in 3 ml because of their large size. In all cases, the highest leakage over the assessment period is reported as the mean ± SE of five or ten individual axes.

3. Results

Table 1. *Water content at 50% loss of viability.*

Species	Drying rate			
	Rapid		Slow	
	Germ	TTZ	Germ	TTZ
P. sativum	na*	na*	0.23	0.22
Q. robur	0.14	0.11	0.61	0.68
S. madagascariensis	0.25	0.12	0.48	0.32
T. emetica	0.33	0.08	0.51	0.22
T. dregeana (year 1)	0.62	0.47	1.24	1.00
T. dregeana (year 2)	0.56	0.39	0.79	0.8
A. marina (year 1)	1.08	na**	1.38	0.29
A. marina (year 2)	1.08	na**	2.00	0.66

* hydrated axes of the orthodox *P. sativum* did not lose viability on rapid drying.

** Axes of *A. marina* did not dry as much as anticipated and viability did not decline to 50%. The data for the TZ test for slowly dried axes are based on a viability or germination of 60, not 50%.

The germination percentage and tetrazolium staining of axes from newly-shed seeds was at least 90% for all species studied. There was a marked decrease in both germination percentage and tetrazolium staining of axes of all species investigated during both fast and slow drying, except the orthodox *P. sativum* which showed a decline on slow drying only. The decline was precipitous, in many cases changing from 100% to 0% in the interval between successive samples. The loss of viability occurred at a lower water contents for fast than slow desiccation in all cases. During hydrated storage a gradual decline in both tests was seen in all species studied. The drying and storage data for *T. emetica* are shown in Figure 1 as an example. To permit comparisons across species and between assay techniques, the water content corresponding to 50% loss of viability was determined from interpolation of the water content/viability relationships and the data are presented in Table 1.

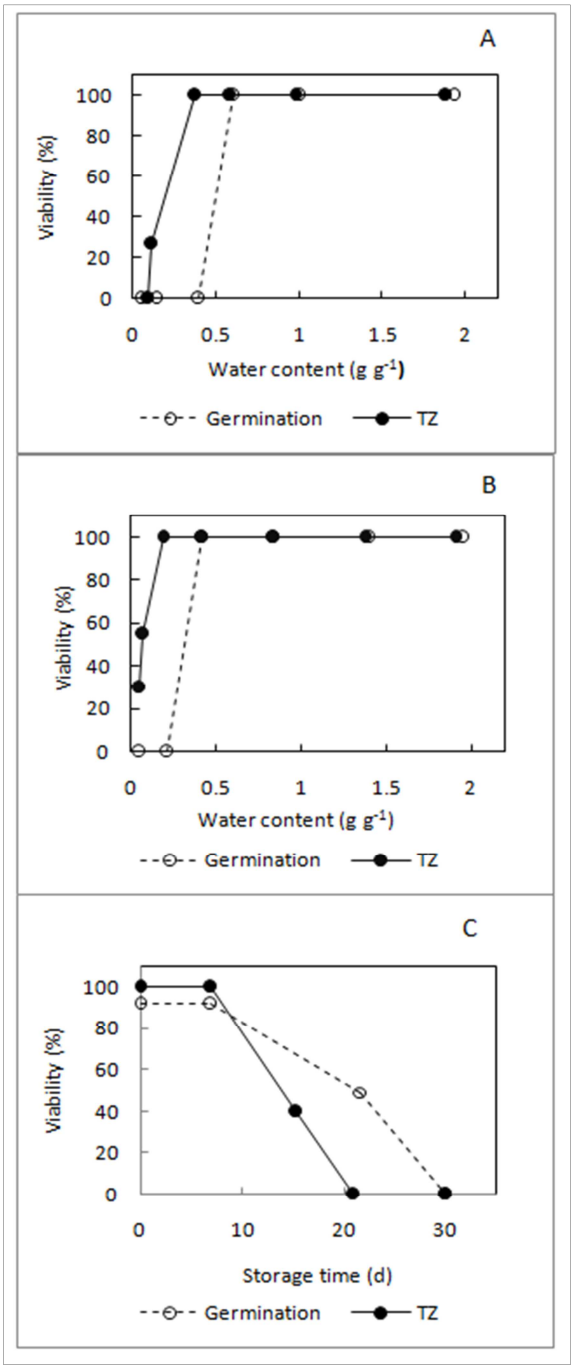
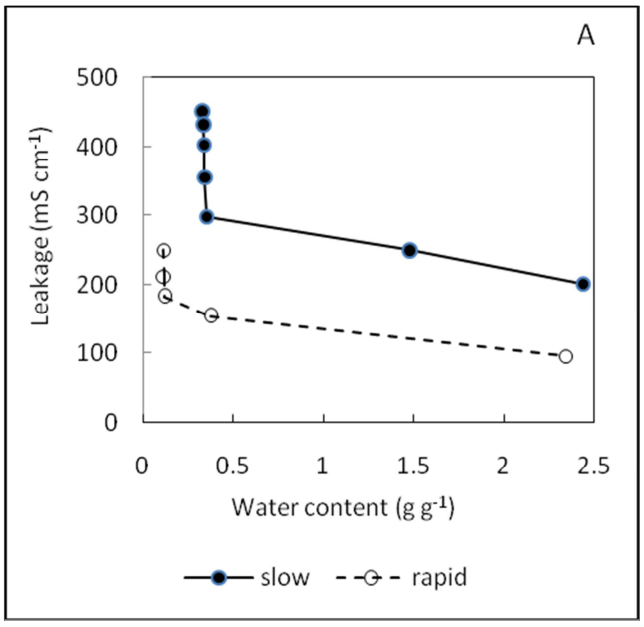


Figure 1. *The influence of water content on viability of axes of Trichilia emetica as assessed by germination (open circles, dashed line) or the tetrazolium test (filled circles, solid line), following slow (A) or rapid (B) drying, and hydrated storage life span as assessed by germination (open circles, dashed line) or the tetrazolium test (filled circles, solid line).*

For the tropical species tested (*S. madagascariensis, T. emetica*, *T. dregeana* and *A. marina*) the water content of the axes corresponding to 50% loss of viability was higher when assessed by germination than by the TZ test, and this was true for replicate harvest of *T. dregeana* and slowly dried *A. marina* taken two years later. The axes of *A. marina* did not dry to the extent anticipated, and the data are based on a reduction to 60% viability or germination for slowly dried material; no data were obtained for the TZ test for rapidly dried axes of *A. marina*. The difference between the results of the germination and TZ tests was apparent under both rapid and slow drying conditions. The axes of the orthodox *P.sativum* did not lose viability on rapid drying, and there were no differences between the results of the test on slow drying. The data for the temperate *Q. robur* were equivocal: on rapid drying the TZ test over-estimated desiccation tolerance (loss of 50% viability at lower water content), with the reverse being the case on slow drying.

For all species there was a gradual decline in viability during hydrated storage (e.g., Figure 1) with no marked differences in the viability assessed by germination or the TZ test.

Electrolyte leakage from axes increased during drying and hydrated storage, with leakage from rapidly dried axes generally being lower than from those slowly dried. Two patterns of leakage vs water content were observed: leakage from axes of *S. madagascaiensis* showed a marked increase commensurate with loss of viability (Figure 2), while in the other species there was a general increase with drying, but no marked change as viability was lost (as an example data for *T. emetica* are shown in Figure 2). During hydrated storage all species showed a general trend of increasing leakage with no marked changes in leakage pattern as viability was lost.



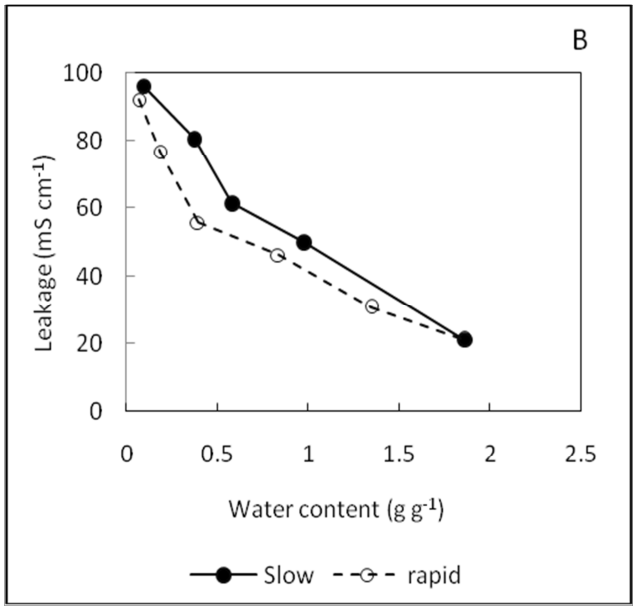


Figure 2. *The influence of water content on the leakage of electrolytes from axes of Strychnos madagascariensis (A) and Trichilia emetic (B) that been dried either slowly (filled circles, solid line) or rapidly (open circles, dashed line).*

4. Discussion

This study was under taken to determine whether tests other than germination could be used to assess the viability of embryonic axes from recalcitrant seeds after the axes had been subjected to drying or storage stresses. Such rapid assessments could be useful in studies on cryopreservatiion of germplasm of species producing recalcitrant seeds.

There was general agreement between the results of the germination assay and the TZ test in that there was a precipitous decline in viability as drying proceeded, and that axes dried rapidly survived to lower water contents than those dried slowly. This phenomenon has been reported frequently (reviewed by Berjak and Pammenter, 2008). However, when the data from the two methods of assessment were compared (water content at 50% viability loss) in four of the six species tested, the TZ test over estimated desiccation tolerance in that it indicated survival to lower water contents than did the germination test, and the effect was apparent with both rapid and slow drying. Such an effect has been observed before in recalcitrant seeds of *Zizania palustris* (Ntuli *et al*., 1997). The reason for this discrepancy is unknown: it could be a consequence of the need for extensive experience in the evaluation of TZ test for each species individually (reviewed by Pritchard 1996). Alternatively, whilst germination is an integrated response of the whole organism, the TZ test assesses activity of the mitochondrial electron transport chain, and it is possible that some other processes led to loss of germinability, whilst there was some residual post-mortem electron flow. In the present study the four species dsplaying this discrepancy between germinability and the TZ tesy were all of tropical origin, but it is unlikely to be a characteristic of embryos or axes from

tropical recalcitrant seeds as the data set is far too small to assess, and *Z. palustris* is not of tropical provenance.

Seeds of *P. sativum* are desiccation tolerant and hydrated axes did not lose viability on rapid drying. It is possible that under slow drying conditions the hydrated axes remained at a sufficiently high water content (relative to dry orthodox seeds) long enough for the processes associated with 'accelerated ageing' to occur, leading to viability loss from ageing rather than desiccation.

During hydrated storage there was a gradual decline in viability, with no marked or consistent differences between the germination and TZ tests. Presumably, under these conditions the degradative processes were sufficiently slow for electron transport to decline in tandem with other deleterious processes leading to viability loss.

Electrolyte conductivity of the leachate from drying axes showed two patters. In *S. madagascariensis* a slow increase as water content declined was followed by a very sharp increase concomitant with loss of viability. Such a pattern has been observed before (Pammenter *et al*., 1991). In the other species the electrical conductivity of the leachate increased regularly as the axes dried, and there was no marked increase at the water content corresponding to viability loss. However, in all cases leakage from slowly dried seeds was higher than from axes dried rapidly, consistent with the other measures of germinability.

In conclusion, this study was undertaken to identify a rapid, reliable assay of the 'condition' of hydrated and partially dried embryonic axes from recalcitrant seeds, as an aid to development of cryopreservation protocols. Germination and the TZ test yielded similar results except that in some species TZ tests indicated viability loss at a lower water content than the germination assays. However, the discrepancy was not large, and the patterns of viability loss were similar, and it is suggested that the TZ test can be taken to provide a reliable indicator (if not an exact measure) of the viability of partially dried embryonic axes excised from recalcitrant seeds. In all species except one electrolyte leakage increased gradually as drying proceeded, and this technique could not be used as a reliable marker of axis viability.

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