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# The influence of rehydration technique on the response of recalcitrant seed embryos to desiccation

**Rosa Perán, N.W. Pammenter*, Janine Naicker and Patricia Berjak**

School of Biological and Conservation Sciences, University of KwaZulu-Natal§, Durban, 4041 South Africa

## Abstract

The concept of 'imbibitional damage' arose when it was observed that considerable leakage of cell contents could occur when dry seed or pollen tissues are plunged directly into water. It is now common practice to imbibe dehydrated tissue slowly, to permit the re-establishment of functional membranes, prior to placing the tissue into liquid water. However, this argument may not hold if the tissue of interest is inherently desiccation-sensitive. Slow drying of desiccation-sensitive (recalcitrant) seeds or excised embryonic axes results in damage at high water contents, because it permits time for aqueous-based deleterious processes to occur. The same argument may apply if partially dried material is re-imbibed slowly, as this technique will also expose the tissue to intermediate water contents for protracted periods. This hypothesis was tested using embryos or axes from seeds of three recalcitrant species (*Artocarpus heterophyllus*, *Podocarpus henkelii* and *Ekebergia capensis*). Excised material was rapidly dried to water contents within the range over which viability is lost during drying, and re-imbibed either rapidly, by plunging directly into water, or slowly, by placing the material on damp filter paper or exposing it to a saturated atmosphere for several hours. Although details of the response differed among species and developmental stage, in all cases direct re-imbibition in water resulted in higher (or similar, but never lower) survival than either of the slow rehydration techniques.

**Keywords: dehydration, desiccation-sensitivity recalcitrant, rehydration, seeds**

*Correspondence
Fax: +27 31 260 1195
Email: pammente@ukzn.ac.za
§Formerly University of Natal.

## Introduction

When dry desiccation-tolerant tissue is imbibed in water, there is a transient leakage of solutes (discussed by Hoekstra *et al*., 1999). If the tissue is very dry, or if imbibition occurs at low temperatures, leakage can be extensive, reducing vigour (Pollock, 1969; Hobbs and Obendorf, 1972; Bramlage *et al*., 1978). These observations led to the concept of 'imbibitional damage', which can be reduced by pre-humidification in water vapour-saturated air prior to imbibition (e.g. Hobbs and Obendorf, 1972; Bramlage *et al*., 1978; Hoekstra and van der Wal, 1988), or by slow rehydration (Vertucci, 1989 for seeds; Kosanke *et al*., 1992 for dried bacteria), and it is now common practice to pre-humidify dry material prior to imbibition.

Recalcitrant seeds are damaged at water contents far in excess of those at which imbibitional damage occurs, and there is little evidence to suggest that such damage is important in desiccation-sensitive material. None the less, on the basis of the 'safety-first' principle, such pre-humidification has been used in some studies (e.g. Berjak *et al*., 1992, 1993; Leprince *et al*., 1998; Pammenter *et al*., 1998). An assumption implicit in this 'safety-first' principle is that slow rehydration, if not beneficial, at least is not damaging to the partially dehydrated tissue. However, this may not be true. It is well known that the rate of drying can affect the water content to which recalcitrant seeds or excised embryonic axes can be dried before loss of viability occurs, with slow drying leading to damage at higher water contents (Farrant *et al*., 1985; Normah *et al*., 1986; Pritchard, 1991; reviewed by Pammenter and Berjak, 1999). It is possible that a similar phenomenon may occur on rehydration; i.e. slow rehydration could lead to the further accumulation of damage. The objective of the study

reported in this paper was to assess this possibility. Embryos or embryonic axes of the recalcitrant seeds of three species were dried rapidly to water contents within the range over which viability is lost, and the effects of three rehydration rates on the subsequent germination were assessed.

## Materials and methods

### *Seed material*

Mature fruits of *Artocarpus heterophyllus* Lamk. (*Moraceae*) were hand-harvested from trees in a domestic garden in Durban (approximately 30°S, 31°E), immature fruits of *Ekebergia capensis* Sparrm. (*Meliaceae*) were collected from street trees in Durban, and mature fruits from street trees in Port Elizabeth (coastal city approximately 34°S, 26°E). Freshly shed seeds of the gymnosperm *Podocarpus henkelii* Stapf ex Dallim. Jacks (*Podocarpaceae*) were collected from below trees in a botanic garden in Pietermaritzburg (80 km inland from Durban). On receipt, seeds of *A. heterophyllus* and *E. capensis* were removed from the fruit, cleaned and surface sterilized by soaking in 1% NaOCl for 10 min, followed by three washings in distilled water. Seeds were kept in plastic bags at 6°C (*A. heterophyllus*) and 16°C (*E. capensis*) until used (maximum of 6 d). Seeds of *P. henkelii* were similarly surface sterilized and stored in sealed buckets elevated on a grid over wet paper at 6°C for periods ranging from 1 to 3 months. Storage at this temperature has been shown to be non-injurious to the seeds (Dodd and van Staden, 1981).

### *Desiccation treatment*

Embryonic axes from seeds of *A. heterophyllus* and *E. capensis*, and whole embryos of *P. henkelii*, were subjected to rapid (flash) drying by placing them on a grid in a jar half-filled with activated silica gel, with air stirred by a small fan (Pammenter *et al.*, 2002). Material was dried to two water contents falling within the range over which viability is lost under these drying conditions (see Results for details). Water contents were determined gravimetrically on individual axes or embryos by drying at 80°C for 48 h, and are expressed on a dry mass basis.

### *Rehydration and germination treatments*

Partially dried axes or embryos were rehydrated by one of three techniques: (1) rapidly by direct complete immersion in an excess of distilled water; (2) at an intermediate rate by placing specimens on moist, but not wet (no free liquid water), Whatman No. 1 filter paper in a Petri dish, such that the upper surface was exposed to the air, for 16 h; or (3) slowly, by exposure to a saturated atmosphere for various times, achieved by suspending the tissues on a mesh over water in a closed container lined with moist filter paper. Additionally, embryonic axes of *E. capensis* were rehydrated rapidly in a solution containing 1 µM $CaCl_2$ and 1 mM $MgCl_2$ (Ca/Mg solution); this treatment has been shown to improve the response of somatic embryos to cryopreservation (Mycock, 1999). The time for dehydrated tissues to rehydrate to their original water contents varied with the size of the tissue. Initial water content was regained within 1.5 h following direct immersion and 2–6 h on moist filter paper. Tissues exposed to a saturated atmosphere did not completely regain water, but reached a plateau after 20–22 h. Drying and rehydration of axes or embryos was conducted at room temperature (air-conditioned to 20–25°C). After rehydration, axes or embryos were surface sterilized as above, transferred to ¼ strength MS medium (Murashige and Skoog, 1962), supplemented with 30 g $l^{-1}$ sucrose and maintained at 25°C under a 16-hour photoperiod of intensity approximately 65 µmol $m^{-2}$ $s^{-1}$ (minimum of 20 axes or embryos per treatment). Germination was assessed as root growth after 30 d. As a control, undried material was subjected to the same rehydration and germination procedures. Within any one water content for each species, significance differences in germination were assessed using the chi-square test.

## Results

When embryonic axes of *A. heterophyllus* were rapidly dehydrated from an initial water content of 2.19 g $g^{-1}$, there was a loss of viability that increased as drying increased (Fig. 1). However, at each water content (except hydrated controls) germinability of material rehydrated in a saturated atmosphere was lower than that of axes rehydrated by the other techniques. As dehydration progressed, this phenomenon became more pronounced, and at a water content as high as 0.39 g $g^{-1}$, no germination occurred in axes rehydrated in a saturated atmosphere. Furthermore, axes so treated for 36 h showed lower germination than those treated for 16 h. At the lowest water content tested, germination of axes rehydrated by direct immersion was greater than that of axes rehydrated on moist filter paper.

A similar effect was observed with embryos of *P. henkelii* (Fig. 2), but it became more pronounced with increasing time of storage of seeds before testing the effects of rehydration treatment. When embryos extracted from seeds stored for 1 month were dried to a water content of 0.57 g $g^{-1}$, germinability decreased in the order of rehydration by direct immersion, moist

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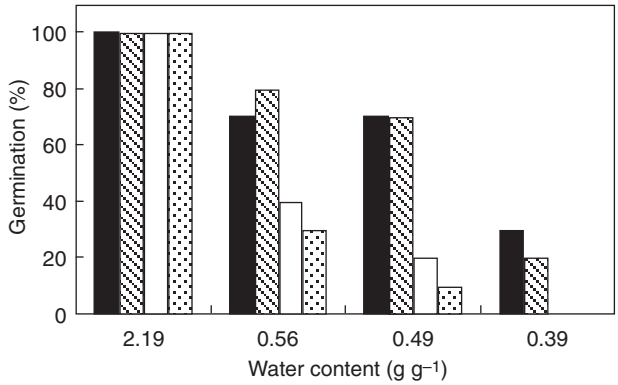


**Figure 1.** The influence of rehydration technique on the germination of embryonic axes of *Artocarpus heterophyllus* dried to the indicated water contents. Solid bar, direct immersion in water; diagonal-hatched bar, placed on moist filter paper; open bar, exposed to a saturated atmosphere for 16 h; stippled bar, exposed to a saturated atmosphere for 36 h. Within each water content (except for fully hydrated) the effect of rehydration treatment was significant (chi-squared, $P < 0.001$).

filter paper and exposure to a saturated atmosphere for 16 h, although different responses among rehydration techniques did not occur at the higher water contents (Fig. 2a). A similar pattern was apparent with drying of embryos from seeds stored for 2 months, although absolute values of germination were lower (Fig. 2b). In embryos from seeds stored for 3 months, differences among rehydration techniques manifested themselves at the relative high water content of 0.78 g $g^{-1}$, and under these conditions there was no germination of seeds rehydrated by exposure to a saturated atmosphere (Fig. 2c). Some, but poor, germination occurred in axes dried to the lower water content, irrespective of rehydration technique.

The response of embryonic axes of *E. capensis* to drying depended upon the stage of maturity of the fruits at the time of harvest. Fully hydrated axes of early season immature fruits were hardly germinable, except those exposed to a saturated atmosphere for 16 h (Fig. 3). Partial drying to 0.48 g $g^{-1}$ resulted in an increase in germination of axes rehydrated rapidly by direct immersion in either water or a Ca/Mg solution, but not of axes rehydrated more slowly. More severe drying to 0.16 g $g^{-1}$ reduced germinability, and at this water content axes rehydrated in a saturated atmosphere failed to germinate. At the higher water content, axes rehydrated in the Ca/Mg solution showed higher germination than those rehydrated in water, but this was not apparent in the axes dried to the lower water content.

Embryonic axes extracted from late-season, fully mature fruits of *E. capensis* showed slightly different responses compared with those of immature fruits

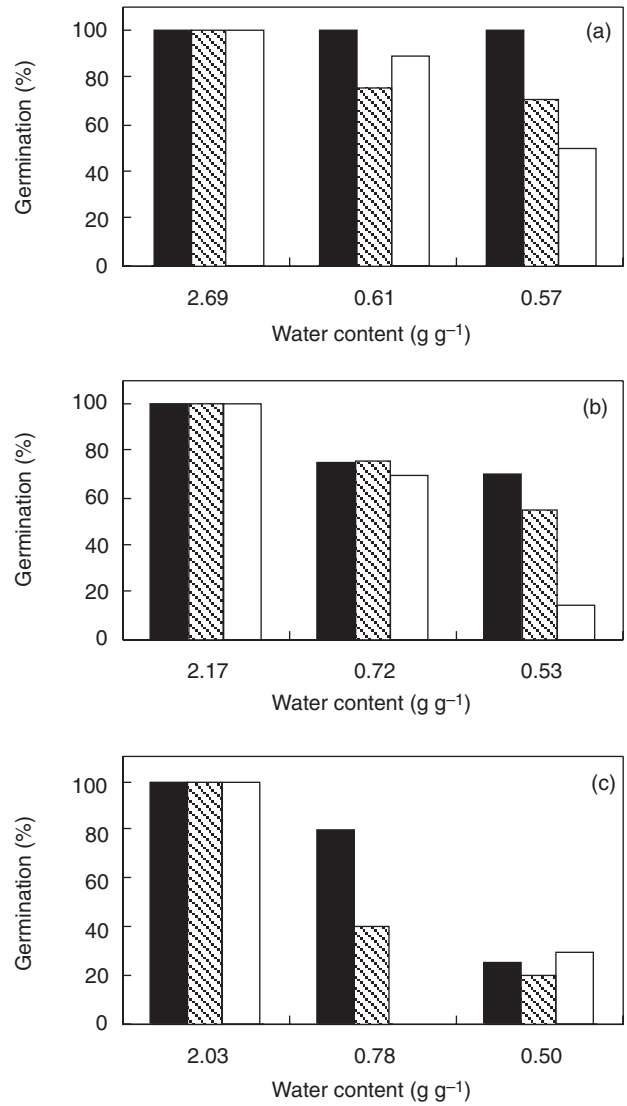


**Figure 2.** The influence of rehydration technique on the germination of embryos of *Podocarpus henkelii* dried to the indicated water contents after the seeds had been stored in the fully hydrated condition at 6°C for (a) 1 month, (b) 2 months or (c) 3 months. The highest water content for each trial is that of the embryo when the seeds were removed from storage. Solid bar, direct immersion in water; diagonal-hatched bar, placed on moist filter paper; open bar, exposed to a saturated atmosphere for 16 h. [In (c) the germination percentage of embryos dried to 0.78 g $g^{-1}$ and rehydrated in a saturated atmosphere was zero.] In (a) and (b) the effect of rehydration treatment was significant at the lower water content, and in (c) at the intermediate water content (chi-squared, $P < 0.001$).

(assessed as total axis survival, Fig. 4a). Partial drying slightly reduced survival of axes rehydrated directly in water and considerably reduced survival of axes rehydrated on moist filter paper; but, contrary to other species and immature fruits of this species,

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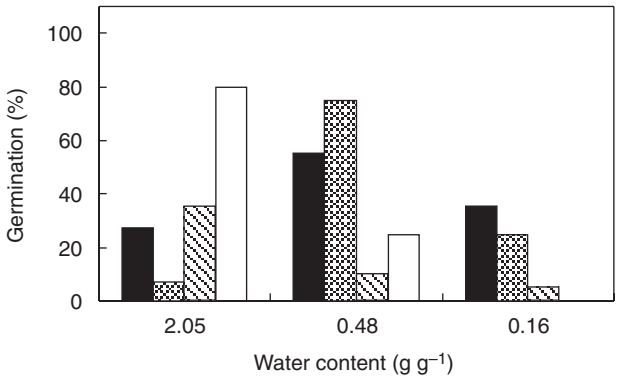


**Figure 3.** The influence of rehydration technique on the germination of embryonic axes of *Ekebergia capensis* removed from immature fruit and then dried to the indicated water contents. Solid bar, direct immersion in water; cross-hatched bar, immersed in a 1 µM $CaCl_2$/1 mM $MgCl_2$ solution; diagonal-hatched bar, placed on moist filter paper; open bar, exposed to a saturated atmosphere for 16 h. Within each water content, the effect of rehydration treatment was significant (chi-squared, $P < 0.001$).

rehydration in a saturated atmosphere was not deleterious relative to rapid rehydration. However, it was noted during collection of germination data that many axes initially scored positive for germination developed callus tissue, but further root growth did not occur. Figure 4a shows the percentage of axes initially scored as germinated (i.e. survival) and Fig. 4b the proportion of these 'germinated' axes that formed callus tissue. As the dehydration stress increased, so did the proportion of axes developing callus tissue. At the lowest water content, axes rehydrated directly in water or in a saturated atmosphere both showed higher rates of callus formation than those rehydrated by the other techniques. From the percentage of axes surviving and the proportion of these that developed callus tissue, the percentage of axes that showed 'successful germination' could be calculated as the (percentage survival) × (1 – proportion callusing) (Fig. 4c). At the intermediate water content ($0.40\ g\ g^{-1}$), axes rehydrated on moist filter paper showed very low successful germination, while there was no difference in germination success among the other rehydration techniques. At the low water content germination success was poor overall, but axes rehydrated in the Ca/Mg solution performed marginally better.

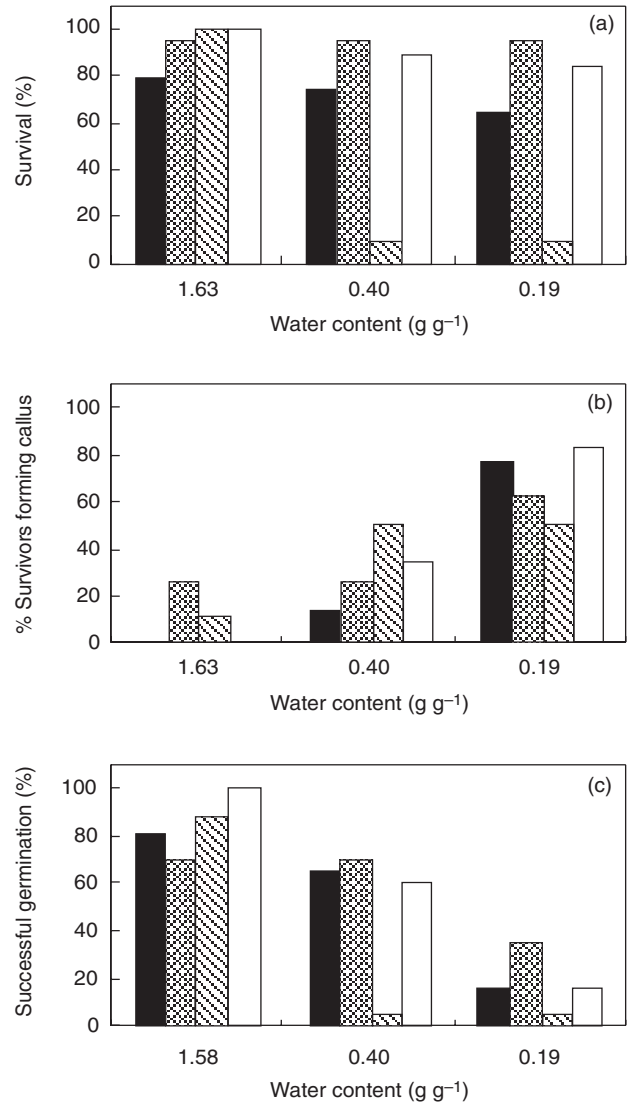


**Figure 4.** The influence of rehydration technique on the germination of, and callus formation by, embryonic axes of *Ekebergia capensis* removed from mature fruit and then dried to the indicated water contents. (a) Total survival (i.e. germinated normally or formed callus); (b) proportion of survivors that initiated callus; (c) successful germination (product of total survival and proportion not callusing). Solid bar, direct immersion in water; cross-hatched bar, immersed in a 1 µM $CaCl_2$/1 mM $MgCl_2$ solution; diagonal-hatched bar, placed on moist filter paper; open bar, exposed to a saturated atmosphere for 16 h. Rehydration treatment significantly affected the proportion of axes surviving after partial drying to both water contents, the proportion of survivors forming callus at all water contents, and true germination after both drying treatments (chi-squared, $P < 0.001$).

## Discussion

The influence of rehydration technique on the response of embryos or embryonic axes of recalcitrant seeds was tested on three species. For one species the influence of storage time, and on another the effect of seed maturity were also assessed. The general pattern observed was that slow rehydration in a saturated atmosphere resulted in lower germination than rapid rehydration by direct immersion.

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The rapid, and potentially damaging, leakage of solutes from orthodox seeds and pollen is observed at low water contents, when the membranes are presumed to be less than fully functional (Simon, 1974; Bramlage *et al*., 1978; Hoekstra and van der Wal, 1988; Hoekstra *et al*., 1999), and this leakage decreases as water content increases and membranes become functional. The situation with respect to recalcitrant seeds is somewhat different. If these seeds are dried to water contents characterizing those of orthodox seeds, they will die and leakage will continue, as membranes are never fully reconstituted. However, many studies on recalcitrant seeds are concerned with water contents higher than those associated with orthodox seeds, and it is at these intermediate water contents that aqueous-based degradative processes can occur (Vertucci and Farrant, 1995; Walters *et al*., 2001, 2002). The longer time that tissue spends at these intermediate water contents, the greater the damage that will accrue (Pammenter *et al*., 1998; Pammenter and Berjak, 1999), explaining the often observed effect of dehydration rate on the response of recalcitrant tissue to drying (e.g. Normah *et al*., 1986; Pammenter *et al*., 1991, 1998; Pritchard, 1991; Liang and Sun, 2000). The importance of the rehydration phase in the response of recalcitrant seeds to drying has been demonstrated in the studies of Leprince *et al.* (1998). They showed that coalescence of oil bodies in cotyledons of recalcitrant cocoa seeds occurred during reimbibition on damp filter paper, rather than drying. It is suggested that the effect of rehydration rate has a similar basis to the effect of drying rate: during slow rehydration the tissue spends extended time periods at intermediate water contents, permitting further damage to occur, whereas rapid rehydration prevents or slows this accumulation of damage. An alternative or additional effect could be that that the slow rehydration techniques used permit free access of oxygen to the tissue (unlike complete immersion), thereby enhancing the rate of production of damaging reactive oxygen species.

The effect of rate of rehydration on *P. henkelii* embryos became more pronounced with increasing seed storage time prior to the dehydration/rehydration studies (Fig. 2). Many species of recalcitrant seeds undergo developmental processes associated with germination while in storage (e.g. Pammenter *et al*., 1984; Farrant *et al*., 1989). The degree of desiccation sensitivity is associated with the degree of development (Farrant *et al*., 1997), and so desiccation sensitivity can increase in storage (Farrant *et al*., 1986; Berjak *et al*., 1992, 1993; Finch-Savage *et al*., 1996; Tompsett and Pritchard, 1998). *P. henkelii* seeds have been observed to be metabolically active and, if scarified, will germinate after several months in storage (Dodd and van Staden, 1981); presumably the associated continued development that leads to increased desiccation sensitivity accounted for the enhanced deleterious effect of rehydration rate with increased storage time in this species.

The influence of the stage of pre-shedding development was apparent in the studies on *E. capensis*. Embryonic axes from immature fruits were not fully germinable (Fig. 3). It has been shown that a mild dehydration stress on the seeds of this species enhances germination rate (Pammenter *et al*., 1998); the enhancement of germination observed in axes dried to 0.48 g $g^{-1}$ and rapidly rehydrated could be a manifestation of this effect. Similarly, the high germination obtained when fully hydrated axes were rehydrated in a saturated atmosphere could be a consequence of mild stress. It is possible that the atmosphere of the rehydration chamber was not fully saturated when the axes were introduced; this could have given rise to slight, initial and transient dehydration (rather than rehydration) of the fully hydrated tissue, leading to enhanced germination. Axes from mature fruit presented contrasting results, in that rehydration in a saturated atmosphere was not as damaging as on moist filter paper, even after taking into account the effect of callus formation (Fig. 4c). However, there are indications that rapid rehydration in a Ca/Mg solution gave rise to slightly better germination than rehydration in water of axes severely dried to 0.19 g $g^{-1}$. This phenomenon has been observed for cryopreserved somatic embryos (Mycock, 1999).

Irrespective of the confounding influences of the extent of pre- and post-shedding development, rapid rehydration of rapidly and partially dried embryos or axes of recalcitrant seeds gave rise to better (or no worse) germination than slow rehydration. This is ascribed to the increased opportunity for an aqueous-based deleterious process to occur during slow drying. Whatever the reason, the results of germination tests on partially dehydrated recalcitrant material can be influenced by the method of rehydration. Therefore, rehydration should be achieved as rapidly as possible during the course of initial studies of a given species. However, as in many attributes of desiccation-sensitive seeds, there is considerable inter-species variation, and ideally each species should be investigated individually. There are indications that use of a Ca/Mg solution may give rise to better germination than rehydration in water.

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