Sub-Imbibed Storage of Recalcitrant Seeds of Four Species

by:

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PREFACE

The experimental work as stated in this Masters thesis was carried out at the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, under the supervision of Professor P. Berjak, Professor N.W. Pammenter and Mr D Erdey.

This study represents original work by the author and no part of this work has been submitted in any form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

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ABSTRACT

The seeds of *Trichilia dregeana*, *Trichilia emetica*, *Podocarpus henkelii* and *Syzygium cuminii* display the characteristics typical of recalcitrant seeds. It is the phenomena of ongoing metabolic activity and desiccation sensitivity that render them unsuitable for storage by the conventional methods used for orthodox seeds.

Investigations on the storage responses of 'sub-imbibed' (partially dried) and fully hydrated seeds of all four species were carried out to study the effects of partial drying on viability and subsequent storage lifespan; i.e. to assess whether 'sub-imbibed' storage is feasible for these species. The outcome of this investigation was proposed to contribute to the resolution of the argument that storing recalcitrant seeds at lowered water contents might extend their longevity; i.e. storage at a relatively high water content but below the fully hydrated level, might prevent germination but would not be sufficient to be injurious to the seed.

Seeds of *T.* dregeana, *T. emetica*, *P. henkelii* and *S. cuminii* were dried to various target moisture contents (which were determined for each species in the initial drying experiment) and then subjected to storage for 3-22 weeks at 6, 16 and 25°C (in sealed containers). In parallel, seeds of each species were stored at the shedding water content. The seeds were periodically removed for sampling, and assessed for water content, germination, respiration, electrolyte leakage and microscopical features.

Storage temperature appeared to affect viability of seeds of *T. emetica* and *T. dregeana* which displayed characteristics of chilling sensitivity. Storage at 6°C was detrimental (when compared with seeds stored under the same conditions at 16 and 25°C), but regardless of whether the seeds were undried or partially dried prior to storage. The seeds of *P. henkelii* did not demonstrate chilling sensitivity, the viability not being compromised at 6°C compared with those seeds stored at 16 and 25°C.
Syzygium cuminii seeds were not subject to storage at 6°C because previous work indicated that they would be chilling-sensitive.

Storage of 'sub-imbibed' seeds of *T. dregeana*, *T. emetica*, *P. henkelii* and *Syzygium cuminii* does not to confer any benefit over seeds stored in the fully hydrated state; rather it appears to be deleterious to seed survival during storage. This was apparent from the assessment of viability, electrolyte leakage and respiration. Vigour and viability of the 'sub-imbibed' seeds of all species declined more rapidly than the fully hydrated seeds. The only exception was *P. henkelii* seeds stored at 25°C, the fully hydrated seeds showed no survival after 11 weeks in storage, while 88% of the 'sub-imbibed' seeds survived this period. These results were, however, attributed to the proliferation of fungi on the fully hydrated seeds at 25°C.

Although ultrastructural observations were made only on the *T. emetica* seeds, it was apparent that the cells from the 'sub-imbibed' seeds (after storage at 16 and 25°C) showed extensive degradation, with the intra-cellular components being largely unrecognisable. The cells from the seeds stored in the fully hydrated condition at 16 and 25°C maintained integrity and appeared metabolically active. In keeping with the suggestion that *T. emetica* seeds are chilling sensitive, the ultrastructure of the cells from both the 'sub-imbibed' and fully hydrated seeds showed deteriorative changes.

All the results of the present study indicated that storage in the 'sub-imbibed' state is deleterious to seed survival. It is apparent that the removal of water, however small a proportion, accelerates seed deterioration during storage. Thus 'sub-imbibed' storage has no practical application for the storage of recalcitrant seeds.

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1. INTRODUCTION

1.1 Recalcitrant and Orthodox Seeds

The terms, recalcitrant and orthodox, have been applied to seeds for the past 30 years since being introduced by Roberts (1973). It is widely accepted that orthodox seeds are those which acquire desiccation tolerance during development and undergo maturation drying, which is associated with extensive metabolic shutdown as the final phase of pre-shedding development (Berjak and Pammenter, 1997). The seeds acquire desiccation tolerance before they are exposed to severe water loss, and are able to withstand further post-shedding dehydration. In this dehydrated state the seeds are relatively quiescent and cannot entrain germinative metabolism, which will begin only when water becomes available and all environmental conditions are favourable (Bewley and Black, 1994). Orthodox seeds can be stored for extended periods that can be predicted under defined conditions of relative humidity (which determine seed water content) and temperature (Ellis and Roberts, 1980). It has been observed for orthodox seeds generally, that low water contents[1] and temperatures result in increased longevity, thus facilitating long-term storage (Roberts and Ellis, 1989). Orthodox seeds will withstand desiccation to water contents as low as 0.053 $g.g^{-1}$ (5% fmb), (Hong and Ellis, 1996). Conventional storage consequently, is based on the maintenance of seeds at low RH and low temperature.

At the other extreme, recalcitrant seeds undergo little or no maturation drying as a final phase in development (Farrant *et al.*, 1992; Tompsett and Prithchard, 1993; Lin and Chen, 1995; Pammenter and Berjak, 1999) and are thus hydrated at shedding. Under natural conditions recalcitrant seeds of many species, will initiate germination as soon as they fall to the ground, with no immediate requirement for additional water.+3 Shedding water content, which is a variable species characteristic, generally ranges from 0.43 - 4.0 $g.g^{-1}$ but can be even higher (Berjak and Pammenter, 1999). However, seeds of some species that are not recalcitrant may be shed at high water contents.

[1] Throughout this thesis, water contents are generally expressed as g H_2O g^{-1} dry mass ($g.g^{-1}$) and occasionally as a percentage on a fresh/wet mass basis.

Therefore shedding water content alone is not a defining feature; rather it must be seen with respect to the response of seeds to desiccation and storage (Berjak and Pammenter, 2001b).

Recalcitrant seeds begin development and progress through the stages of histodifferentiation and reserve deposition, as do orthodox seeds. It is after this point that development does not follow the same pattern (Farrant *et al.*, 1993). Recalcitrant seeds do not undergo the process of maturation drying. It appears that seeds do not enter this phase because the suite of mechanisms or processes that are operative in orthodox seeds is wholly or partially lacking (Pammenter and Berjak, 1999). As well as being shed at high water contents recalcitrant seeds are metabolically active at this stage (Berjak *et al.*, 1989; Chien and Lin, 1997; Farrant *et al.*, 1997; reviewed by: Finch-Savage, 1996). The seeds remain metabolically active for a physiologically-defined period which varies from one species to another, provided there is no substantial water loss. In some species, germination associated events are initiated shortly after, and occasionally even prior to, shedding (Farrant *et al.*, 1986; Farrant *et al.*, 1988; Motete *et al.*, 1997) and as these events progress, the seeds become increasingly more sensitive because they require more water to complete the process (Farrant *et al.*, 1989, Pammenter *et al.*, 1991; Berjak *et al.*, 1992; Berjak *et al.*, 1993). Initial removal of water after shedding may transiently result in enhanced metabolic activity (Erdey, pers. comm.[2]) which is expressed as an increase in totality and rate of germination. However further dehydration rapidly results in viability loss (Berjak *et al.*, 1984).

Because they are desiccation sensitive, recalcitrant seeds cannot be dried to the water contents suitable for conventional storage. A further complication is that non-orthodox seeds of many species do not tolerate storage at low temperatures. A study on hydrated seeds of *Azadirachta indica* originating from Mombasa, Kenya; showed that a decline in viability accompanied by deranged axis cell ultrastructrure, occured in response to chilling (Berjak *et al.*, 1995), and those of *Avicennia marina* are rapidly killed at 6°C. Seeds of

[2] D.P. Erdey, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa.

Theobroma cacao (cocoa) appear to be damaged at temperatures below 15°C (Chin and Roberts, 1980). Seeds of many tropical species may similarly be chilling-sensitive at temperatures well above 0°C (Corbineau and Côme, 1988; Chin *et al.*, 1989; Tompsett, 1994 and Ajayi, 2006). However temperate recalcitrant seeds (e.g. *Quercus* spp., *Aesculus hippocastunum*) generally do tolerate chilling (Chin and Roberts, 1980).

The chilling sensitivity of fruits and leaves of tropical plants has been attributed to a conformational change in cell membranes from liquid crystalline to gel phase (Wang *et al.*, 1982) The transition temperature of membranes in chilling sensitive tropical plants has been estimated at 10°C (Crowe *et al.*, 1989), which is considerably above that of chilling tolerant plant species. The basis of chilling injury in seeds is not well understood but is suggested to include several factors. Respiratory capacity and coordination can become impaired when enzymes are cold-labile (Guy, 1990 and Salahas *et al.*, 2002) or when the mitochondrial inner membrane changes from the liquid crystalline to the gel state (Bedi and Basra, 1993; Berjak *et al.*, 1995). At low temperatures the cytoskeletal elements dismantle (in chilling-sensitive plants) (Raison and Orr, 1990), resulting in derangement of metabolism as certain biochemical pathways operate as multi-enzyme complexes anchored to the cytoskeleton (Masters, 1984 and Masters, 1996). Impaired or unregulated metabolism over an extended time period is suggested to lead to uncontrolled free radical generation, cellular death and ultimately death of the seed (Pammenter *et al.*, 1994).

The phenomenon of seed recalcitrance occurs in both gymnosperms and angiosperms (dicotyledenous and monocotyledonous species). There are some families where all investigated species have desiccation-tolerant seeds (Chenopodiaceae, Compositae, Labiatae, Solanaceae and Pinaccae) and other families where all species investigated produce desiccation-sensitive seeds (Rhizophoraceae and Dipterocarpaceae) (Hong and Ellis, 1996). However within the gymnosperms and the angiosperms there are also single genera which have individual species producing either orthodox or recalcitrant seeds (e.g. *Podocarpus*). Although there are no clear taxomonical relationships regarding the occurrence of the phenomenon, seed recalcitrance has been suggested to be the ancestral seed condition (von Teichman and van Wyk, 1994; Pammenter and Berjak, 2000).

According to those authors it is likely that desiccation tolerance of seeds evolved early because selective pressures would have been strong and the disadvantages not significant. However it is the opinion of Farnsworth (2000) and Dickie and Pritchard (2002) that the desiccation-tolerant seed condition was ancestral and that recalcitrance has been derived independently several times.

Ellis *et al.* (1990) found the post-harvest behaviour of coffee seeds to be inconsistent with that expected of either orthodox or recalcitrant seeds. The seeds were able to survive to considerably lower water contents than those observed for recalcitrant seeds, but did not survive the degree of desiccation that would be tolerated by orthodox types. Additionally, a reduction in temperature actually decreased viability, which is not found to be the case with orthodox seeds (Ellis *et al.*, 1990). The seeds were described as intermediate between recalcitrant and orthodox in terms of the responses to conventional storage techniques (Ellis *et al.*, 1990; Hong and Ellis, 1996), thus adding a third grouping in which seeds could be categorised according to their post-harvest behaviour and response to conventional storage techniques. Hong and Ellis (1996) stated that seeds characterised as intermediate lose viability when desiccated below water contents of 0.14 - 0.11 $g.g^{-1}$. However, regardless of the relationship between viability and declining water content, it may be misleading, to describe a 'critical water content' below which seeds lose viability as the response to desiccation depends on several factors such as temperature and rate of drying (e.g. Pammenter *et al.*, 1998).

As more species have been studied, however, it has become more apparent that there is a spectrum of responses to desiccation and therefore there are many seeds which do not fit into these distinct categories. Berjak and Pammenter (1999) expressed the opinion that categorisation of seeds as orthodox, intermediate and recalcitrant is an over-simplification. A variable response to water loss is seen even among orthodox species, some withstanding more severe dehydration than others (Walters, 1998). The concept of a continuum of post-harvest seed behaviour is favoured by Berjak and Pammenter (1999), which accounts for those seeds which do not fit into the distinct categories. At the one extreme are those species that produce highly recalcitrant seeds that cannot withstand any

significant water loss without their viability being compromised (Berjak and Pammenter, 1999). At the other end of the continuum are the highly desiccation-tolerant orthodox seeds that can withstand substantial water loss and remain viable. According to those authors, seeds across the species probably fall along the continuum, between the two extremes, according to their specific response to desiccation and other characteristics. Even within the recalcitrant seed category, there is marked variation in the response to desiccation, which has led to the recognition of highly, moderately and minimally recalcitrant seeds (Farrant *et al.*, 1988; Berjak *et al.*, 1989). As a further complication there can be considerable variation within a species, with regard to the extent of desiccation they can survive, if the seeds are harvested at different stages of maturity. This is particularly apparent in seeds that show orthodox storage behaviour, since immature seeds of orthodox species are desiccation intolerant. Studies of the acquisition and loss of desiccation tolerance during embryogenesis and germination have demonstrated that tolerance to desiccation progressively increases with seed maturation. While this is particularly so for orthodox seeds, even most of those that are recalcitrant are relatively least desiccation sensitive at, or near, shedding (Berjak *et al.*, 1990; Berjak *et al.*, 1992; Berjak *et al.*, 1993; Finch-Savage, 1992; Farrant *et al.*, 1993; reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak, 1999) and decreases with germination (Senaratna and McKersie, 1983; Leprince *et al.*, 1990).

The differing responses among recalcitrant seeds may depend on the habitat from which the seeds originate, as well as the rate at which post-harvest germinative events proceed. Minimally recalcitrant seeds are able to withstand more water loss than highly recalcitrant seeds probably because germinative events proceed more slowly (Farrant *et al.*, 1988). The slower progress of germination at the shedding water content may have a selective advantage because it allows the seeds to remain viable for a longer period in the natural environment and this criterion can be exploited as it should facilitate more extended hydrated storage. Such seeds generally occur in areas that are not continuously conducive to seedling establishment. Highly recalcitrant seeds usually occur in habitats where water is abundant and conditions are continuously favourable for seedling establishment. In such species, germination commences rapidly and these seeds cannot withstand any

significant water loss. In the majority of cases, highly recalcitrant seeds are, in effect, seedlings (Berjak *et al.*, 1989). It would be more appropriate then to compare them with seedlings or young plants grown from orthodox seeds, than with the seeds themselves.

Germinated orthodox seeds can be a useful model system for studies of recalcitrance (Sun, 1998) as, like recalcitrant seeds, they are desiccation sensitive. The critical water content (defined as the water content where viability is lost) of germinated orthodox seeds is also affected by developmental stage, drying rate and drying temperature (Hong and Ellis, 1990; Pammenter *et al.*, 1991; Leprince *et al.*, 1995). In both recalcitrant seeds and orthodox seedlings, desiccation sensitivity is associated with a high metabolic rate and injury has been suggested to be related to free radical-mediated oxidative damage (Farrant *et al.*, 1988; Hendry *et al.*, 1992; Leprince *et al.*, 1995). Recalcitrant seeds cannot be maintained for extended periods in hydrated storage, because they are metabolically active and undergo germination-associated changes (Pammenter *et al.*, 1994). The storage stability of germinated orthodox seeds is also significantly reduced compared with control seeds at the same water content (Hong and Ellis, 1992).

The significant property that allows orthodox seeds to withstand water removal is that of the acquisition and maintenance of desiccation tolerance (Leprince *et al.*, 1993). Desiccation tolerance ensures the survival of the species over periods when environmental conditions are not favourable for seedling establishment and survival (Leprince *et al*, 1993). It also permits the invasion of areas subject to disturbance and periodic stress, including relatively dry habitats.

1.2 The Strategies that Contribute to Desiccation Tolerance

The biological basis for recalcitrant seed behaviour was described as being largely unknown, and possibly not the same for all species (Chin, 1988). However, several factors have since been identified as distinguishing the physiology of recalcitrant seeds from that of orthodox types. The acquisition of desiccation tolerance is a complex, programmed developmental event. Oliver and Bewley (1997) made the point that a distinction can be drawn between desiccation tolerance achieved through processes of

repair mechanisms (during rehydration) or through protection of membrane and cellular constituents (that limit damage during water removal) and this concept is further explored by Oliver *et al.* (2000). For orthodox seeds, studies on desiccation tolerance mechanisms suggest that protective mechanisms are significant and probably more prevalent (Pammenter and Berjak, 1999). According to those authors there is no single process that is responsible for desiccation tolerance. Rather, a suite of cellular and intracellular processes has been suggested to confer desiccation tolerance. The effective expression and functioning of all of the processes detailed by Pammenter and Berjak (1999) is suggested to allow orthodox seeds to withstand water loss and a significant level of dehydration. Therefore, the extremely desiccation tolerant types would be expected to possess the full suite of mechanisms, while the recalcitrant types showing sensitivity to desiccation are thought to be lacking in at least one - but probably several - of the necessary components (Pammenter *et al.*, 1991; Pammenter and Berjak, 1999). This would account for the relative degree of desiccation sensitivity shown by seeds of different species.

Some of the strategies that have been suggested to contribute to desiccation tolerance include:

1) **Accumulation of insoluble reserves and a reduction in the degree of vacuolation** (Farrant *et al.*, 1993; Vertucci and Farrant, 1995; Farrant *et al.*, 1997): This would increase mechanical resilience of the cells to dehydration. Farrant *et al.* (1997) examined seeds of *Avicennia marina* (highly recalcitrant), *Aesculus hippocastanum* (moderately recalcitrant) and *Phaseolus vulgaris* (orthodox) prior to maturation drying. Those authors found the different degrees of vacuolation and insoluble reserve deposition to correlate with the degree of desiccation tolerance.

2) **The reaction of the cytoskeleton** (Pammenter and Berjak, 1999; Mycock *et al.*, 2000; Berjak and Pammenter, 2001a; Gumede *et al.*, 2003): The cytoskeleton provides integrated intracellular support. Upon dehydration the network must disassemble (Mycock *et al.*, 2000) but also re-assemble in an orderly manner, upon imbibition in orthodox seeds. This does not occur after dehydration to levels affecting viability of recalcitrant

seeds (Mycock *et al.*, 2000; Gumede *et al.*, 2003), leading to loss in intracellular support and structural organisation (Pammenter and Berjak, 1999; Mycock *et al.*, 2000). Thus failure of the cytoskeleton to re-assemble is probably a major damaging factor in such seeds. The nucleoskeleton too, has been found to be similarly affected by dehydration in the recalcitrant seeds of *Bridelia micrantha* (Merhar *et al.*, 2004).

3) **The conformation of the DNA** (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter 2001a): Dehydration affects the integrity of the nuclear architecture, including the nucleoskeleton as mentioned above, as well as the genetic material (Boubriak *et al.*, 2000). It is vital that the integrity of the DNA is maintained in the desiccated state or repaired rapidly during re-hydration (Pammenter and Berjak, 1999). However, for recalcitrant seeds there is little information available on this aspect.

4) **Intracellular de-differentiation** (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001b): Mature orthodox seeds characteristically display intracellular de-differentiation (simplification and minimisation of intracellular structures [reviewed by Vertucci and Farrant, 1995]). De-differentiation prior to, or simultaneous with, dehydration in orthodox seeds strongly suggests that membranes (and cytoskeletal components) are susceptible to dehydration. De-differentiation in an ordered manner is considered to be a pre-requisite for the survival of dehydration (Pammenter and Berjak, 1999).

5) **The accumulation of protective molecules including late embryogenic abundant proteins (LEAs) and non-reducing sugars** (Blackman *et al.*, 1991; reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter 2001a, b): These proteins are involved in the acquisition and maintenance of desiccation tolerance. LEA proteins are ubiquitous in both dicotyledonous and monocotyledonous orthodox seeds (Thomas, 1993): however, they are not uniformly absent among recalcitrant seed species. The expression or absence of LEAs cannot be taken in isolation as an indication that a particular species will or will not withstand dehydration (Kermode, 1997). However, in association with the presence or absence of other factors, the absence of LEAs may contribute to the degree of desiccation tolerance.

6) **The presence and efficient operation of free radical scavenging systems** (Leprince *et al.*, 1993; reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001b): Orthodox seeds exhibit a range of anti-oxidant processes (e.g. Hendry, 1993; Leprince *et al.*, 1993) that operate efficiently under conditions of water stress. During dehydration of recalcitrant seeds damage, probably ascribable to uncontrolled free-radical production, occurs (Berjak and Pammenter, 2001a) as was observed in recalcitrant seeds of *Quercus robar*, *Castanea sativa* and *Aesculus hippocastanum* (Finch-Savage *et al.*, 1994). This suggests not only that there is production of free radicals, but also they are not being removed or quenched because of ineffective anti-oxidant systems. These systems are present in desiccation-sensitive tissues (Hendry *et al.*, 1992; Finch-Savage *et al.*, 1993), but possibly become impaired under conditions of water stress (Smith and Berjak, 1995).

7) **'Switching off' of metabolism** (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter 2001a, b): If metabolism continues unabated during dehydration, it must become unbalanced which could lead to uncontrolled production of free radicals. 'Switching off' of metabolism would counteract such damage. In developing orthodox seeds of *Phaseolus vulgaris*, Rogerson and Matthews (1977) observed that a substantial decline in respiratory rate occurs which they suggested was caused by the sharp decline in respiratory substrates, recorded to accompany maturation drying. Those authors suggested that this decline is a vital event which allows orthodox seeds to tolerate substantial loss of water. Farrant *et al.* (1997) indicate that the decline in respiratory rate correlated with mitochondrial dedifferentiation, occurs prior to maturation drying in *P. vulgaris* seeds. In desiccation-sensitive seeds, lethal damage occurs in the water content range 0.45 - 0.25 $g.g^{-1}$ where metabolism continues, but in an uncontrolled and unbalanced manner (Vertucci and Farrant, 1995).

8) **The presence of an effective oleosin layer around lipid bodies** (Leprince *et al.*, 1998): The role of oleosins in desiccation tolerance has been suggested to be of importance primarily in those seeds which have a high lipid content or in seeds such as *Azadirachta indica* (Berjak *et al.*, 1995) where oil bodies are large (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a). One of the consequences of deterioration in seeds is that the lipid bodies in the cells coalesce (Smith and Berjak,

1995). Oleosins are specialised proteins bounding, and maintaining the integrity of, the lipid bodies. In their study on desiccation-tolerant and -sensitive oil seeds, Leprince *et al.* (1998) suggested that there was a lack of effective oleosins bounding oil bodies in the sensitive types. According to those authors, no oleosins were detected in the highly desiccation sensitive seeds of *Theobroma cacao* and in both *Quercus rubra* and *Azaditachta indica* the oleosin: oil body ratio was very small. However, an intermediate species (*Coffea arabica*), showed a higher ratio similar to the oil rich orthodox seeds of *Brassica napa*. Upon rehydration, recalcitrant seeds show coalescence of lipid bodies as one of the abnormalities resulting from dehydration (Leprince *et al.*, 1998), which could result from the deficiency of the oleosins surrounding the oil bodies. It has been suggested by those authors that oleosins may be particularly important in stabilising the oil bodies of seeds in which these intracellular compartments are very large.

9) **The role of sugars in the acquisition of desiccation tolerance has been extensively considered** (reviewed by Vertucci and Farrant, 1995; Berjak and Pammenter, 1997): It has been observed that maturing orthodox seeds accumulate non-reducing sugars, especially sucrose and oligosaccharides of the raffinose series (Koster and Leopold, 1988; Leprince *et al.*, 1990) or galactosyl cyclitols (Horbowicz and Obendorf, 1994; Obendorf, 1997). These sugars have been implicated in the desiccation tolerance in two major ways in orthodox seeds: Firstly, the "Water Replacement Hypothesis" received prominence (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a). It should be noted, however, that little *in vivo* evidence has ever been reported for the "Water Replacement Hypothesis", which is consequently no longer considered to be a feasible explanation for the role of sugars in desiccation tolerance (Williams and Leopold, 1989; Leopold *et al.*, 1994; reviewed by Hoekstra *et al.*, 1997). Secondly, is the implication of sugars in the formation of the glassy (vitrified) state, which involves the vitrification of the aqueous phase, which may reduce the deleterious effects of unbalanced metabolism (Koster, 1991; Leopold *et al*, 1994; reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a). As water is removed, the sugars are held to form high-viscosity, amorphous super-saturated solutions. The presence of glasses has been implicated in the maintenance of viability for extended periods in the dry state, rather than conferring desiccation tolerance. In addition, the formation of oligosaccharides results in a depletion

of monosaccharides, which depletes respiratory substrates, resulting in a dramatic decrease in the damaging reactions that can occur (Rogerson and Matthews, 1977; Koster and Leopold, 1988). It should be noted, however, that Buitink (2000) is of the opinion that proteins are the major effectors of the highly viscous intracellular condition in dry, orthodox seeds.

10) **The presence and operation of repair mechanisms during re-hydration** (reviewed by Pammenter and Berjak, 1999; Berjak and Pammenter, 2001a,b): While protection may be predominant in mosses and certain resurrection plants (Oliver and Bewley, 1997) during dehydration, there is evidence that repair mechanisms constitute a major mechanism when orthodox seeds are rehydrated. In recalcitrant seeds, the necessary repair mechanisms may be present but unable to operate beyond certain levels of dehydration.

The challenge of storing recalcitrant seeds resides in their desiccation sensitivity, which prevents the use of conventional low relative humidity (RH) storage. Unlike orthodox seeds, recalcitrant seeds are shed with sufficient water for germinative metabolism to be initiated without the need for additional water (Motete *et al.*, 1997). Thus ongoing metabolism results - sooner or later, depending on the species - in the onset of germination when seeds are stored at the water content characteristic of the newly-shed state. However, once germination proceeds beyond a certain point, there is a requirement for a higher water concentration (Farrant *et al.*, 1988; Berjak *et al.*, 1989), thus tissue and intracellular water concentration becomes limiting, and if exogenous water is not supplied, the seeds will die (Pammenter *et al.*, 1994).

1.3 Dehydration of Recalcitrant Seeds

Generally, recalcitrant seeds tolerate very little water loss. However, it has been noted that a far more substantial water loss can be tolerated without compromising viability, for individual species, the more rapidly water is removed (Farrant *et al.*, 1986; Berjak *et al.*, 1989; Berjak *et al.*, 1990). This phenomenon has been reported for *Landolphia kirkii*, *Castanospermum australe*, *Scadoxus membranacues*, *Hevea brasiliensis*, *Camellia*

sinensis and a variety of other species (Berjak *et al.*, 1989). With orthodox seeds, better desiccation tolerance is achieved with slower drying rates (Bewley and Black, 1994; Oliver and Bewley, 1997), which are thought to allow sufficient time for the induction and operation of protection and repair mechanisms (Pammenter *et al.*, 2000). In contrast, recalcitrant material does not appear to possess adequate mechanisms that would be effective during slow drying. While rapid drying increases the extent of water loss tolerated by recalcitrant material, slow drying is thought to allow sufficient time for continuation of metabolic activity (before water is removed to impose a limitation on these events) which results in unbalanced aqueous-based reactions leading ultimately to membrane degradation and viability loss (Berjak *et al.*, 1989; Pammenter *et al.*, 1991; Berjak *et al.*, 1993; Pammenter *et al.*, 1998; Walters *et al.*, 2001). Additionally, the products of the damaging reactions that occur may accumulate while the tissues are exposed to unfavourable water content ranges for extended periods (Pammenter *et al.*, 1998). Rapidly dried seeds can survive to lower water contents because metabolism is swiftly halted by dehydration (Pammenter *et al.*, 1998), i.e. insufficient time is spent at intermediate water contents for metabolism-linked damage to accumulate (reviewed by: Vertucci and Farrant, 1995; Pammenter and Berjak, 1999; Walters *et al.*, 2001). Pammenter *et al.* (1998) suggested that a slow drying rate removes water relatively evenly from the various tissues while rapid drying results in an uneven removal of water across the tissue, facilitating retention of non-damaging water concentrations in the meristems, thus perhaps also contributing to the survival of the rapidly-dried seeds. It has further been suggested that appropriately slow dehydration of metabolically active recalcitrant seeds allows them time (before damage predominates) to progress further towards germination, when they become more desiccation sensitive, thus losing viability at higher water contents (Farrant *et al.*, 1985; Berjak *et al.*, 1989, 1992, 1993). However, this would occur only if the drying rate were slow enough not to curtail ongoing germination (Pammenter and Berjak, 1999). In general, the harmful consequences of relatively slow drying are more likely to be a result of unbalanced metabolism at 'intermediate' water contents, than a consequence of increasing desiccation sensitivity as a result of the progression of germination. A restriction is generally imposed on the drying rate that can be achieved, because most recalcitrant seeds are relatively large.

Rapid drying does not impart desiccation tolerance to recalcitrant axes, which are intrinsically desiccation sensitive. Although rapid drying generally enables recalcitrant material to tolerate drying to lower water contents than if slowly dried, these seeds or axes will still not survive dehydration beyond a lower limit, which is always considerably higher than the water content to which orthodox or intermediate seeds can be dried (Pammenter and Berjak, 1999). This limit is usually close to the water content below which the remaining water is structure-associated and non-freezable (Pammenter *et al.*, 1993). Nevertheless, even if this lower water content limit is not exceeded, rapidly dried axes will not remain viable for any appreciable period under ambient or chilled conditions (Walters *et al.*, 2001).

Another factor appears to affect the extent to which recalcitrant seeds can withstand dehydration is the temperature at which dehydration occurs (e.g. Kovach and Bradford, 1992). It is therefore important to take dehydration rate and temperature into account when attempting to determine a 'critical water content' beyond which subsequent dehydration will result in viability loss for that particular species.

1.4 Storage of Recalcitrant Seeds

Species producing recalcitrant seeds occur primarily in the hot, humid, tropical and sub-tropical regions of the world, although a few originate in the temperate regions. The number of plants known to produce recalcitrant seeds has steadily grown (Tweddle *et al.*, 2003) and there is increasing evidence that orthodox seeds behaviour might not be the norm, as has previously widely been held. Many economically important species produce recalcitrant seeds, including tea (Berjak *et al.*, 1993), lychee, mango and commercial rubber (Chin and Roberts, 1980) as well as jackfruit, oil palm, almond, cocoa, coconut, walnut and avocado (Chandel *et al.*, 1996). Furthermore, with extended research, increasing numbers of tropical forest tree species have been established as producing recalcitrant seeds (e.g. *Trichilia* spp. [Maghembe and Msanga, 1988; Choinsky, 1990] and *Shorea* spp. [Krishna and Naithani, 1998; Tsan, 2000; Berjak and Pammenter, 2004]).

Awareness about loss of plant genetic resources and the need for environmental protection has increased, thus great efforts are being made to ensure the conservation of plant genetic resources. Conservation of orthodox seeds has been assisted by accumulated knowledge about their storage behaviour, for which technology is well developed (Chin, 1995). However, finding suitable methods for successful storage of recalcitrant seeds for the short-, medium- or long-term poses a great challenge. The most likely solution has, already for 20 years, been considered to be cryostorage (at -196°C) of the germplasm in liquid nitrogen (Roberts *et al.*, 1984; Chmielarz, 1997; Berjak *et al.*, 1999; Berjak *et al.*, 2000; Engelmann, 2000; Wesley-Smith, 2002).

1.4.1 Cryopreservation

Cryopreservation protocols are determined empirically for each species because the response to the various protocols varies considerably, depending on the species and the explants used (Kioko *et al.*, 1998). Germplasm of a few desiccation-sensitive species has been successfully cryopreserved e.g. *Quercus robur* (Berjak *et al.*, 1999) *Ekebergia capensis* (Walker, 2000) and *Acer saccharum* (Wesley-Smith, 2003). However, for many species a successful protocol remains elusive. In general, cryopreservation involves the storage of tissues at ultra-low temperatures usually in liquid nitrogen (-196°C). Various manipulations can determine the success or failure of the protocol, viz. surface sterilisation, use of cryoprotectants, the extent and rate of dehydration, cooling (freezing) rates (Kioko *et al.*, 1998; Berjak *et al.*, 1999), as well as the medium used for rehydration (Berjak and Mycock, 2004).

The dehydration step is vital as it reduces the amount of free water available, if freezing is carried out at high water contents lethal ice crystal formation may occur as the tissue is passed through the critical temperature ranges (Becwar *et al.*, 1983). Whole seeds can usually not be dried to the levels required for successful cryopreservation. Isolated embryonic axes, somatic embryos and meristems are more suitable as the small size allows for fast dehydration and rapid freezing (Pammenter and Berjak, 1999).

Drying methods have been developed to optimise desiccation of recalcitrant seed material. In 1990 Berjak *et al.* developed a rapid dehydration technique they called 'flash drying' by which the excised embryonic axis is exposed to a stream of air (approximately 10 $l.min^{-1}$). The development of this technique allows dehydration to relatively low water contents to be rapidly achieved in a non-injurious manner. Wesley-Smith *et al.* (1999) modified the apparatus to achieve even more rapid dehydration. A study on *Trichilia dregeana* demonstrated that axis flash drying is a far more effective technique than is slow drying of axes within the seeds (Kioko *et al.*, 1998). Flash drying serves as an effective method to provide minimally destructive dehydration resulting in axes in a condition that should facilitate cryopreservation (Vertucci *et al.*, 1991). It should be noted that even if there is some cell death, the degree of dehydration must ensure survival of a critical number of potentially meristematic cells (Berjak *et al.*, 1999).

The rate at which the tissue is cooled is also a critical factor in determining the success of cryopreservation protocol. As with drying rate, more rapid cooling is usually found to be less injurious to the axes (Wesley-Smith *et al.*, 1999). Rapid freezing facilitates cryopreservation especially of tissues that are still at relatively high water contents after dehydration. The explants are taken through the temperatures where ice is formed so quickly that ice crystals either do not form or they do not grow large enough to be injurious (Wesley-Smith *et al.*, 1992). Rapid cooling is accomplished by direct immersion into liquid nitrogen or another cryogen, achieving cooling rates of several hundred degrees per second (Wesley-Smith *et al.*, 1999). Thus what has emerged is that, for each species, there appear to be optimal drying and cooling rates, although these are unlikely to be exclusive to individual species. Following cryopreservation the frozen tissue must be thawed in such a manner as to avoid injury due to ice crystal formation during warming. As with cooling rate, the faster the intracellular solution passes through the temperature range where crystallisation occurs, the higher the survival as less damage is caused by ice crystals (Wesley-Smith, 2003). The composition of the rehydration medium too, has been found to be critical for normal seedling production (Berjak and Mycock, 2004)

Cryoprotectants are used extensively for explants other than zygotic axes, to lower the risk of ice formation. However, these compounds may cause dehydration injury and osmotic damage during the freezing and thawing steps, from the limited information available for zygotic axes (Berjak *et al.*, 1996). Ultimately, success of cryopreservation protocols is determined by the recovery of plants from the cryopreserved material, thus the non-injurious optimisation of every step in the procedure is essential.

Cryopreservation has great potential as a tool for the long-term conservation of plant germplasm, especially for species producing recalcitrant seeds which cannot be stored by conventional methods. However, the use of this technology is presently limited as its development is still in the experimental stages. Furthermore, as can be appreciated from what has been said above, cryopreservation is labour-intensive and technologically challenging as well as expensive, and is generally considered primarily as a means of longer-term storage. Hence it is important that research aimed at optimisation of the storage of the genetic resources of species producing recalcitrant seeds, in a more convenient manner continues, which would facilitate short- to medium-term conservation of planting material.

1.4.2 Hydrated Storage

This method of storage maintains the seeds at the original water content, which should avoid any dehydration-associated effects. However, this has proven to be ineffective as a long-term option. Recalcitrant seeds stored in the hydrated state will lose viability relatively rapidly, their lifespan ranging from a few days to months, depending on the species and the condition of the seeds (Chin and Roberts, 1980; Berjak *et al.*, 1989; Pammenter *et al.*, 1994; Berjak, 1996). Mycock and Berjak (1990) pointed out that, even if the hydrated seeds should inherently retain viability for weeks to months, their more rapid deterioration is almost inevitable because of seed-associated micro-organisms, particularly fungi. Surface sterilisation does not provide a long-term solution because most often the contaminants are internally located within the seed tissues. Fungal contamination is a major obstacle because the conditions necessary for hydrated storage

(high humidity and high temperatures) also favour the proliferation of fungi (Berjak, 1996).

Recalcitrant seeds, particularly those from the tropics and sub-tropics, harbour a range of fungi and bacteria even when freshly hand harvested (reviewed by Sutherland *et al.*, 2002). Visible manifestations are often not evident in fresh seeds or in those seeds which have been in hydrated storage for only a short period of time. Studies on *Avicennia marina* showed that the resilience of the seeds against fungi in the short-term is associated with enhanced metabolic activity indicative of the onset of germination (Calistru *et al.*, 2000; Anguelova-Merhar *et al.*, 2003). However, according to those authors, this is a short-term effect only. This is probably because the seeds become more susceptible to fungal attack as they deteriorate due to the mild, but sustained, stress imposed by wet storage conditions (Pammenter *et al.*, 1994; Motete *et al.*, 1997). The dominance of particular fungal species changes with storage time; irrespective of the species, however, fungal presence in and on the seeds in the saturated storage environment definitely accelerates seed deterioration and consequently shortens the time for which they can be stored in this manner (Mycock and Berjak, 1990).

Methods can be employed to minimise the presence, and therefore the effects, of fungi. Calistru *et al.* (2000) found that the hydrated storage life-span of *A. marina* seeds was significantly extended when fungal proliferation was curtailed, and this was probably also the basis of extended storage lifespan of gel-encapsulated *Avicennia marina* seeds reported by Motete *et al.* (1997) (see below). The longevity of *Havea brasiliensis* seeds was extended from 3 to 12 months when the seeds were coated with 0.3% (w/w) Benlate® a benomyl fungicide (Chin, 1988). However, this does not provide a long-term solution as the seeds will inevitably lose viability too rapidly to achieve the practical objectives of long-term seed storage (Pammenter and Berjak, 1999). Nevertheless, curtailing the activity of, or eliminating, the seed-associated mycoflora is highly significant in the context of short- to medium-term storage of recalcitrant seeds.

Recalcitrant seeds undergo ongoing development, grading into germination-associated events during storage. Berjak *et al.* (1989) suggested that the ultimate loss of viability of recalcitrant seeds in hydrated storage is an inevitable consequence of the progress of germination. Recalcitrant seeds have sufficient water for the initial stages of germination to occur (Farrant *et al.*, 1986; Berjak *et al.*, 1989). However, depending on the species, germinative metabolism will be initiated immediately, or the developmental events grading into the onset of germination will proceed (Berjak *et al.*, 1989), and the longevity of the seeds in hydrated storage is suggested to be inversely related to the rate at which germination-associated events proceed. As germination continues unabated, a point is reached where additional water is required (Pammenter *et al.*, 1994). Generally once cell division and extensive vacuolation has been initiated during radicle extension, the seeds will require exogenous water. If this water is not available, and, under hydrated storage conditions no exogenous water is supplied (Berjak *et al.*, 1992), the seeds will lose vigour, and ultimately viability.

Hydrated storage does not impose an immediate water stress, but with increased time, the seeds develop a water requirement which has been suggested to give rise to a mild, but prolonged, water stress (Pammenter *et al.*, 1994). This could result in damage accumulation associated with the deterioration of the seeds. The nature of the damage is thought to be similar to that caused by dehydration except that it takes considerably longer to reach lethal levels (Pammenter *et al.*, 1994). At the point where water becomes limiting, metabolism becomes restricted, but this would not be similar to the finely controlled process that occurs in orthodox seeds prior to maturation drying. The consequence of this is that the metabolism that does occur is proposed to be uncontrolled and deranged, and as during slow dehydration of the seeds, has lethal consequences (Vertucci and Farrant, 1995; Pammenter *et al.*, 1998). Damage, which must accumulate with time in storage, may include uncontrolled free radical production resulting in peroxidation of lipids, proteins and other macromolecules. Free radical production occurs during normal cell metabolism, but these are rapidly and efficiently quenched by anti-oxidants and enzymes (Hendry, 1993). However, free radical production in water-stressed recalcitrant seeds or axes, may increase dramatically and/or the mechanisms which would

normally serve to remove them from the system may be damaged, and therefore not function efficiently (Hendry, 1993).

It has so far not been possible to differentiate between the damage caused by intracellular deterioration and damage induced by fungi. However, studies on *Avicennia marina* revealed that it may be possible to differentiate between the two effects based on the timing (Calistru *et al.*, 2000).

The storage lifespan of chilling-tolerant seeds in hydrated storage can be extended (not indefinitely) by reducing storage temperature (King and Roberts, 1980). A decrease in storage temperature has been shown to be effective in reducing the metabolic rate of the seeds, which is, in turn, effective in extending storage lifespan as a longer time is taken for the seeds to reach the point where additional water is required (Pritchard *et al.*, 1995). The use of this approach is, however, limited because many species are chilling-sensitive and cannot withstand effective reduction in storage temperature (below 15-10°C depending on the species), (Corbineau and Côme, 1988; Pammenter and Berjak, 1999). Attempts to manipulate metabolism by changing the atmospheric composition of the storage environment have yielded mixed results. Tompsett (1983) showed the importance of oxygen availability (for respiration) for retention of viability of seeds of *Araucaria hunsteinii*. In contrast Sowa *et al.* (1991) showed that treatment of *Litchi chinensis* and *Dimocarpus longan* seeds with nitrous oxide, which reduced metabolic rate, increased storage lifespan. Edwards and Mumford (1985) also showed an increase in longevity of sour orange (*Citrus aurantium*) as cumulative respiratory activity decreased.

Motete *et al.* (1997) developed one approach that resulted in dramatic increase in storage lifespan for *A. marina* seeds, involving encapsulation of surface-sterilised seeds in crude alginate gel: the seeds retained viability at a high level for 70-80 d (a more than four-fold increase in lifespan). Those authors could not identify depression of any specific

metabolic pathway, despite the fact that ultrastructural studies indicated that metabolism in the gel-encapsulated seeds had slowed. However, it was evident that there was a significant reduction in the rate of fungal proliferation on the encapsulated seeds and in the total number of seeds that became visibly infected (Motete *et al.*, 1997). This method certainly provides some promise in extending storage lifespan of those seeds which are particularly short lived. Encapsulation has the potential increase the chances of successful transport of many species (Pammenter and Berjak, 1999). However it still remains to be firmly resolved whether or not metabolism of recalcitrant seeds can be diminished non-injuriously, to successfully prolong storage lifespan.

Hydrated storage is an important method of maintaining viability and vigour of seeds in the short term. Perennially produced seeds such as those of rubber (*Hevea brasiliensis)* have been successfully stored in this manner for about 3 months, while, unless encapsulated, the highly recalcitrant *A. marina* seeds survive only 2-3 weeks in storage. Clearly, hydrated storage can not provide the solution to long term conservation of genetic resources, and it is implicit that the effects of seed-associated mycoflora must be eliminated or at least minimised, to optimise the storage potential of the hydrated seeds.

1.4.3 Sub-imbibed Storage

All recalcitrant seeds are metabolically active at shedding, with development progressing without an obvious marker event, into germination (Berjak, *et al.*, 1989; Farrant *et al.*, 1997; Chien and Lin, 1997). There is substantial variation, however, in the rate at which germination occurs. Lowering the water content slightly has been suggested to be one approach to slowing or curtailing germinative metabolism in a non-injurious manner, thus prolonging storage lifespan. King and Roberts (1980) proposed that it may be possible to store recalcitrant seeds at a relatively high water content but below the fully hydrated level, a procedure termed 'sub-imbibed' storage. It was thought that this would prevent germination from continuing at the normal rate during storage, but would permit ongoing vital metabolism (King and Roberts, 1980). In view of the recent evidence (reviewed above), the preclusion of germination might prevent the occurrence of unbalanced

metabolism and hence of damaging reactions. It was suggested (King and Roberts, 1980) that this might be achieved without the loss of physiological integrity associated with the fully imbibed state.

However, it is equally possible that the seeds will not tolerate the removal of this water the consequence of which could be a mild, prolonged water stress during storage and, even in 1980, King and Roberts cautioned that the rate that the rate of degeneration could increase with a decrease in seed water content. Thus, if damage occurs as a result of such partial dehydration and continues to accumulate, 'sub-imbibed' storage may not provide the solution to long-term storage. If 'sub-imbibed' conditions induce the evidence suggests that recalcitrant seeds would not deal effectively with intracellular damage probably because of the absence or ineffective operation of repair mechanisms (Pammenter and Berjak, 1999). Another advantage proposed 'sub-imbibed' storage is that it might reduce the proliferation of microorganisms (Hong and Ellis, 1996).

Studies on *Trichilia dregeana* seeds showed that storage at 'sub-optimal' water contents to be so injurious as to shorten lifespan relative to storage in the fully hydrated state (Drew *et al.*, 2000), but, as reported by those workers, the seeds were not in an optimal state at the outset of the experiment. Similarly, Corbineau and Côme (1986, 1988) found that partial drying resulted in shortening of longevity in the recalcitrant seeds of four tropical species. However, as the suggestion of 'sub-imbibed' storage affording an extension of seed lifespan has been made periodically since 1980 and as recently as 1996 by Hong and Ellis, this possibility needs to be systematically investigated further.

1.5 The Present Study

The present investigation was aimed at testing the feasibility of 'sub-imbibed' storage for recalcitrant seeds of three tropical dicotyledonous tree species, and one gymnosperm that is widely distributed in Southern Africa. These were *Trichilia dregeana* (Sond.) and *T. emetica* (Vahl.) (Meliaceae), *Syzygium cuminii* (L.) (Skeels) (Myrtaceae) and the gymnosperm *Podocarpus henkelii* (Stapf.) (Podocarpaceae). The seeds were dehydrated to the lowest water contents at which 100% viability was maintained, and then subjected to storage at three different temperatures. The seeds ('sub-imbibed' and fully-hydrated) were periodically removed for sampling and assessed for water content, germination, respiration, electrolyte leakage and ultrastructural features.

The seeds were set to germinate to determine whether there were changes in vigour and viability during storage. Loss of membrane integrity is an indication of desiccation damage in seeds, and can be expressed in terms of the rate and extent of electrolyte leakage from the tissues, as solutes will leak from damaged tissues faster than when undamaged. Thus measuring electrolyte leakage from the seeds provides valuable information. The rate of CO_2 evolution can be a useful indicator of the course of metabolic changes during storage. Observation of the ultrastructure provides valuable information of the internal status of the seeds. Thus these assessments provided useful information about the condition of the seeds after 'sub-imbibed' and hydrated storage.

The objective of the present study was to determine whether 'sub-imbibed' storage has a practical application for the storage of recalcitrant seeds.

2. Materials and Methods

2.1 The seeds

2.1.1 *Trichilia dregeana* (Sond.) and *Trichilia emetica* (Vahl.)

This genus belongs to the family Meliaceae. Both species are recalcitrant-seed-producing forest trees, with spreading crowns, occupying largely the same ecological area. *Trichilia dregeana* and *T. emetica* are indigenous to most of tropical and sub-tropical Africa (Pooley, 1994), being the only two species of this genus that occur in South Africa. The physical resemblance between trees of the two species is striking, and has led to many workers using the names incorrectly and interchangeably. However, closer examination shows that the two species can be distinguished by the morphology of the leaves and the fruits, and to some extent, the seeds.

Trichilia dregeana is commonly known as the Forest Mahogany. It is a tall evergreen tree (10 - 35 m) found at medium to high altitude (Van Wyk and Van Wyk, 1997) in the high rainfall, evergreen coastal and montane forests on the eastern coast of Africa (Pooley, 1994). The leaves are compound, very dark green and shiny above but paler and almost hairless beneath (Pooley, 1994). The tips of the leaflets are usually sharply pointed (Van Wyk and Van Wyk, 1997). The flowers (produced from October to December) are whitish green, with a sweet scent and form in dense bunches among the leaves. The fruit (which reaches maturity March/May) is a rounded dehiscent capsule (30 - 50 mm) which is joined directly to the stalk (Van Wyk and Van Wyk, 1997). In contrast, the fruits of *T. emetica* are connected to the stalk by a sharply differentiated neck (stipe) (Van Wyk and Van Wyk, 1997). The fruit ripens from pale pinkish green to brown, when it splits into three segments to reveal six large black seeds each of which is almost completely covered by a bright red aril (Pooley, 1994). *Trichilia dregeana* is a very useful tree, first recorded to have been used in the 1800s to repair ships entering Durban harbour (Pooley, 1994).

The tree is cultivated as an attractive ornamental and has been widely utilised as a street tree in Durban, South Africa. The tree provides useful timber and the bark and roots are used extensively for traditional medicinal purposes. Ants utilise the aril, while the seeds are eaten by birds and processed by humans, The oil extracted from the seeds provides superior cooking oil and is used in production of soaps and cosmetics.

The seeds of this species were originally reported by Choinski (1990) to be recalcitrant and chilling sensitive.

Trichilia emetica is commonly known as the Natal Mahogany. It is a medium to tall evergreen tree (5 - 20 m) (Pooley, 1994) occurring at medium to low altitudes, and is widespread in coastal areas, riverine forest and bushveld (Pooley, 1994). The large compound leaves are shiny dark green with brownish hairs beneath and each leaflet has a rounded tip. (In contrast, the leaves of *T. dregeana* are almost hairless and individual leaflets have a pointed tip.) The flowers are silvery-green. The fruit is a round dehiscent capsule (18 - 25 mm) (Van Wyk and Van Wyk, 1997), which ripens from grey-green to pale brown. The fruit splits to expose black seeds, each almost completely enveloped by a scarlet aril (Pooley, 1994). Mature seeds are produced in January/February.

This tree, like *T. dregeana* has a wide variety of uses. It is a popular ornamental species, valued for its shade. According to Pooley (1994) the fruits are eaten by baboons, monkeys and antelope, and the seeds by birds. The scarlet arils can be removed and crushed to extract a milky juice which is used as a sweet drink or with side dishes (Pooley, 1994). The oil extracted from the seeds also provides raw materials for use in cosmetics and soaps. Particularly the bark of *T. emetica* is used in herbal medicine, for treatment of a number of ailments.

There is little published information on the seed behaviour of *T. emetica*, but the seeds have been categorised as recalcitrant (Maghembe and Msanga, 1988) and unstorable. The seeds are reported to have short longevity and are recommended to be sown within three days of collection (Hines and Eckman, 1993).

For both species of *Trichilia*, mature, newly-opened fruits were harvested from trees at various locations on the KwaZulu-Natal North coast. The seeds were removed from the fruit capsules and placed in plastic bags. Upon arrival in the laboratory the red aril and seed coat were removed manually, within 48h of collection, using a scalpel. The cleaned seeds were soaked for 1.5 h and 30 min for *T. dregeana* and *T. emetica* respectively, in a systemic anti-fungal cocktail consisting of 2.5ml.l^{-1} Previcur N® (active ingredient: propamocarb-HC, AgrEvo, South Africa) and 0.2ml.l^{-1} Early Impact® (active ingredients: Flutriafol [triazole] and carbendazim [benzamidazole] Zeneca, Agrochemicals, South Africa). After rinsing the seeds were placed in a laminar flow cabinet and dried back to the original fresh weight. The seeds were then dusted with a Benomyl fungicide, Benlate (Fundazol WP, Sanachem, South Africa) and placed on a mesh above water in sealed buckets to prevent dehydration. The bucket and the mesh had been sterilised with sodium hypochlorite before use to remove fungal spores and other inoculum. The experimental approach used is illustrated in Figure 1 (*T. dregeana*) and 2 (*T. emetica*).

2.1.2 *Podocarpus henkelii* (Stapf.)

Podocarpus is the largest genus of the gymnospermous family Podocarpaceae, and is the dominating conifer of the southern hemisphere. *Podocarpus henkelii* is one of the species indigenous to Southern Africa. These trees are locally common in the montane forests of the Northern Natal Drakensberg. *Podocarpus henkelii* is a tall straight stemmed tree reaching 20 - 40 m in height. The leaves are glossy dark green, long and narrow and taper to a point. The seeds are olive green (up to 25 x 20 mm), ripening during early winter (Pooley, 1994).

Mature seeds are shed at a water content of up to 62% (fmb) and when incubated on a moist substrate display slow and sporadic germination (Palmer and Pitman, 1972). According to Dodd and van Staden (1981) the epimatium (coat) not only constitutes a barrier to germination by restricting water uptake, but also permits rapid and uncontrolled

water loss, which can lead to ultimate loss of viability. Short-term storage of the seeds has been reported (for up to 18 months) by maintaining the hydrated seeds at 4°C (Dodd and Van Staden, 1981). However, subsequent findings in our laboratory showed all similarly-stored seeds to have germinated in the storage container within a year (Berjak, pers. comm.[3]). It was also noted by Dodd and Van Staden (1980) that the seeds were metabolically active over a 16 week storage period, indicative of further embryo growth during storage. The seeds of *P. henkelii* have the characteristics typical of recalcitrant seeds.

Mature seeds of *P. henkelii* were hand-harvested from the ground below the trees (in late May and early June) by the staff at the National Botanical Gardens in Pietermaritzburg. The seeds were placed in plastic bags, boxed and conveyed to Durban within a few days of collection. Once the seeds arrived in the laboratory they were removed from the bags and placed temporarily on aerated racks and lightly covered with a plastic sheet to curtail moisture loss in the seed storage room at 16°C until used. Surface sterilisation was carried out by rinsing the seeds and placing them in 1% sodium hypochlorite for 20 min. The seeds were then rinsed 3 times with distilled water and dried with paper towel, once dry the seeds were dusted with a Benomyl fungicide (Fundazol. WP, Sanachem, South Africa). The experimental approach used is illustrated in Figure 3.

2.1.3 *Syzygium cuminii* (L.) Skeels

This genus belongs to the family Myrtaceae. *Syzygium cuminii* is an important tree in the tropics where the wood is used for timber and firewood and the fruit is eaten by humans and other animals. *Syzygium cuminii* is commonly known as the Jabalon plum or Jamun. It is not indigenous to South Africa but was introduced to the continent from India and tropical Asia (Pooley, 1994). The mature fruit is a fleshy purple berry which can be up to 25 mm long and 20 mm in diameter (Mbuya *et al.*, 1994). Each fruit contains a single

[3] P. Berjak, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa.

seed. The seeds are polyembryonic; however, the degree of polyembrony in this species is strongly correlated with seed size. Small seeds produce only one seedling, while larger seeds may produce up to 3 seedlings (Srimathi *et al.*, 1997; Kader *et al.*, 2000). The seeds of *S. cuminii* are recalcitrant, being shed at high water contents and lose viability relatively soon after shedding (2-20 weeks) (Rawat and Nautiyal, 1997; Mittal *et al.*, 1999; Ouedraogo *et al.*, 1999). Storage of seeds at the shedding water content resulted in germination during storage (Mittal *et al.*, 1999). Survival of the seeds during storage is dependent on the storage temperature. Seeds stored at 16°C maintained viability longer than those stored at 25 - 30°C (Rawat and Nautiyal, 1997; Mittal *et al.*, 1999; Srimathi *et al.*, 1999).

The ripe fruits were collected from trees in Tanzania. The seeds were extracted from the fruits manually and rinsed in water, the seed coat was left intact. The seeds were packaged in perforated polyethylene bags and transported by air via a courier service. The experimental approach used is illustrated in Figure 4.

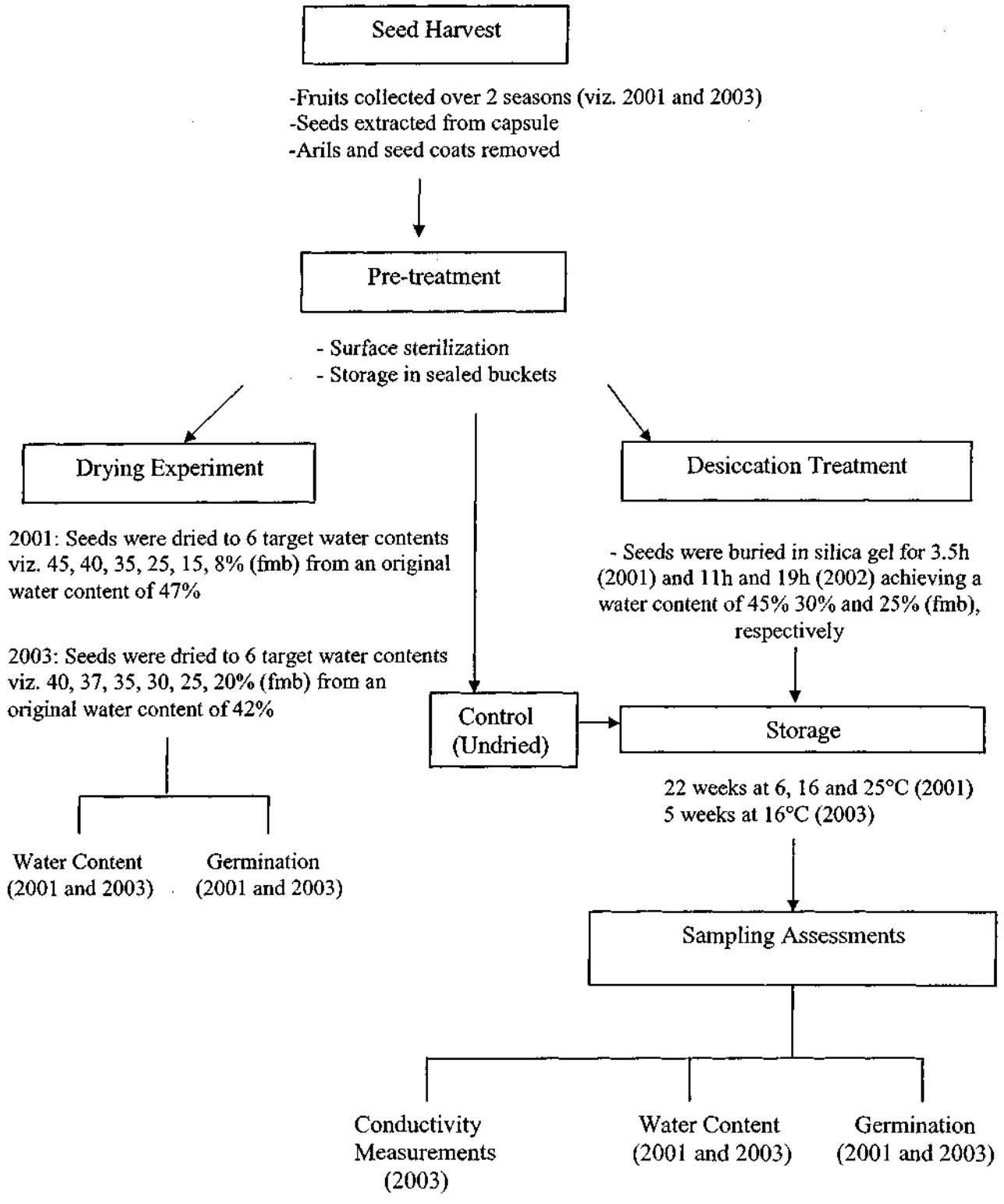


Figure 1: Schematic diagram to represent the experimental approach used for *Trichilia dregeana* seeds harvested in 2001 and 2003.

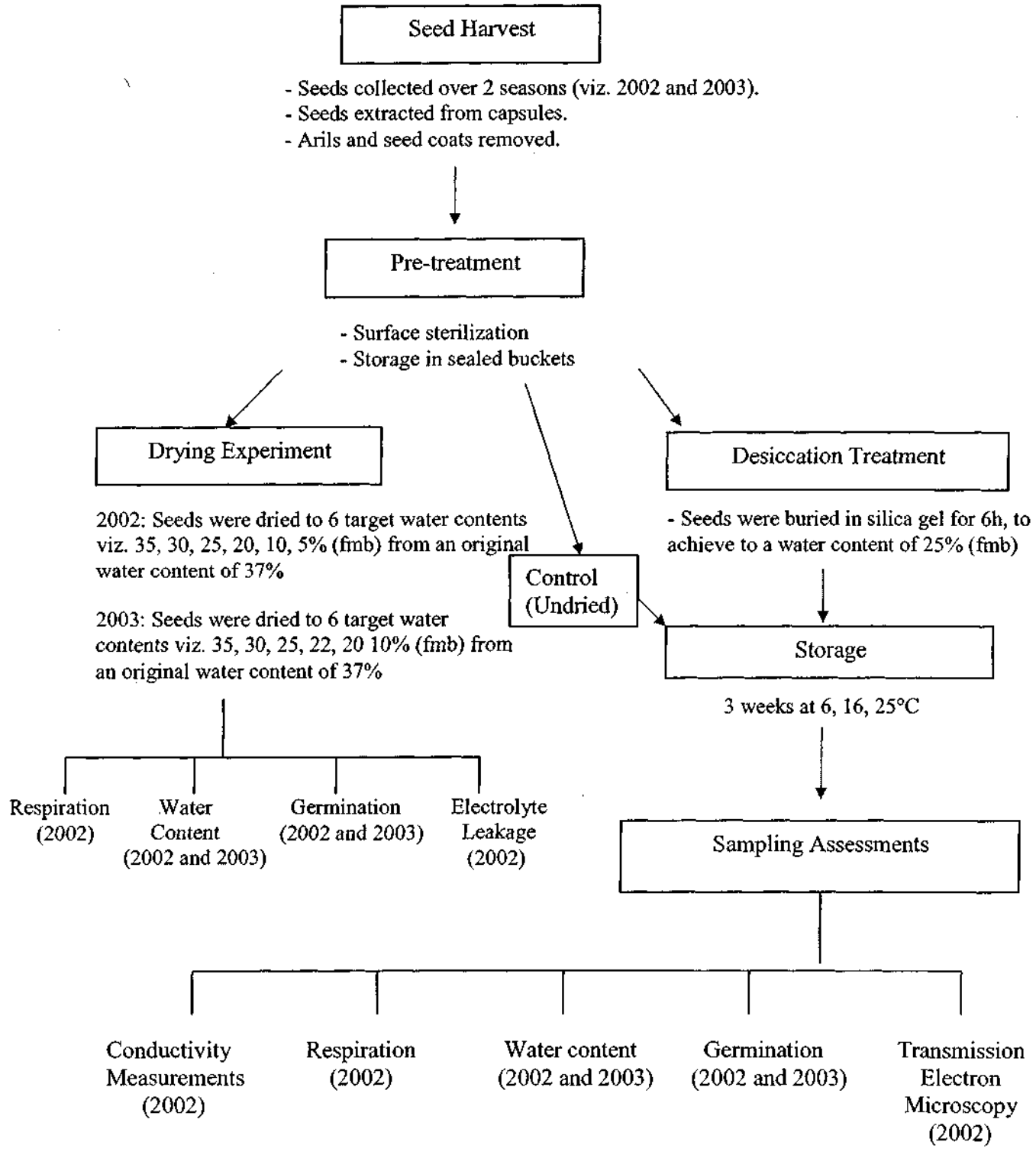


Figure 2: Schematic diagram to represent the experimental approach used for *Trichilia emetica* seeds harvested in 2002 and 2003.

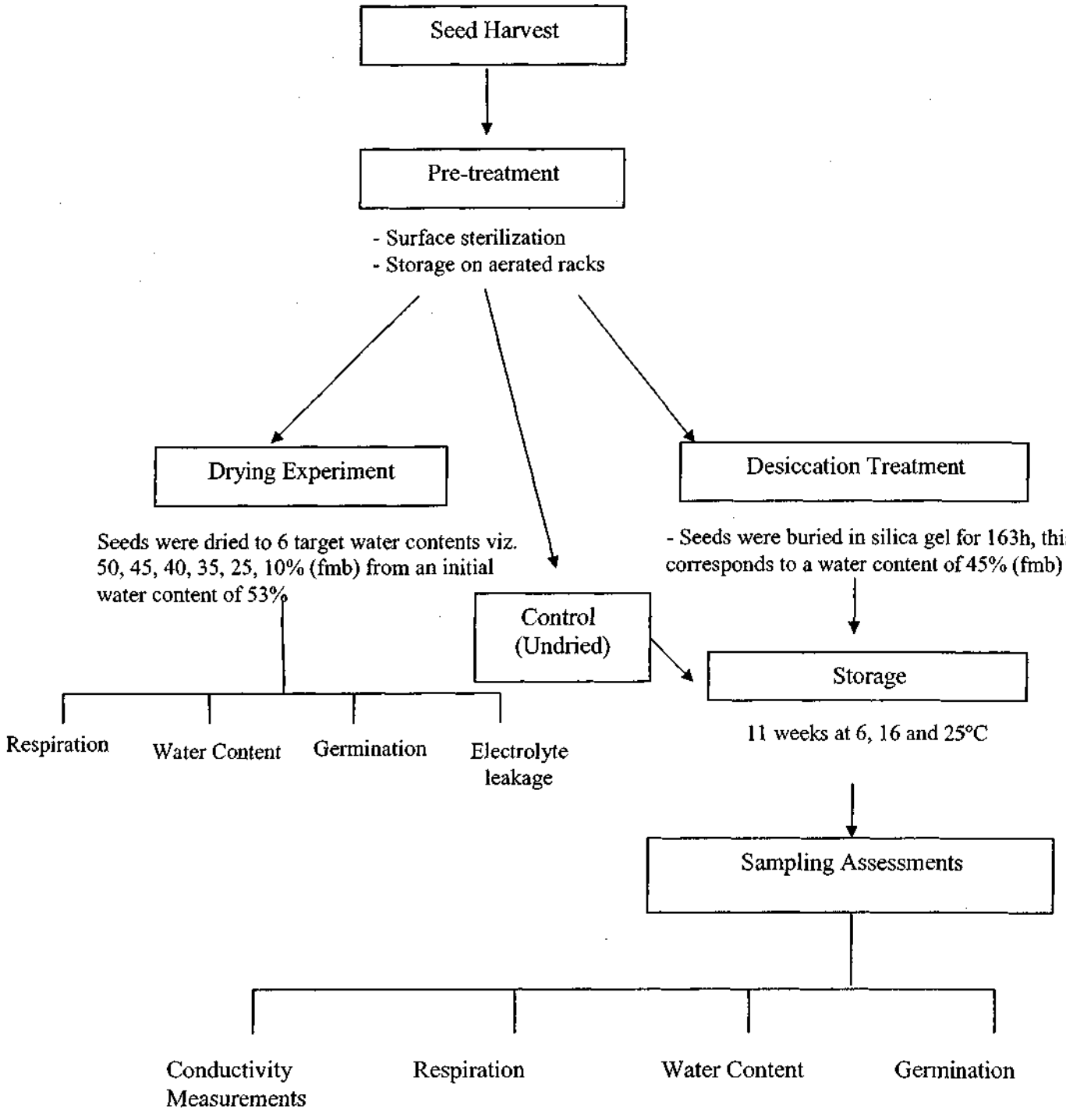


Figure3: Schematic diagram to represent the experimental approach used for *Podocarpus henkelii* seeds.

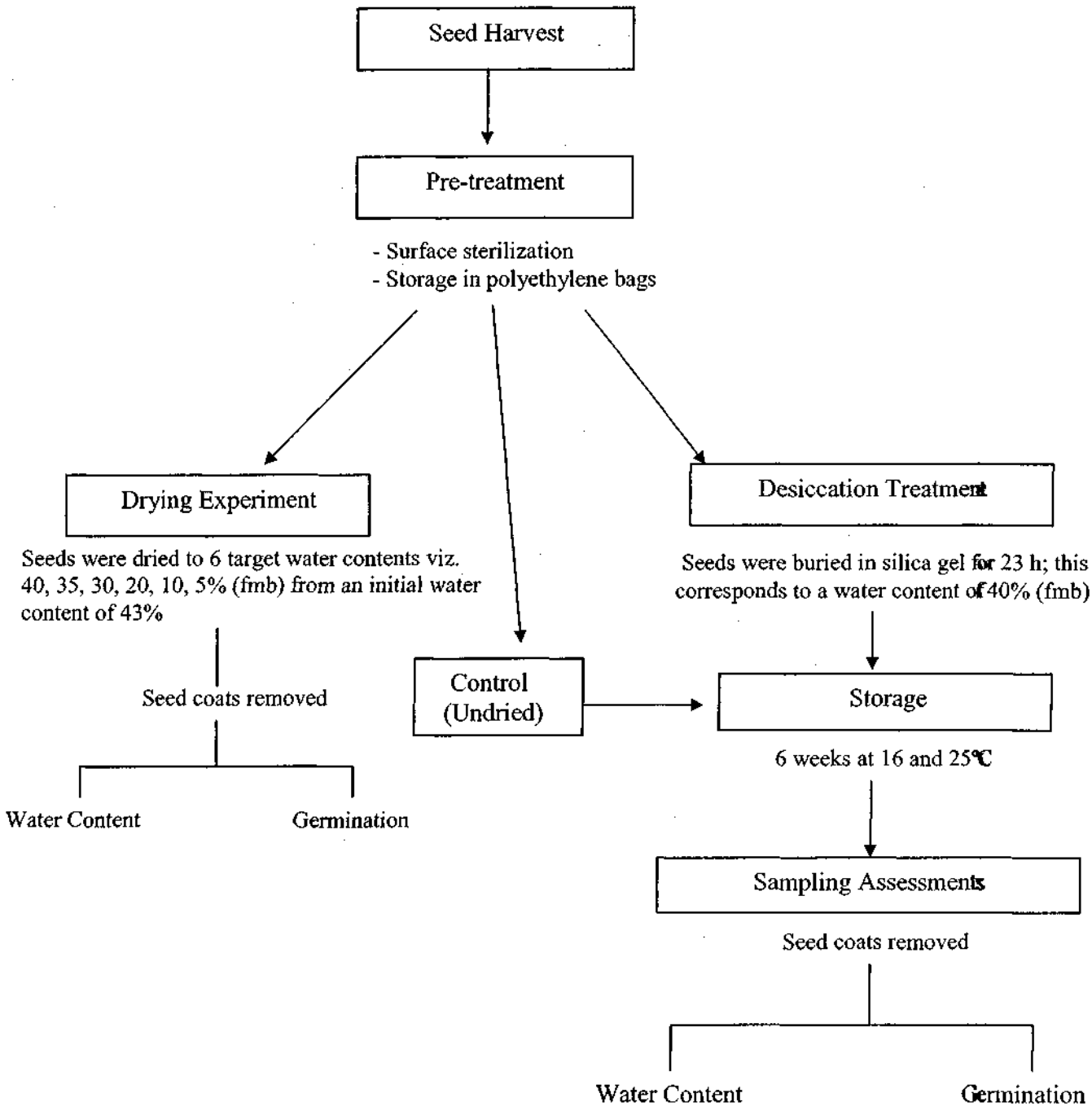


Figure 4: Schematic diagram to represent the experimental approach used for *Syzygium cuminii* seeds.

2.2 Drying Experiment

Unless otherwise specified, individual treatments described below (2.2 - 2.8) were the same for seeds of all the species. This experiment was performed in order to determine the target water content ([target moisture content] Hong and Ellis, 1996) for the main experiment and the drying time required for this target water content to be reached. The seeds were removed from buckets and sampled immediately. Water contents determinations were performed on the whole seeds, and the axis and cotyledons (gametophyte tissue in *P. henkelii* seeds) separately. Six water contents were chosen as the target water contents. These were chosen according to the IPGRI desiccation and storage protocol and are dependent on the water content of the whole seeds. The corresponding target weight was calculated for each water content using the following formula: Wt of seed (g) @ TMC = [(100-MC after processing)/(100-TMC)]*initial wt. Dehydration of the seeds to the various target water contents was achieved by burying the seeds in a known amount of silica gel (300g/30 seeds) in sealed Ziploc® bags. The dehydrating seeds were aerated daily and the silica gel was changed when it lost its intense blue colour. The seeds were periodically removed from the silica gel and weighed. When the seeds reached the weight corresponding to the target water content, they were removed for sampling.

2.3 Desiccation and Storage Treatments

The target water content for this experiment was chosen as the lowest water content where 100% survival was achieved. After dehydration (see above) to the chosen target water content, the seeds were removed from the silica gel and were subsequently placed in buckets which had been sterilised with 1% sodium hypochlorite and sprayed with ethanol. The buckets containing the seeds were then filled with vermiculite, after mixing the buckets were sealed. The seeds were placed at 3 different temperatures viz. 6°C, 16°C and 25°C. Fully hydrated seeds were also placed in buckets and put into storage and each temperature. At each temperature there was one bucket for 'sub-imbibed' seeds and one bucket for fully hydrated seeds. Samples were randomly selected from each bucket and removed for assessment after periodic intervals.

2.4 Germination Assessment

Samples of 25 seeds were used for the germination trials. The seeds were placed in 1% sodium hypochlorite for 20 min for surface sterilisation and were rinsed thoroughly in distilled water. The seeds were partially buried in moistened vermiculite within germination trays and placed to germinate at 26°C (day) and 18°C (night) with a 16 h photoperiod. Germination was as scored positive when the seeds produced a radicle of a 5 mm or more. Each day the number of germinated seeds was recorded to enable germination rate to be calculated. The number of seeds producing shoots was recorded daily after the first shoot was visible. At any sign of infection the seeds were sprayed with the Previcur/Impact fungicidal cocktail.

2.5 Water Content Determination

Ten seeds were sampled in each case in order to determine water content. Water contents of the fresh and treated seeds were determined gravimetrically by drying the individual axes and storage tissue (i.e. cotyledons in the case of *T. emetica*, *T. dregeana*, and *S. cuminii* and gametophyte tissue in the case of *P. henkelii* seeds) in the oven at 80°C for 48 h. Water contents were calculated and expressed on a dry mass basis ($g.g^{-1}$) ± standard deviation.

2.6 Respiration Measurements

Respiration rate was determined by CO_2 production, using an Infra Red Gas Analyser (IRGA)(ADC-225-MK 3, The analytical development Co. Ltd. Hoddeson, England). Ten seeds were sampled in each case. A respiration time course was determined for each species in order to determine a suitable incubation period, which depends on the respiratory rate. Whole seeds (embryos in the case of *P. henkelii*) were placed in sealed chambers within a water bath at 25°C. The chambers were flushed with CO_2-free air and, after 90 min, a 1ml air sample was removed from each chamber and injected into the IRGA. The output was represented as a peak on the graph, the height of the peak was recorded. The chambers were purged with CO_2-free air and left for 60 min before a second sample was removed. This was repeated for 45, 30 and 15 min intervals. The peak height was converted into [CO_2] ppm (using the standard curve) and plotted against

incubation time (time course). Respiration was determined from a sample of 10 seeds, after each incubation period two air samples were removed. A suitable incubation period was chosen from this experiment. After each drying period or storage interval the specimens were placed in the chambers and incubated for the pre-determined time. Gas samples were injected into the IRGRA and readings were taken, and converted into [CO_2] ppm. This was then converted into μmol $CO_2.g^{-1}.dm^{-1}.min^{-1}$, thus it was possible to determine changes in CO_2 production during the drying or storage period.

2.7 Processing for Microscopy

The embryonic axes of five seeds (for each treatment) were removed from the covering structures and the terminal 2 mm of the radicle excised and prepared for examination (In the case of *P. henkelii* The terminal 1mm of the embryo was removed, and the next 2 mm excised). The specimens were placed in 2.5% phosphate-buffered glutaraldehyde (0.1M, pH 7.2) containing 0.3% caffeine, for 24 hours. The fixative was removed and the specimens washed with three changes of phosphate buffer and post fixed with 1% aqueous osmium tetroxide (OsO_4) for 1 h. The specimens were again rinsed with phosphate buffer and dehydrated through an acetone series, followed by infiltration and embedding in a low viscosity epoxy resin, which was polymerised at 70°C for 8 h. The samples were sectioned using glass knives on a Reichert Ultracut E microtome, post stained with lead citrate and viewed with a Jeol 1010 TEM.

2.8 Conductivity Measurements

The electrolyte leakage from cotyledons and axes was recorded separately (embryos for *P. henkelii*) using the CM 100 conductivity meter (Reid and Associates, Durban, S.A.). Ten seeds were sampled in each case. The wells of the trays were thoroughly rinsed with distilled water. Each well was then filled with 3 ml of distilled water. The seeds were rinsed with distilled water and the separated cotyledons, axes or embryos were placed in the wells (axes and cotyledons from the same seeds were placed in corresponding wells). Conductivity was measured at a setting of 4 V, and programmed for 36 measurements over 18 h, at 30 min intervals. Thereafter the dry mass of each specimen was determined

individually, as described above (2.5). Electrolyte leakage was expressed per g dry mass over time using the recorded conductivity measurements - i.e. $\mu S.cm^{-3}.g^{-1}.min^{-1}$.

3. RESULTS

Note that where water/moisture contents are given as percentages, these are on a fresh mass basis (fmb). Water contents on a dry mass basis are expressed as g water per g dry mass ($g.g^{-1}$). Error bars on graphs indicate ± standard deviation.

3.1 Drying Experiments

These experiments involved drying whole seeds of *Trichilia dregeana*, *T. emetica*, *Podocarpus henkelii* and *Syzygium cuminii* to various pre-determined target water contents (TMCs) according to the protocol devised by IPGRI[1] and DFSC[2], in order to obtain the non-lethally reduced water content for each species, and the drying time required to reach these water contents, prior to 'sub-imbibed' storage. Additionally, for *T. emetica*, respiratory activity and electrolyte leakage were assessed in relation to dehydration.

3.1.1 *Trichilia dregeana*

3.1.1.1 Water Content

Trichilia dregeana seeds were collected in 2001 and 2003. The axis and cotyledonary water contents (2.92 ± 0.16 and 0.82 ± 0.09 $g.g^{-1}$, respectively) of seeds harvested in 2001 were similar to those of seeds collected in 2003 (2.81 ± 0.10 and 0.8 ± 0.06 $g.g^{-1}$, respectively) (Figure 5A). In both cases, the water content of the axes of freshly harvested seeds was markedly higher than the water content of the corresponding cotyledons (Figure 5).

Trichilia dregeana seeds collected in 2001 were buried in activated silica gel for various durations (determined by the time taken to reach each target water content) up to, but not

[1] IPGRI – International Plant Genetic Resources Institute, Rome
[2] DFSC- Danida Forest Seed Centre, Humlebaek, Denmark

exceeding, 120 h (Figure 5A). The removal of water from the axes and cotyledons of these seeds was most rapid during the initial stages of dehydration. While there was little or no change in cotyledonary water content (0.82 ± 0.09 $g.g^{-1}$) after 3.5 h drying time, the mean water content of the axes decreased from 2.92 ± 0.16 to 2.16 ± 0.35 $g.g^{-1}$, when compared with freshly harvested, undried seeds. Axis and cotyledonary water contents decreased to 1.73 ± 0.33 and 0.53 ± 0.14 $g.g^{-1}$, respectively, after only 6 h drying (Figure 5A). Thereafter, water loss by the seed tissues was more gradual, as the water contents of the axes and cotyledons decreased to 0.87 ± 0.10 and 0.14 ± 0.02 $g.g^{-1}$, respectively, after drying for an additional 60 h (Figure 5A).

A similar trend of water loss during desiccation was evident for the component tissues of *T. dregeana* seeds harvested in 2003 (Figure 5B). Water loss was most rapid during the first 10 h of dehydration, and although drying time did not exceed 72 h, the rate of dehydration by the axes and cotyledons of seeds harvested in 2003 was similar to that of seeds harvested in 2001, reaching similar axis water contents after drying for 72 h (i.e. 0.9 ± 0.17 $g.g^{-1}$) (Figure 5B).

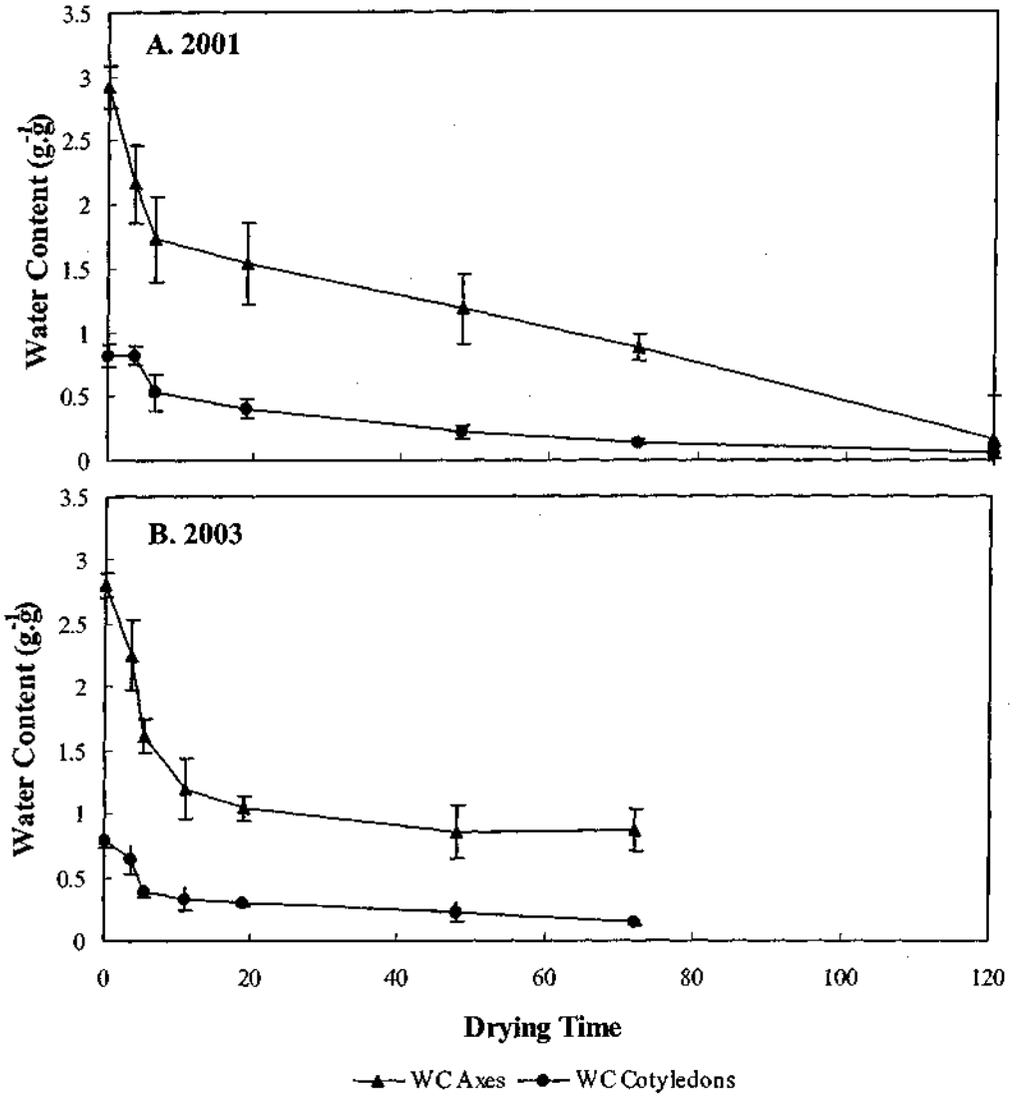


Figure 5: The relationship between drying time and water content of the embryonic axes (▲) and cotyledons (●) of *T. dregeana* seeds harvested in 2001 (A) and 2003 (B). For both harvests, whole seeds were buried in activated silica gel for various durations (determined by the time taken to reach the requisite target water contents), and the seed component water contents compared with those of undried, freshly harvested seeds (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.1.2 Germination

All freshly harvested *T. dregeana* seeds collected in 2001 and 2003 germinated (Figure 6A), and, while not determined for the former, all undried seeds from the 2003 harvest produced apparently normal seedlings (Figure 6B). The survival of *T. dregeana* seeds following desiccation, however, was different between the two seed lots. The viability of *T. dregeana* seeds collected in 2001 decreased gradually when dried to axis water contents below 1.5 ± 0.28 $g.g^{-1}$ (Figure 6A). In contrast, seeds harvested in 2003 showed a marked decline in germination only below an axis water content of 1.0 ± 0.10 $g.g^{-1}$ (Figure 6A). For both harvests, however, substantial loss of viability was recorded at axis water contents around 0.85 ± 0.20 $g.g^{-1}$ (Figure 6A). The percentage of seeds (2003 harvest) that produced normal seedlings also decreased precipitously after drying to axis water contents below 1.0 ± 0.01 $g.g^{-1}$ (Figure 6B).

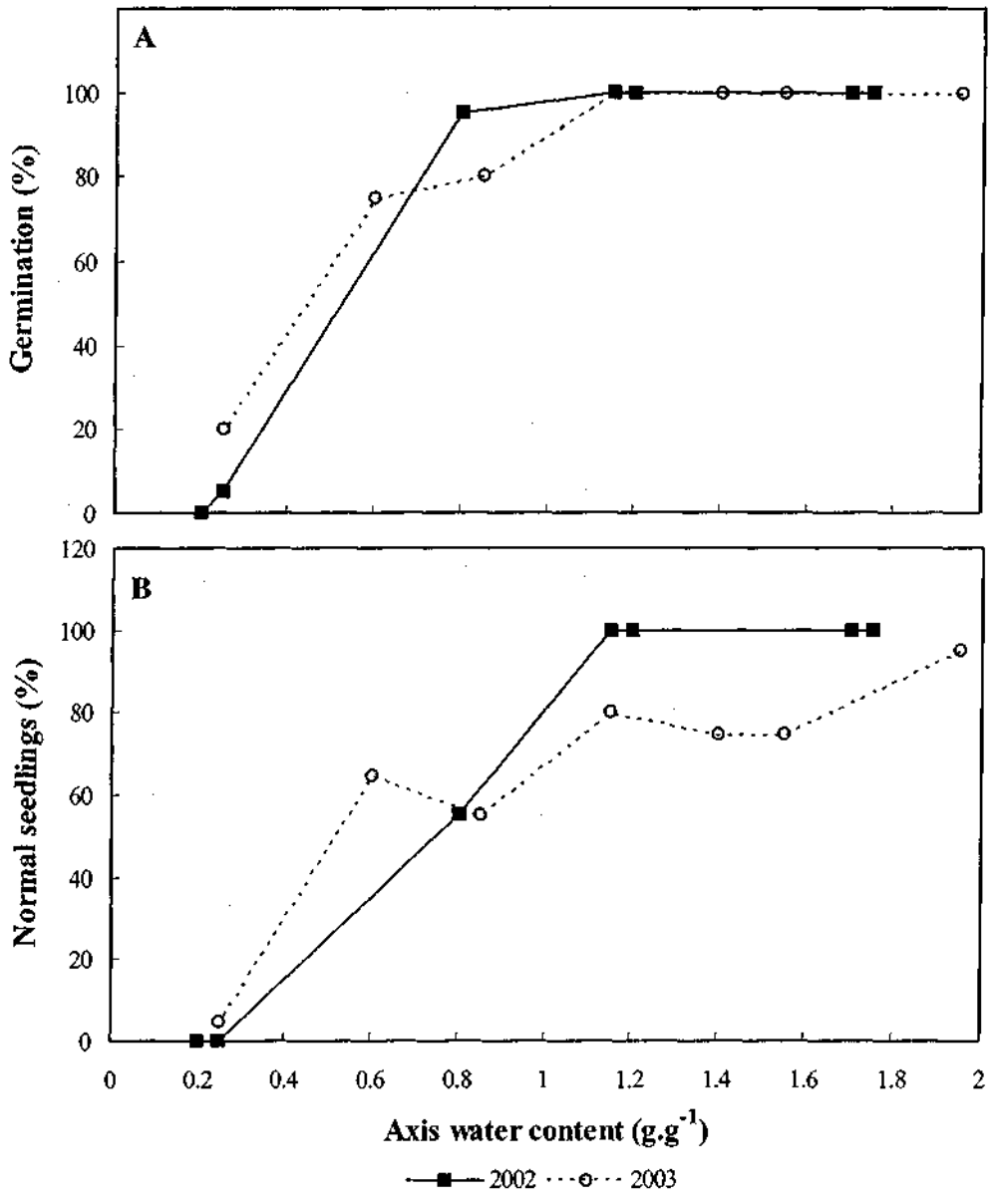


Figure 6: Viability (A) of *T. dregeana* seeds collected in 2001 (■) and 2003 (○), and normal shoot (seedling) production (B, 2003 only), over a range of axis water contents (n = 25 seeds).

The germination rates of freshly harvested *T. dregeana* seeds collected in 2001 and 2003 were similar. In both cases, radicle emergence was initiated 4 to 5 days after the seeds had been placed to germinate, and all the seeds from both lots had germinated within 13

to 14 days (Figure 7). Despite there being no apparent difference in the germination rate, of fresh seeds from the two harvests, those seeds harvested in 2003 appeared to be more vigorous. Following 19 h dehydration, seeds harvested in 2001 took up to 30 days to achieve maximum germination, compared with 20 days for those from the 2003 harvest (Figure 7). Furthermore, while 100% germination was attained by the 2003 harvest after this drying period, maximum germination was diminished to 80% for the 2001 seeds, which were at a higher axis water content than those harvested in 2003. In contrast though, only 5% of the 2003 seeds germinated when dried for 48 h (which resulted in an axis water content of 0.85 ± 0.21 g.g-1), while 55% the seeds harvested in 2001 germinated when dried for 48 h (which resulted in an axis water content of 1.18 ± 0.28 $g.g^{-1}$) (Figure 7). It should be noted, however, that after 48 h drying, the axes of the seeds from the two harvests had reached different water contents, as indicated.

Drying seeds for 3.5 h had little or no effect on the rate of germination of seeds harvested in 2003 (Figure 7B), which was similar to the germination profile of freshly harvested seeds from the same seed lot. In contrast, however, after 3.5 h dehydration seeds harvested in 2001 germinated more slowly than fresh seeds (Figure 7A). Despite the apparent slight lag in germination rate, however, all those seeds germinated.

Based on these results, 3.5 h was selected as the appropriate drying regime to apply prior to the 'sub-imbibed' storage trial of *T. dregeana* seeds harvested in 2001. In contrast, for the 2003 harvest, as seed viability was unaffected after desiccation in activated silica gel for up to 19 h, two drying regimes of longer duration (*viz.* 11 and 19 h) were selected in preparation for 'sub-imbibed' storage.

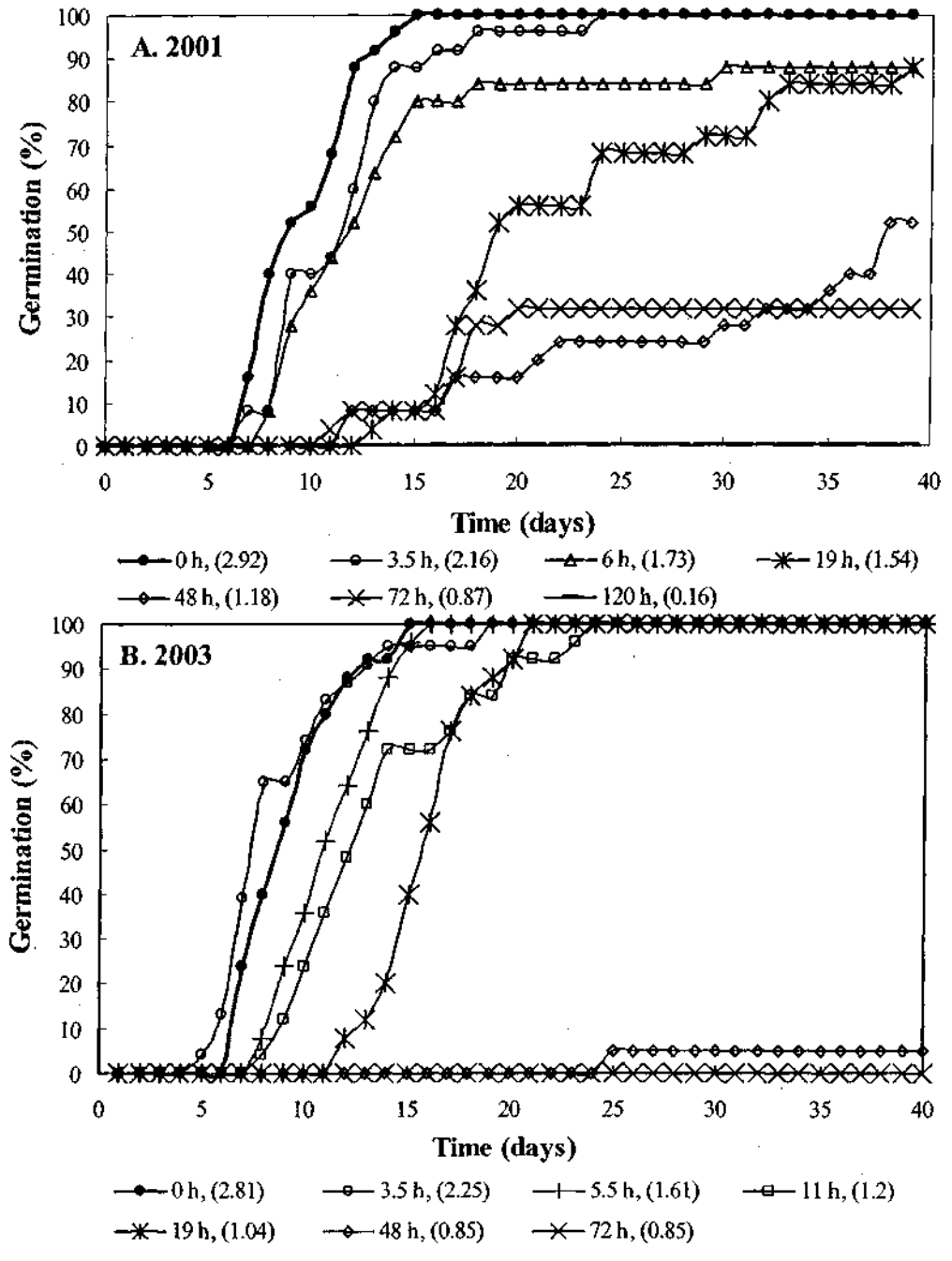


Figure 7: The germination time-course of freshly harvested (undried) *T. dregeana* seeds and seeds dried in activated silica gel for various durations from the 2001 (A) and 2003 (B) harvests. Keys indicate axis water contents (in brackets, units: $g.g^{-1}$) after specified drying times (ref. Figure 5) (n = 25 seeds).

3.1.2 *Trichilia emetica*

3.1.2.1 Water content

Trichilia emetica seeds were collected from the same site over two consecutive seasons, 2002 and 2003. The initial water content of whole seeds harvested in 2002, as determined individually for 10 freshly harvested seeds was 37% (fmb). The water content of the axes (1.81 ± 0.13 $g.g^{-1}$) was, on average, markedly higher than that of the cotyledons (0.56 ± 0.03 $g.g^{-1}$) and a greater proportion of water was lost from the axes during drying (Figure 8). The initial water content of the whole seeds harvested in 2003 was 38% (fmb), with axis and cotyledon water contents of 1.94 ± 0.23 and 0.47 ± 0.03 $g.g^{-1}$, respectively.

Trichilia emetica seeds collected in 2002 and 2003 were buried in activated silica gel for various durations which were determined by the time taken for the seeds to reach each pre-determined TMC. In 2003, the seeds were dried to six TMCs, which differed slightly from the protocol based on the results of the 2002 seed lot. The former seeds were not dried to 5% (fmb), (over 116 h, Figure 8), as this had resulted in 0% germination for the 2002 harvest (Figure 10). An additional target water content (22%, 15 h) was chosen because, in 2002, the seeds began to lose viability between 6 and 19 h of drying. Figure 8 shows the decrease in water content of the axes and cotyledons during the drying period: water was lost most rapidly during the initial stages of drying (0-6 h) for both axes and cotyledons. After 6 h drying the water content of the axes decreased from 1.81 ± 0.13 to 1.09 ± 0.15 $g.g^{-1}$ (2002 seeds) and from 1.94 ± 0.23 to 1.06 ± 0.06 $g.g^{-1}$, after 6.5 h drying for seeds harvested in 2003. Cotyledonary water contents decreased from 0.56 ± 0.03 (2002) and 0.47 ± 0.03 (2003) to 0.27 ± 0.03 and 0.3 ± 0.05 $g.g^{-1}$, respectively; after 6.5 h drying (Figure 8).

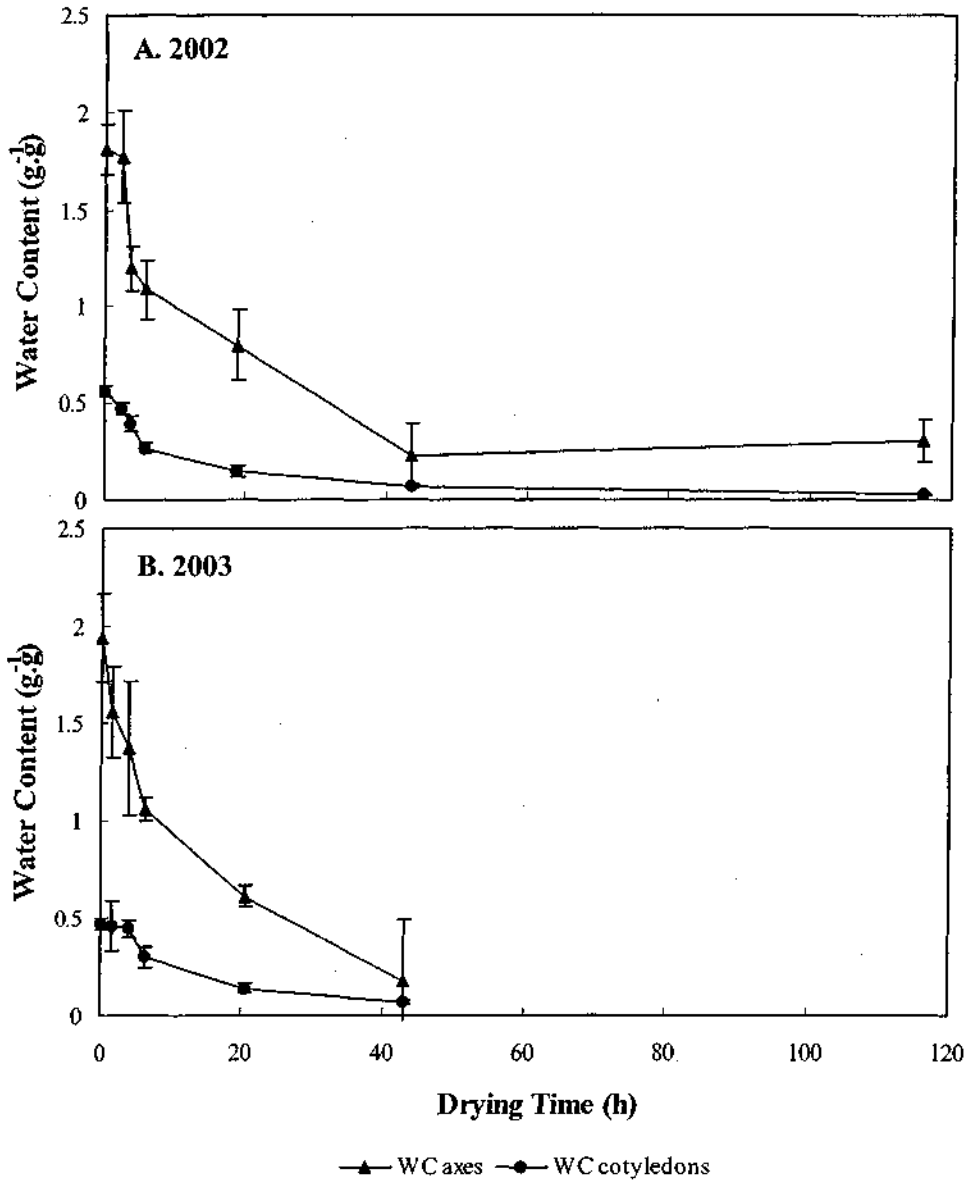


Figure 8: The relationship between drying time and water content of embryonic axes (▲) and cotyledons (●) of *T. emetica* seeds harvested in 2002 (A) and 2003 (B). In both harvests, whole seeds were buried in activated silica gel for various durations (determined by the time taken to reach the requisite target water contents), and the seed component water contents compared with those of undried, freshly harvested seeds (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.2.2 Germination

All the seeds (2002 and 2003 harvests) germinated at the shedding water content, which was essentially similar for both harvests (Figure 9A). All the undried seeds in 2002 and 95% from the 2003 harvests produced apparently normal seedlings (Figure 9B). The response of the two seed harvests to desiccation was similar, in both cases seed viability being unaffected by drying to a mean axis water content of 1.09 $g.g^{-1}$ (over 6 h). While 95% of the seeds harvested in 2002 survived desiccation to an axis water content of 0.85 ± 0.18 $g.g^{-1}$, the germination of the seeds harvested in 2003 decreased to 80% when dried to similar levels (Figure 9A). All seeds from both harvests lost viability when dried to mean axis water contents below 0.25 $g.g^{-1}$ (Figure 9A). The ability of the plants to produce normal shoots and seedlings was more adversely affected by desiccation, than was radicle protrusion. The seeds collected during 2002 showed a marked decline in seedling production when dried below an axis water content of 1.37 ± 0.34 $g.g^{-1}$, and those collected in 2003 were even more adversely affected. The percentage of 2003 seeds that produced normal seedlings decreased even after the shortest drying time when mean water content had declined from 1.94 ± 0.23 to 1.56 ± 0.24 $g.g^{-1}$ (Figure 9B). A drying time of 6 h, corresponding to mean axis water contents in the range 1.06 to 1.09 $g.g^{-1}$ was chosen for the 'sub-imbibed' storage (both harvests), as this was the longest drying time (lowest water content) which did not affect viability (Figure 9A).

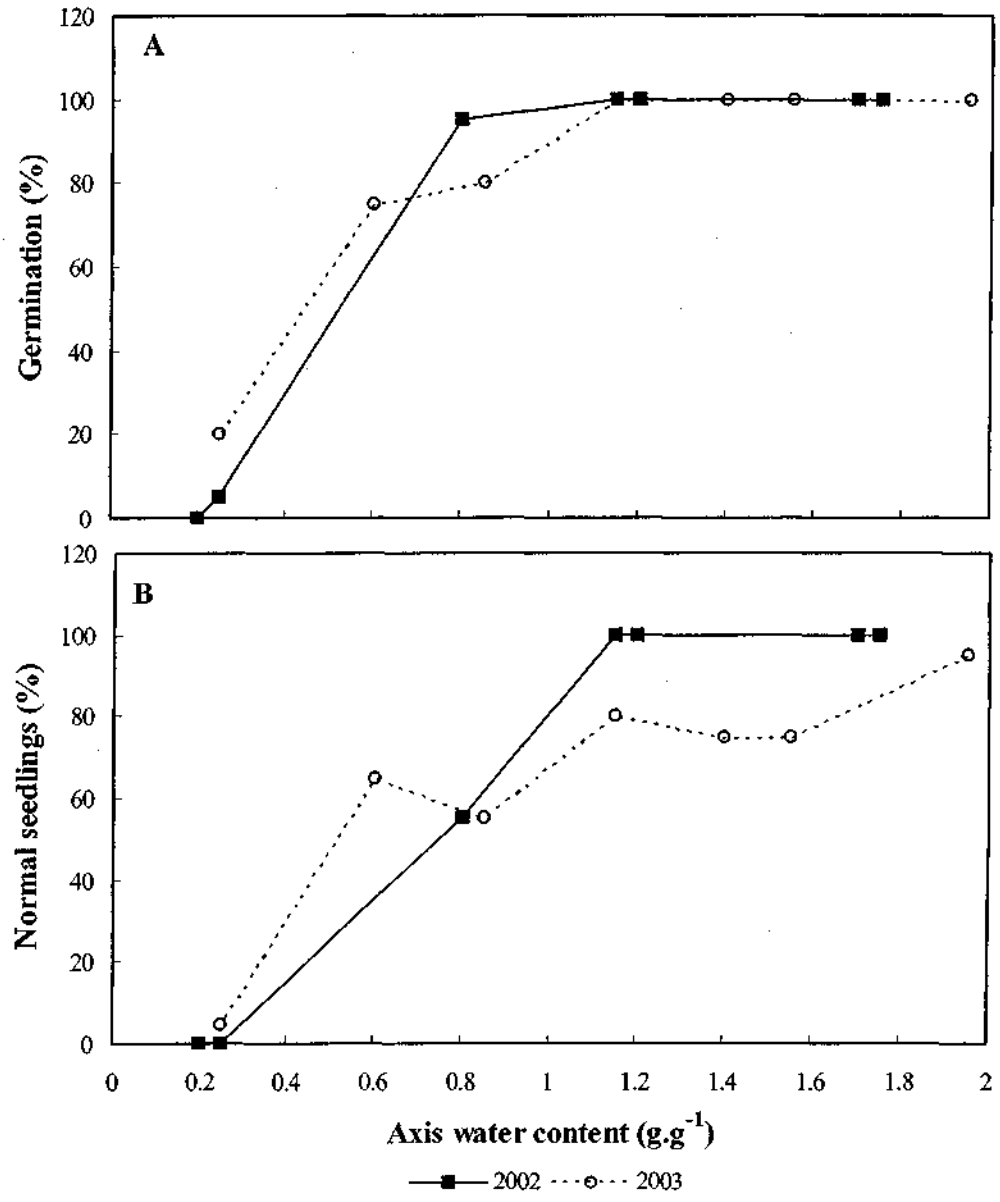


Figure 9: Viability (A) and percentage normal shoot production (B) of *T. emetica* seeds collected in 2002 (■) and 2003 (o), over a range of axis water contents (n = 25 seeds).

There was a difference in the germination rates of the freshly harvested seeds collected in 2002 and 2003. The seeds from the 2002 harvest germinated more slowly, radicle protrusion was initiated 2 days after being placed to germinate and 100% germination of

the seed batch was reached after 6 days. In contrast, more seeds from the 2003 harvest showed radicle protrusion within 2 days, and 100% of the seeds had germinated by the fourth day (Figure 10). The germination rate of *T. emetica* seeds collected in 2002 increased after short-term, partial desiccation for durations not exceeding 6 h, after there was decreased vigour and viability). This increase in vigour was particularly evident in those seeds that had been dried for 6 h (to an axis water content of 1.09 ± 0.15 $g.g^{-1}$), as 80% of the sample had germinated within 3 days, while only 30% of the freshly harvested seeds had germinated within the same period (Figure 10A). Interestingly, the germination curve of the seeds collected in 2002 showing enhanced germination after 6 h drying, was similar to the germination curve of freshly harvested seeds collected in 2003 (Figure 10).

The seeds collected in 2003 did show an enhancement of germination rate following partial desiccation; however the extent of invigoration was less marked than that for the seeds collected in 2002. In addition, 52% of the seeds that were dried for 4 h to a water content of 1.37 ± 0.16 $g.g^{-1}$ germinated within the first 2 days, as opposed to only 20% of the freshly harvested seeds germinated for the same duration. Further drying (for 6 h to a water content of 1.06 ± 0.18 $g.g^{-1}$), however, was associated with a slight lag in germination compared with the fresh seeds and those dried for shorter periods (Figure 10).

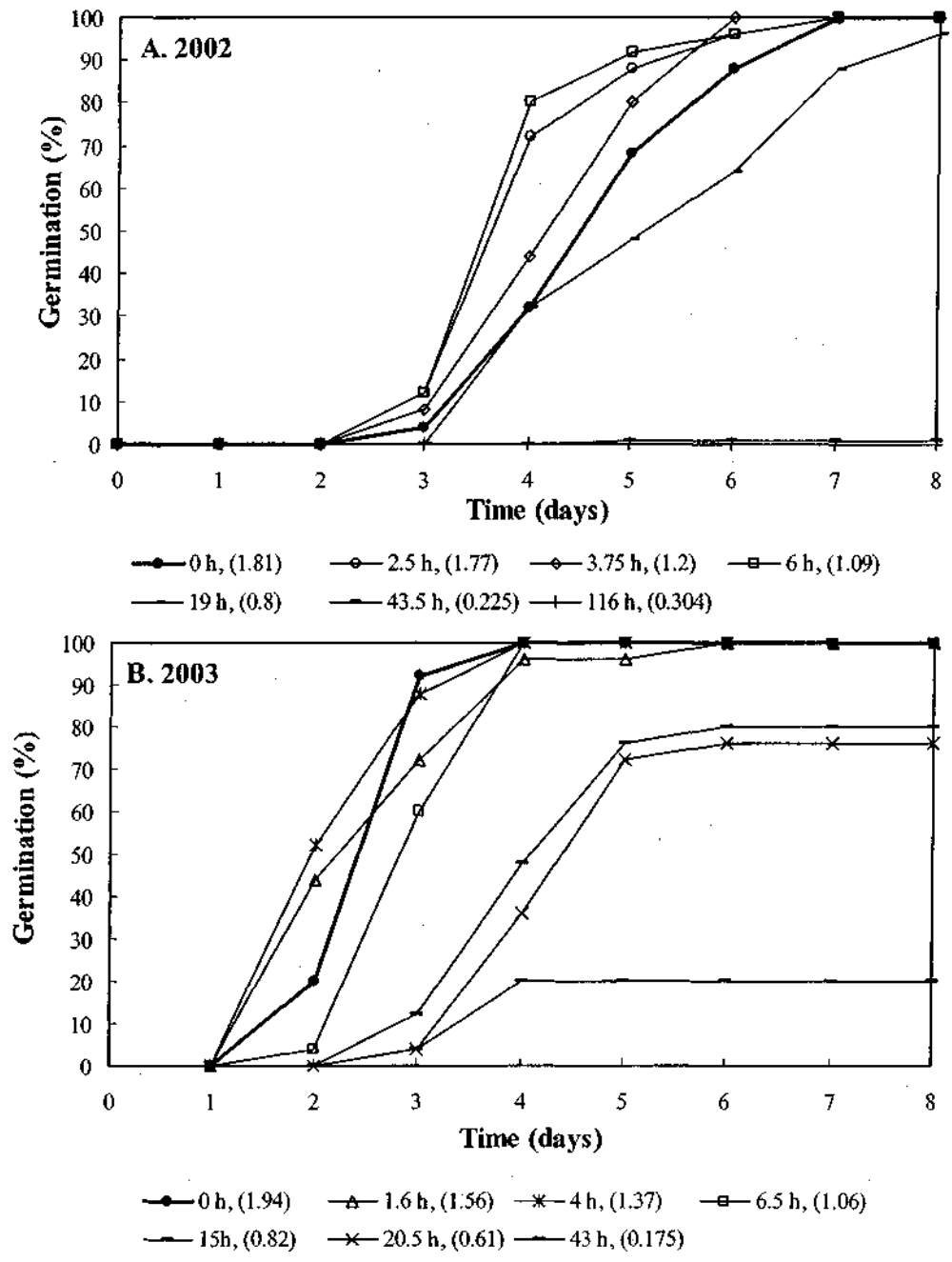


Figure 10: The germination time-course of freshly harvested (undried) *T. emetica* seeds from the 2002 (A) and 2003 (B) harvests, and seeds which had been dried in activated silica gel for various durations corresponding to the time taken to reach each pre-determined target water content. Keys indicate axis water contents (in brackets, units: $g.g^{-1}$) after specified drying times (ref. Figure 8) (n = 25 seeds).

3.1.2.3 Respiration

In order to determine the optimal duration of incubation, a respiration time course was determined for freshly harvested, undried, whole *T. emetica* seeds harvested in 2002 (Figure 11). The relationship between seed respiration and duration of incubation over 90 minutes showed a linear increase in CO_2 evolution. For practical purposes, therefore, a 45 minute incubation period was chosen to determine the effects of desiccation on the respiration of whole *T. emetica* seeds.

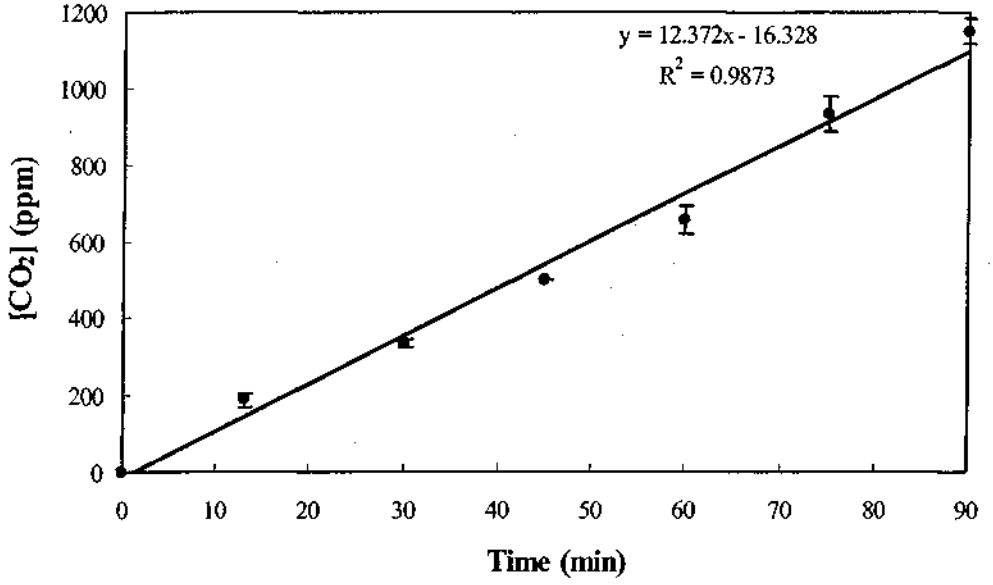


Figure 11: The relationship between incubation time and respiration, assessed by CO_2 accumulation, of freshly harvested (undried) whole *T. emetica* seeds, harvested in 2002 (n = 10 seeds). Bars represent the standard deviation above and below the mean.

The respiration rate of freshly harvested *T. emetica* seeds (axis water content 1.81 ± 0.13 $g.g^{-1}$) was 0.47 ± 0.07 nmol $CO_2.g^{-1}$ $dm.min^{-1}$ (Figure 12). Despite an initial decline in respiration rate to 0.37 ± 0.07 nmol $CO_2.g^{-1}$ $dm.min^{-1}$ when seeds were dried for 2.5 h (to an axis water content of 1.77 ± 0.23 $g.g^{-1}$), drying for 3.75 h (axis water content of 1.2 ± 0.11 $g.g^{-1}$) and 6 h (axis water content of 1.09 ± 0.15 $g.g^{-1}$) was associated with an

increased rate of whole seed respiration increased rate of whole seed respiration as indicated by CO_2 evolution to 0.52 ± 0.1 nmol $CO_2.g^{-1}$ dm.min^{-1}, i.e. to levels higher than that recorded for freshly harvested, undried seeds (Figure 12). Desiccation for durations longer than 6 h resulted in a progressive decline in whole seed respiration, to levels lower than that determined for undried, freshly harvested seeds (Figure 12).

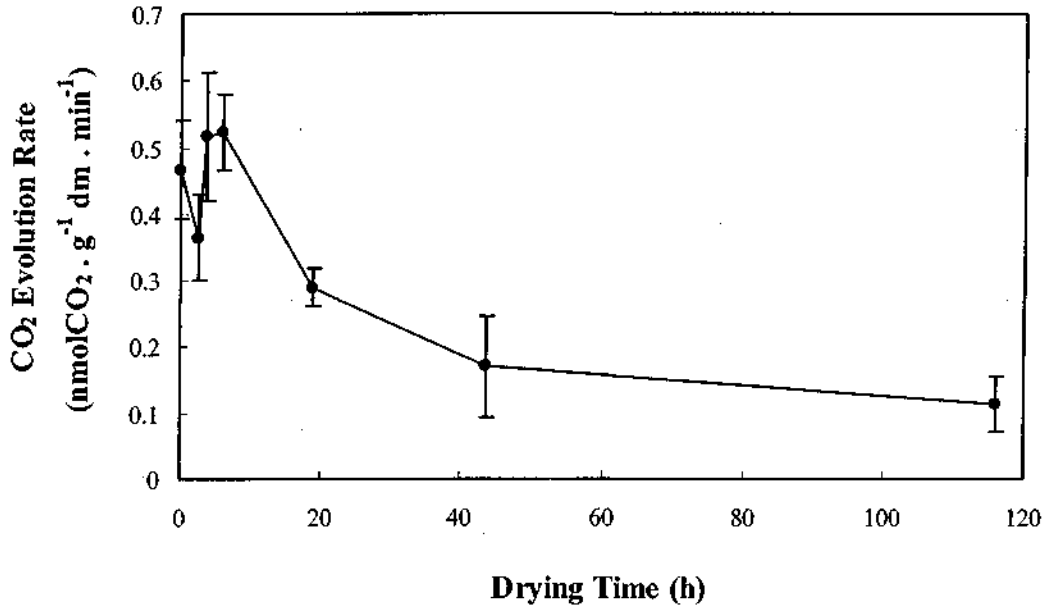


Figure 12: The effect of desiccation on the respiration rate, determined by measuring the rate of CO_2 evolution, of whole *T. emetica* seeds harvested in 2002. (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.2.4 Electrolyte Leakage

The conductivity of the leachate in which *T. emetica* seeds (from the 2002 harvest) were immersed was measured prior to, and after, drying to determine the effect of desiccation on electrolyte leakage. At each sampling, electrolyte leakage was determined for axes and cotyledons, separately. It was immediately apparent that the level of mean electrolyte leakage (expressed as $\mu S.cm^{-1}.g^{-1}.min^{-1}$) of freshly harvested, undried axes was much higher (50-fold) than that of the corresponding cotyledons (Figure 13). This

difference in the level of electrolyte leakage between the seed components was also apparent for those samples taken during desiccation.

Following drying for up to 6 h (water content 1.09 ± 0.15 $g.g^{-1}$), the level of mean electrolyte leakage from *T. emetica* axes was similar to that of freshly harvested, undried seeds where axis water content was 1.81 ± 0.13 $g.g^{-1}$ (Figure 13). Further drying, for durations longer than 6 h, however, greatly increased the level of leakage of electrolytes from the axes. The mean leakage of electrolytes from the cotyledons was unaffected by seed drying in silica gel for up to 3.75 h (when water content had declined from the initial 0.56 ± 0.03 to 0.39 ± 0.04$g.g^{-1}$), further desiccation (for 6 h and more), dramatically increased the level of electrolyte leakage (Figure 13).

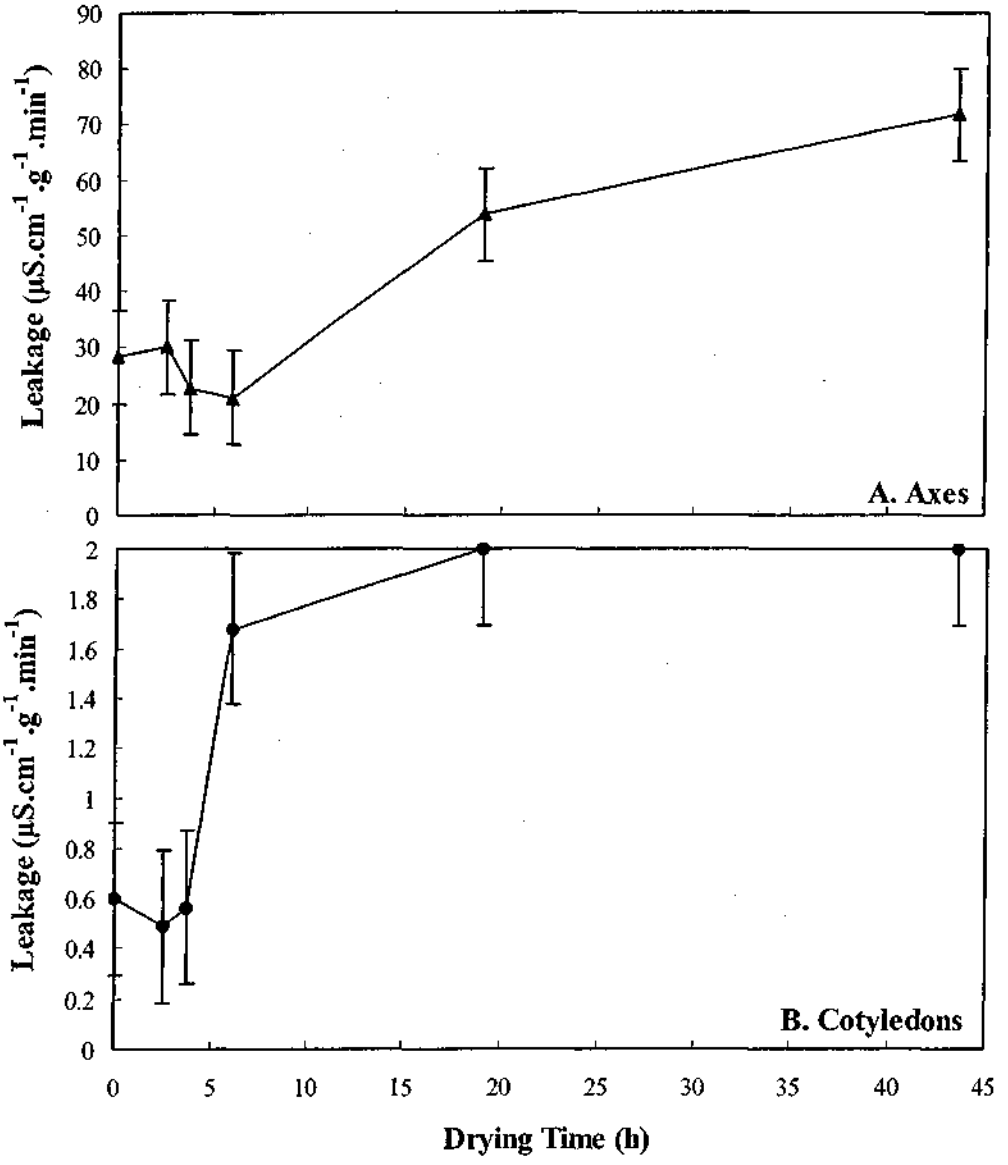


Figure 13: The relationship between drying time and electrolyte leakage from embryonic axes (A) and cotyledons (B) of *T. emetica* seeds harvested in 2002. (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.3 *Podocarpus henkelii*

3.1.3.1 Water content

The seeds of *P. henkelii* were shed at a relatively high water content (53%, fmb). The water content of the embryos (1.8 ± 0.13 $g.g^{-1}$) was higher than that of the megagameteophyte (female gametophyte) (1.03 ± 0.13 $g.g^{-1}$) (Figure 14). There was very little, or no, change in the water contents of the embryo or gametophyte tissue following drying for 22 h, these remaining similar to the water contents determined for the corresponding tissues from the seeds at shedding. The rate of water loss from *P. henkelii* seeds was very slow, despite their being buried in silica gel: it took 168 h (7 days) for the initial water content of the embryos of newly shed, undried seeds to decline from 1.8 ± 0.13 $g.g^{-1}$ to 1.6 ± 0.14 $g.g^{-1}$.

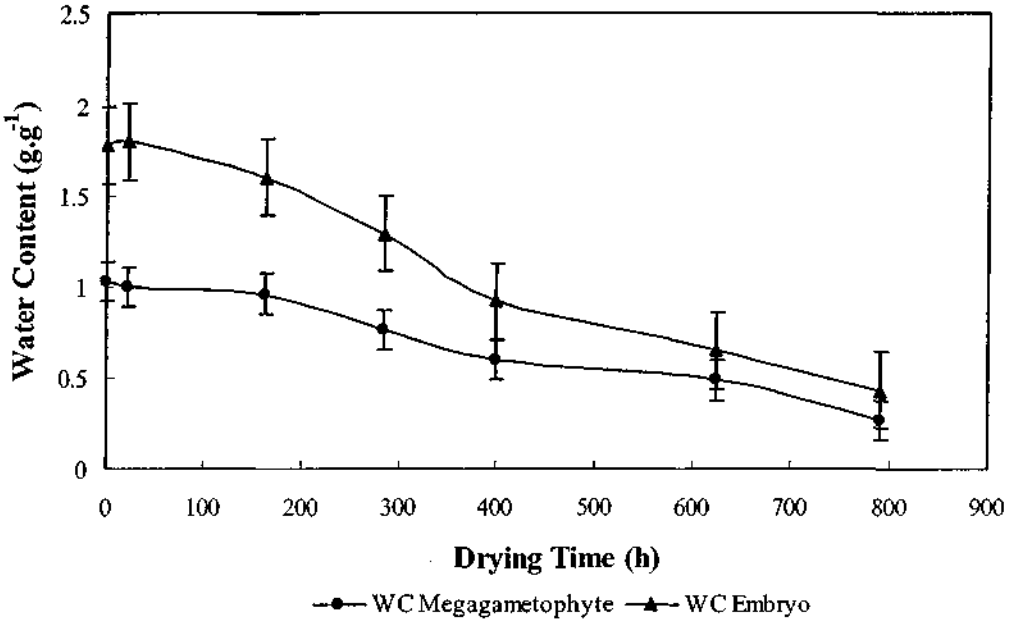


Figure 14: The relationship between drying time and water content of embryos (▲) and megagametophyte (●) taken from whole seeds of *P. henkelii* after drying in silica gel to various target water contents (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.3.2 Germination

The viability of *P. henkelii* seeds was unaffected by drying the seeds to an embryo water content of 1.6 ± 0.14 $g.g^{-1}$ (168 h drying); further desiccation (embryo water content 1.2 ± 0.42 $g.g^{-1}$), however, resulted in a reduction in seed germination by 10%, and viability was completely lost when the seeds were dried to an embryo water content of 0.92 ± 0.14 $g.g^{-1}$ (Figure 15). The ability of the seeds to produce apparently normal shoots and seedlings, was more adversely affected by desiccation than was seed viability measured by radicle protrusion. While all the freshly harvested seeds developed normal seedlings, the proportion of normal seedlings was reduced by 5, 28 and 35% after drying to embryo water contents of 1.75 ± 1.18, 1.6 ± 0.14 and 1.3 ± 0.42 $g.g^{-1}$, respectively (Figure 15).

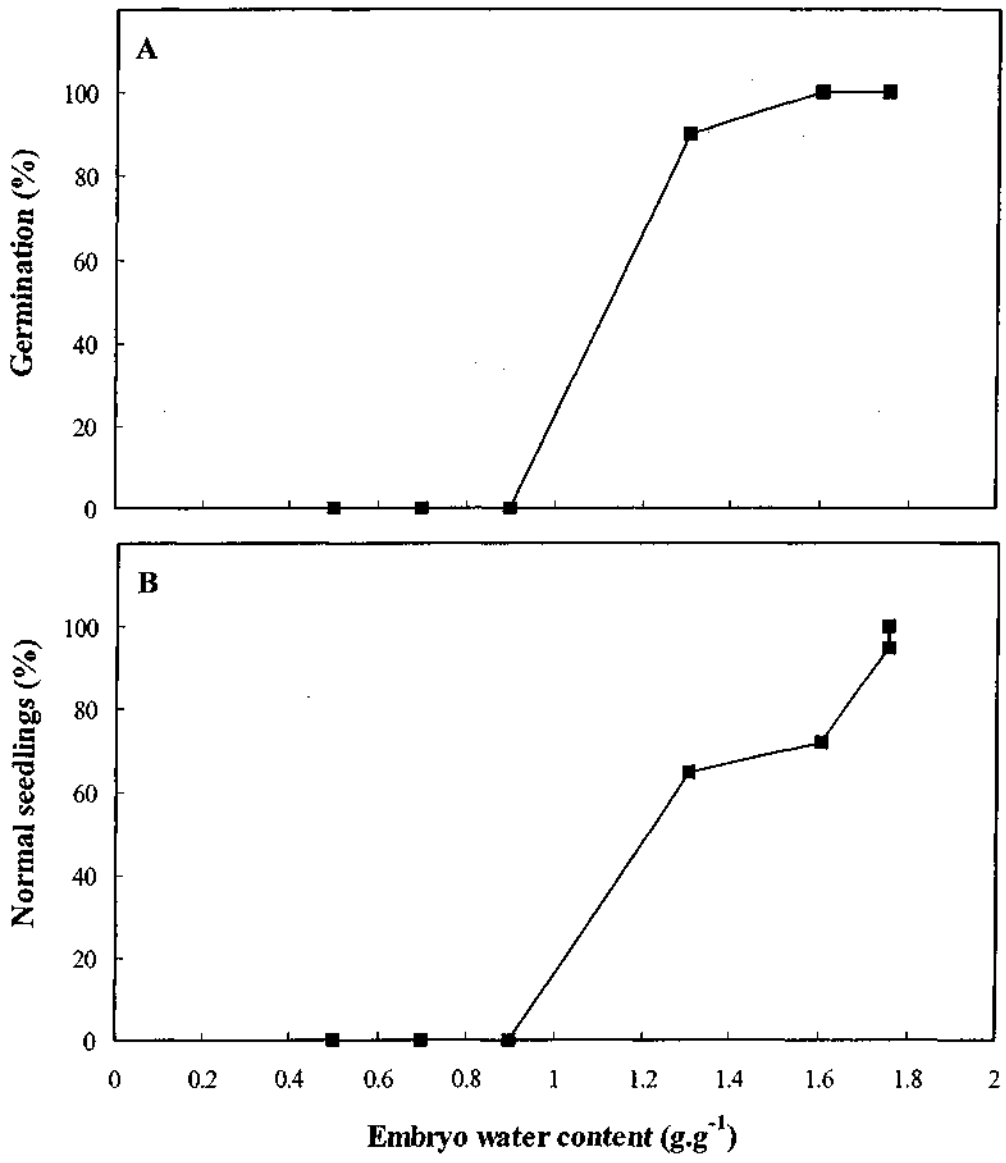


Figure 15: Viability (A) and percentage normal shoot production (B) of *P. henkelii* seeds, over a range of embryo water contents (n = 25 seeds).

The freshly harvested seeds (embryo water content 1.78 ± 0.13 $g.g^{-1}$) had initiated radicle emergence 8 days after been placed to germinate, and all the seeds in the sample had germinated within 17 days (Figure 16). The rate of seed germination was enhanced following 22 h drying, with no measurable associated decline in embryo water content (1.8 ± 0.13 $g.g^{-1}$), as 66% of the seeds had germinated within 9 days, while the fresh

seeds took 12 days to achieve similar levels of germination (Figure 16). Seed viability was unaffected by drying for 163 h (to an embryo water content of 1.6 ± 0.14 $g.g^{-1}$); however, the rate of germination was slower than that of the fresh seeds. Further desiccation (for 284 h) adversely affected both the rate and totality of *P. henkelii* seed germination (Figure 16).

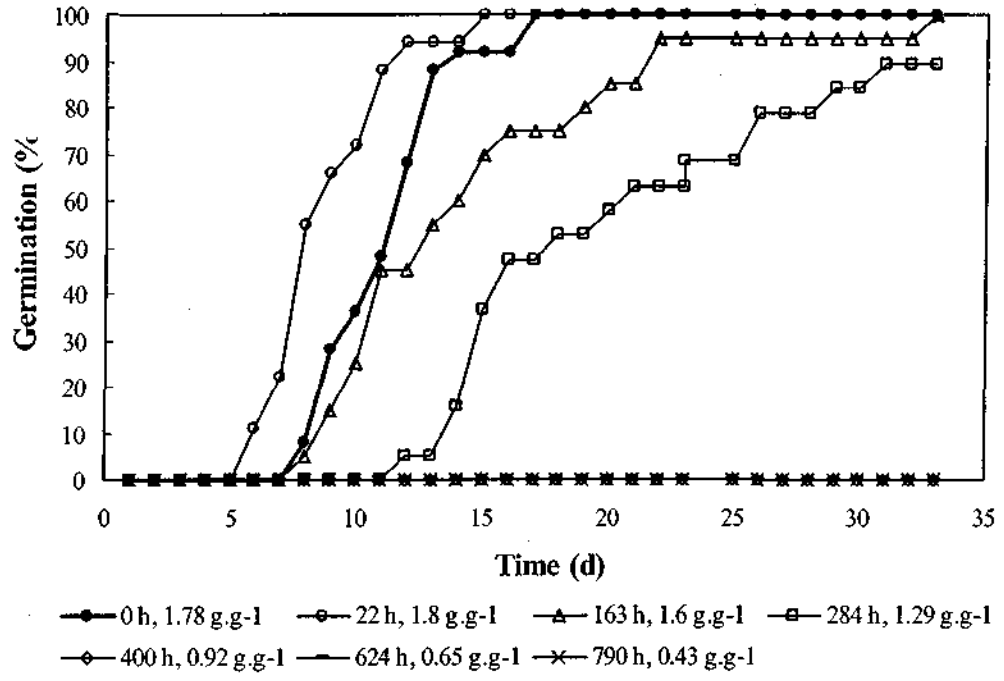


Figure 16: The germination time-course of newly shed (undried) *P. henkelii* seeds and seeds dried in activated silica gel for various durations, corresponding to the time taken to reach each predetermined target water content. Key indicates axis water contents. (n = 25 seeds).

3.1.3.3 Respiration

Because of the large volume of the gametophyte tissue which, in any case, is extra-embryonic in gymnosperm seeds, a respiration time course was determined for the embryos only (Figure 17). The relationship between measured CO_2 concentration (respiration) and incubation time was linear. An incubation time of 60 min was chosen for respiration measurements to be taken during drying and storage experiments, as it was deemed to be within the linear portion of the curve, and would allow for sufficient

incubation time for CO_2 evolution measurements to be taken from seeds that could have been less vigorous, during the drying and storage experiments (Figure 17).

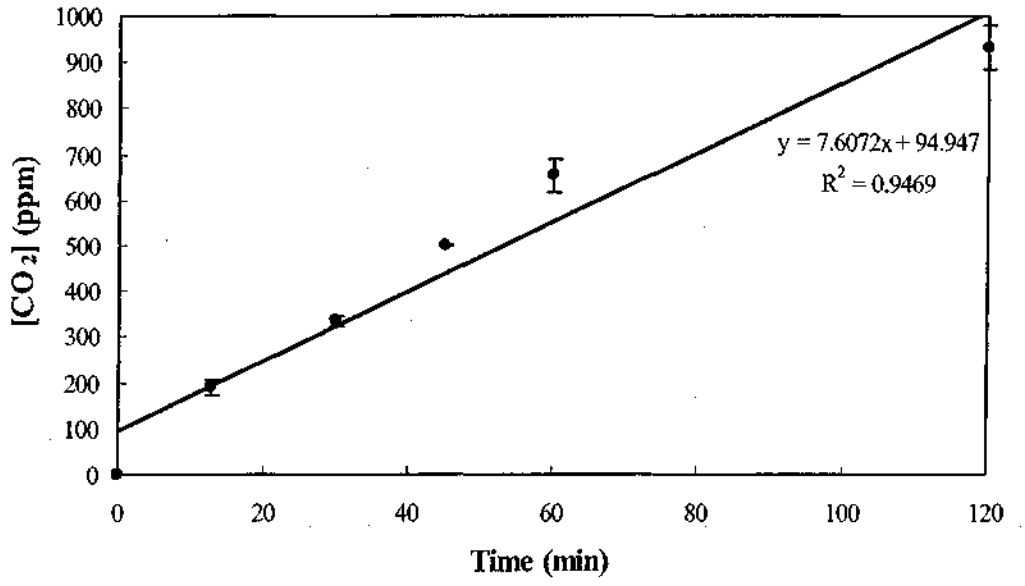


Figure 17: The relationship between incubation time and accumulation of CO_2 in the incubation flask for freshly harvested (undried) embryos of *P. henkelii* seeds (n = 10 seeds). Bars represent the standard deviation above and below of the mean.

Individual embryo respiration measurements were made for a sample of 10 newly shed seeds, and from each sample taken during desiccation. The respiration rate of embryos removed from undried, freshly harvested seeds was 12.5 ± 2.17 nmol CO_2. $g^{-1}dm.min^{-1}$ (Figure 18). Drying for 22 h resulted in a decline in respiration to 3.6 ± 1.22 nmol $CO_2.g^{-1}dm.min^{-1}$ (Figure 18). After this initial decline, the rate of embryo respiration remained low, with only minor fluctuations, during further drying (Figure 18).

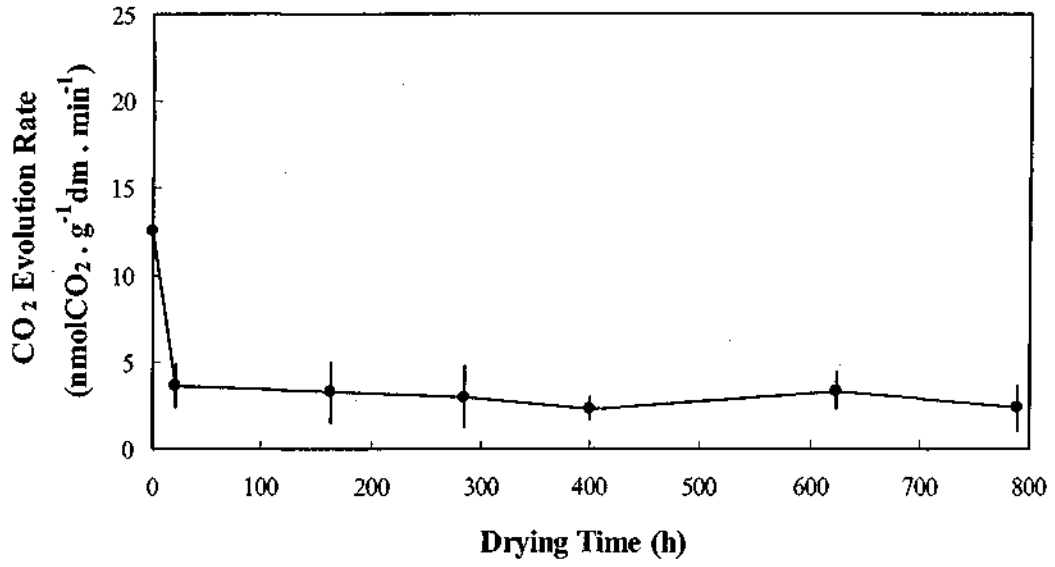


Figure 18: The effect of desiccation on respiration rate, determined by measuring the rate of CO_2 evolution, of *P. henkelii* embryos (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.3.4 Electrolyte Leakage

Electrolyte leakage was determined for the embryos only, prior to, and after, drying, to determine the effect of desiccation on leakage of electrolytes. Mean electrolyte leakage for 10 individual embryos taken from undried, freshly harvested seeds was 9.3 $\mu S.cm^{-1}.g^{-1}.min^{-1}$ (Figure 19). Drying for up to 284 h (to an embryo water content of 1.29 ± 0.42 $g.g^{-1}$) was not associated with a significant change in mean electrolyte leakage. Further drying (400 h, embryo water content 0.92 ± 0.14 $g.g^{-1}$), however, resulted in an increase of leakage to 11.6 $\mu Scm^{-1}.g^{-1}.min^{-1}$ (Figure 19).

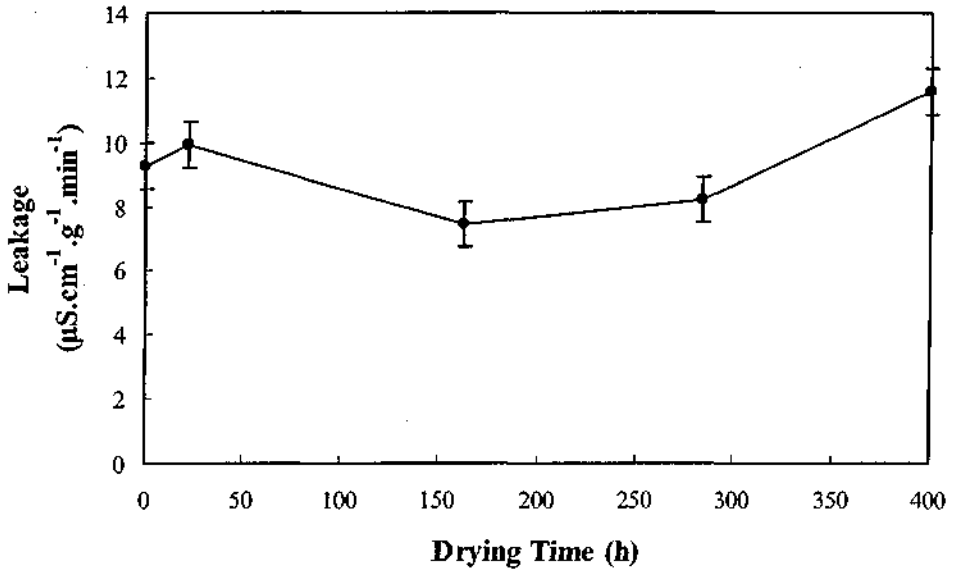


Figure 19: The relationship between drying time and electrolyte leakage of *P. henkelii* embryos (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.4 *Syzygium cuminii*

3.1.4.1 Water Content

The seeds of *Syzygium cuminii* are polyembryonic, some seeds had as many as three embryos but most contained two. For the water content measurements all the embryos were extracted (for seeds containing more than one embryo) and the mean water content was calculated for the embryo components in each seed. The initial whole seed water content at shedding was 43% (fmb), and the water content of the axes ($1.66 \pm 0.31 g.g^{-1}$) was much higher than that of the corresponding cotyledons (0.69 ± 0.03 $g.g^{-1}$) (Figure 20). Despite being buried in activated silica gel, the rate of water loss by the whole seeds was slow, requiring 288 h to reach the target water content of 5%, hence axes and cotyledons were assessed over this prolonged period when ultimately there was little difference between the water contents of the two seed components (Figure 20).

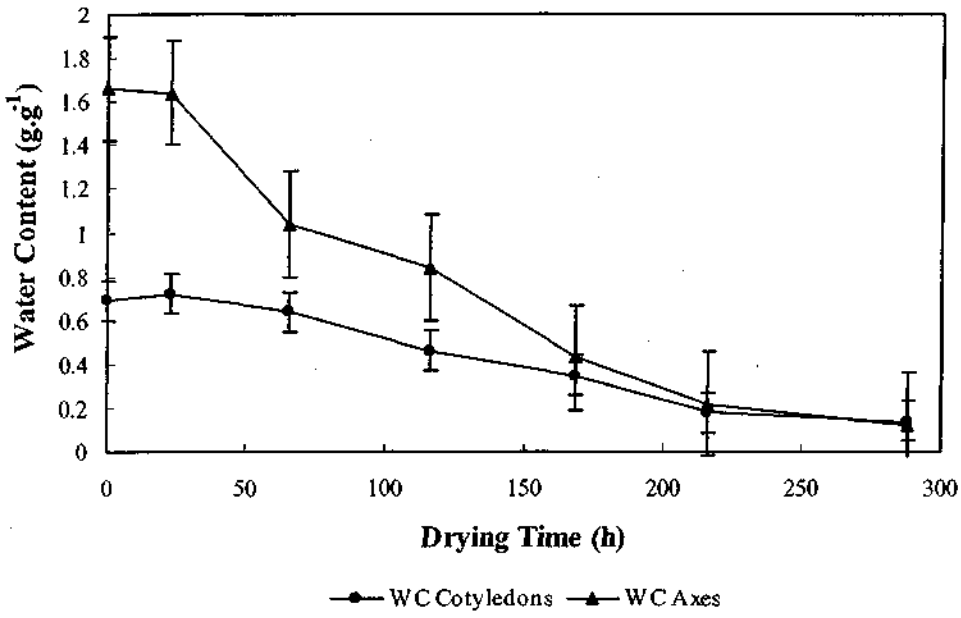


Figure 20: The relationship between drying time and water content of the embryonic axes (▲) and cotyledons (•) taken from whole *S. cuminii* seeds after drying in silica gel to various target water contents (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.1.4.2 Germination

All the freshly harvested *S. cuminii* seeds germinated and all these seeds produced apparently normal seedlings (Figure 21). During germination many seeds produced more than one radicle; however, germination was scored positive after the emergence of at least one radicle. Both seed viability and the percentage of apparently normal seedlings decreased when the mean water contents of the axes were reduced to levels below the initial 1.63 $g.g^{-1}$ (drying for longer than 23 h) (Figure 21). Germination was reduced by 40% after seed drying to an axis water content of 1.05 ± 0.47 $g.g^{-1}$, and viability was completely lost below axis water contents of 0.12 ± 0.02 $g.g^{-1}$. Seedling production was more adversely affected by desiccation than was radicle emergence: while 30% of those seeds that had been dried to an axis water content of 0.8 ± 0.33 $g.g^{-1}$ germinated, only 12% produced apparently normal seedlings (Figure 21).

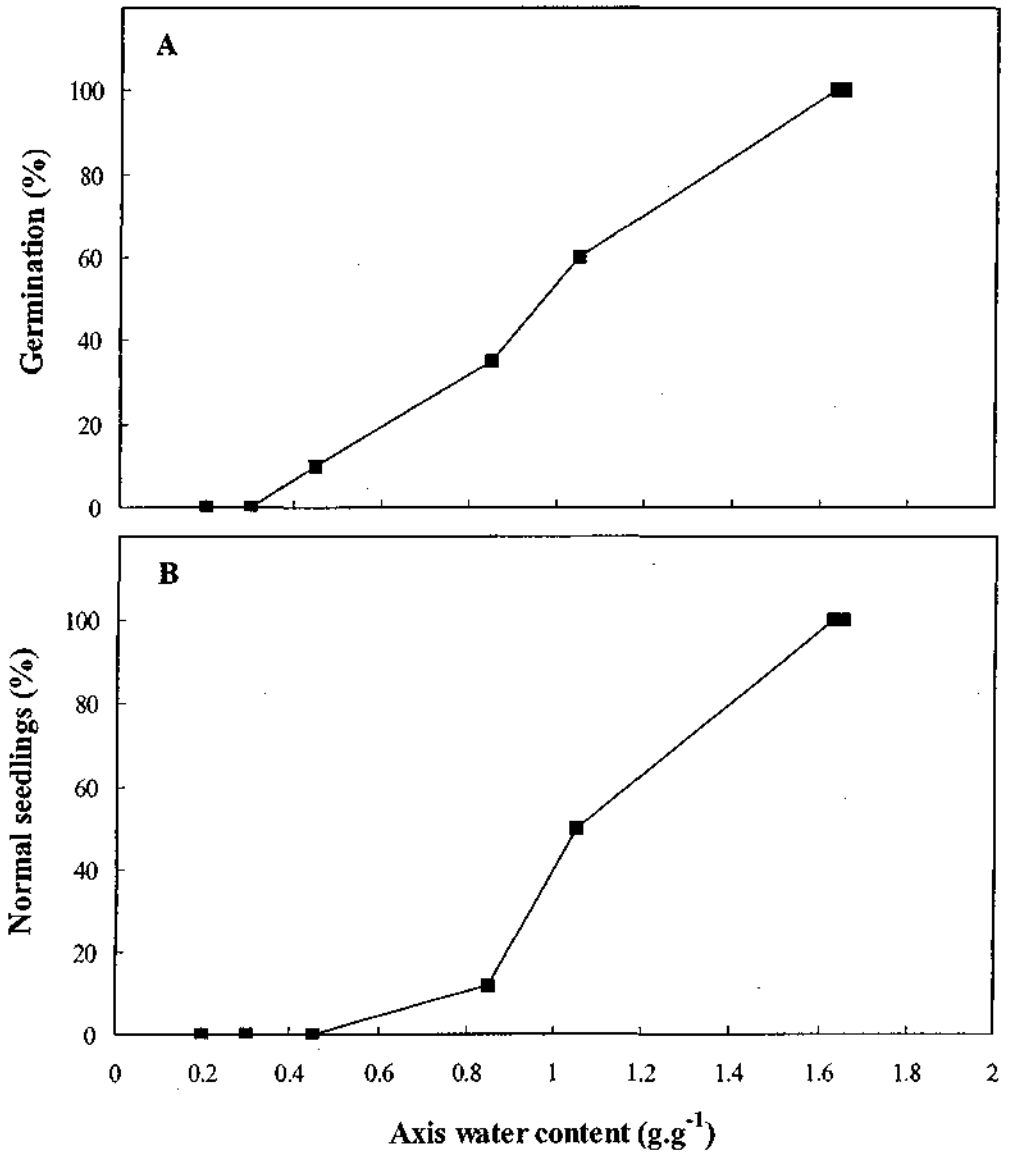


Figure 21: Viability (A) and percentage normal shoot production (B) of *S. cuminii* seeds, over a range of axis water contents (n = 25 seeds).

Fresh (undried) seeds initiated radicle emergence 4 days after being placed to germinate, and all the seeds in the sample germinated within 9 days (Figure 22). Axis water content and seed viability were unchanged following drying for 23 h, but when compared with the fresh, undried material, these seeds germinated slightly more rapidly (Figure 22). Drying *S. cuminii* seeds for durations of 65 h (to an axis water content 1.04 ± 0.47 $g.g^{-1}$)

and longer, however, proved to be deleterious, as both germination totality and rate were considerably reduced (Figure 22).

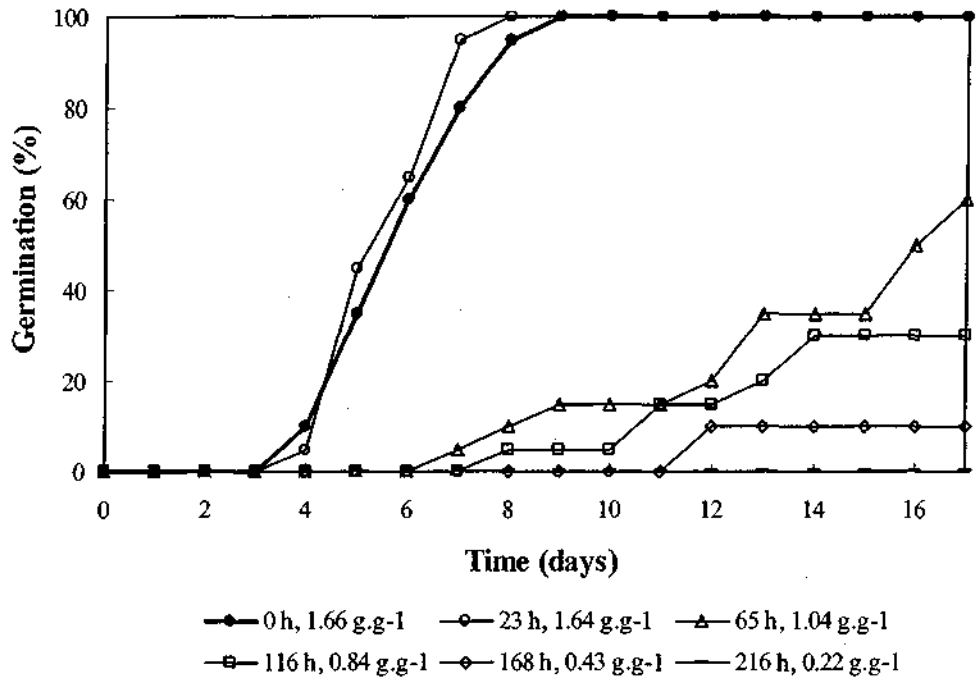


Figure 22: The germination time-course of freshly harvested (undried) *S. cuminii* seeds and seeds dried in activated silica gel for various durations, corresponding to the time taken to reach each predetermined target water content (n = 25 seeds).

3.2 Storage Experiments

These experiments involved storing whole seeds of *Trichilia dregeana*, *T. emetica*, *Podocarpus henkelii* in sealed containers at various temperatures, *viz.* 6, 16 and 25°C, while 6°C storage was omitted for the seeds of *Syzygium cuminii* (as these seeds were previously reported to be chilling sensitive). In all cases, seeds were stored in both the hydrated state (i.e. undried, freshly harvested or shed seeds), henceforth termed hydrated storage, or following partial, short-term desiccation to the target water contents where seed viability was not compromised (i.e. 'sub-imbibed' storage). The requisite duration of desiccation required prior to storage in the 'sub-imbibed' state for each species was previously determined (see section 3.1). The seeds were assessed after various periods in storage. Note that for some species the water contents of the hydrated (undried) seeds prior to storage in these experiments were different from the water contents of the hydrated (undried) seeds used in the initial drying experiments. This is due to the fact that a different batch of seeds was used for the two experiments. Error bars on graphs indicate ± standard deviation.

Trichilia dregeana

Trichilia dregeana seeds were collected in 2001 and 2003. The results following hydrated and 'sub-imbibed' storage of the two seed lots are presented separately.

3.2.1.1 *Trichilia. dregeana* seeds harvested in 2001

3.2.1.1.1 Water Content

Trichilia dregeana seeds harvested in 2001 were stored for a maximum of 22 weeks at 6, 16 and 25°C (Figure 23). When assessed at the start of the storage experiment, the axis water content of those seeds that had been partially dried, in preparation for 'sub-imbibed' storage, was around 25% lower (i.e. 2.16 ± 0.3 $g.g^{-1}$) than that of freshly harvested, undried seeds (i.e. 2.9 ± 0.16 $g.g^{-1}$) (Figure 23A). Essentially the mean axis

water contents of the hydrated and 'sub-imbibed' seeds showed a slow decline during the storage period (Figure 23A). Whether hydrated or 'sub-imbibed', axes of seeds stored at 6°C maintained a higher mean water content than axes of seeds stored at 16 and 25°C, when assessed after 22 weeks in storage (Figure 23A).

The cotyledonary water content of the partially dried seeds (i.e. 0.8 ± 0.08 $g.g^{-1}$) was essentially not lower than the cotyledons of freshly harvested, undried seeds, when assessed at the start of the storage period (0.82 ± 0.09 $g.g^{-1}$) (Figure 23B). During storage, there was a general fluctuation in the mean water content of the cotyledons of seeds maintained in both the hydrated and partially dried states at all three temperatures (Figure 23B). The mean cotyledonary water content of *T. dregeana* seeds after 22 weeks 'sub-imbibed' storage at all three temperatures (i.e. 0.74 $g.g^{-1}$), however, was only slightly lower than that of the seeds (after partial drying) at the start (Figure 23B). There was little or no change in the mean cotyledonary water content during hydrated storage, which after 22 weeks storage at all three temperatures, was similar to that of freshly harvested seeds (Figure 23B).

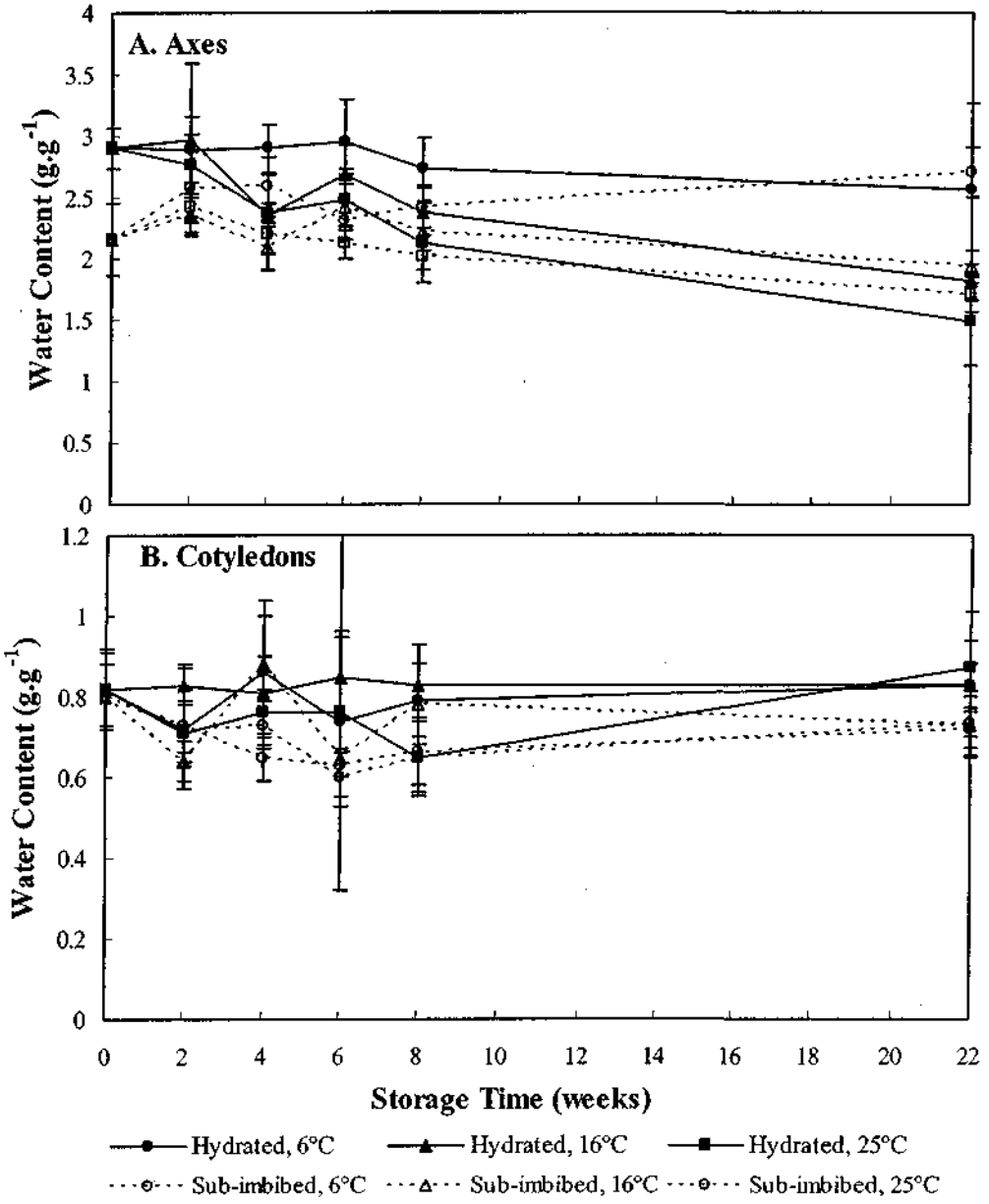


Figure 23: Axis (A) and cotyledonary (B) water contents of *T. dregeana* seeds, harvested in 2001, following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 22 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.2.1.1.2 Germination

The storage behaviour of hydrated and 'sub-imbibed' *T. dregeana* seeds of the 2001 harvest followed similar trends, and was dependent on the temperature of the storage environment. Undried seeds maintained at the hydration level at harvest (i.e. hydrated storage), and seeds maintained at essentially constant lowered axis water contents (i.e. 'sub-imbibed' storage) remained viable following storage for 22 weeks at 16 and 25°C, as all the seeds germinated (Figures 24B and 24C). In contrast, the germination of seeds stored at 6°C decreased to 12% following hydrated and 'sub-imbibed' storage for 22 weeks (Figure 24A). While all freshly harvested and partially dried seeds stored at 16 and 25°C germinated, the rate of seed germination varied with partial drying, storage temperature and duration (Figures 24B and 24C).

When assessed prior to being placed into storage, seeds that had been partially dried germinated more slowly under the same conditions compared with the freshly harvested, undried, seeds. The onset of germination was delayed from day 8 to day 11, and 96% of the freshly harvested seeds had germinated within 17 days, while seeds that had been partially dried achieved similar levels of germination within 20 days (Figures 24A-C). The reduced vigour of those seeds that had been partially dried continued to be manifested during 'sub-imbibed' storage, as the germination rate of these seeds was always slower than that of seeds maintained in a hydrated state, when assessed at each sampling, during storage at 16 and 25°C (Figures 24B and 24C).

The best storage temperature was 16°C, as both germination rate and totality were the least adversely affected following hydrated storage for 22 weeks, when compared with those seeds stored in the same condition at 6 and 25°C. In fact, storage for 8 weeks at 16°C was associated with an improved germination rate of the hydrated *T. dregeana* seeds, as 96% had germinated within 10-12 days, while freshly harvested, undried seeds, when assessed prior to storage, achieved similar levels of germination within only 16 days (Figure 24B).

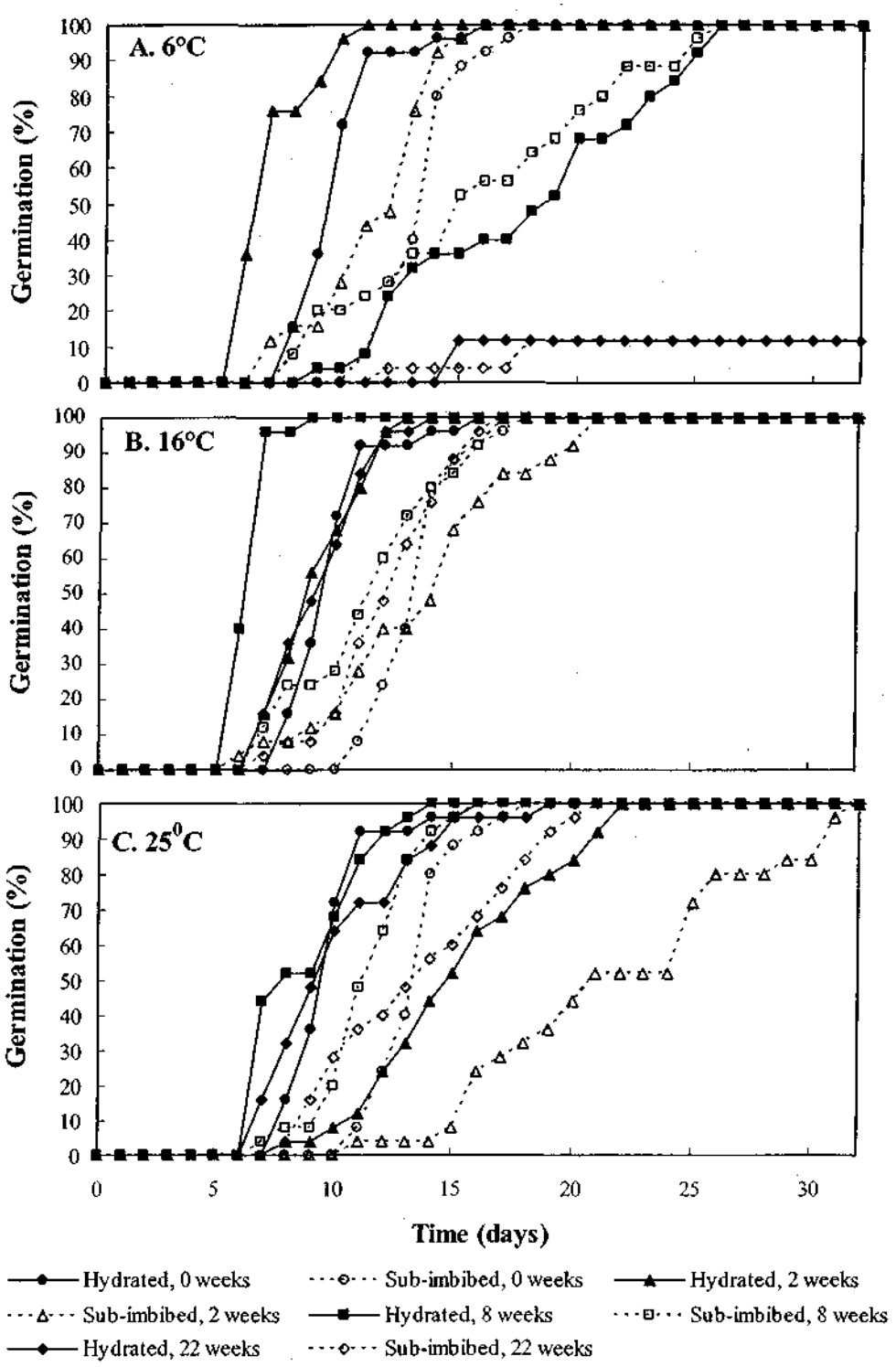


Figure 24: The effect of hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 22 weeks at 6 (A), 16 (B) or 25°C (C) on the vigour and germinability of *T. dregeana* seeds, harvested in 2001 (n = 25 seeds).

3.2.1.2. *Trichilia dregeana* seeds harvested in 2003

3.2.1.2.1 Water Content

To determine the effects of lowered water contents on the storage behaviour of *T. dregeana* seeds during 'sub-imbibed' storage, two water contents (*viz.* 30 and 35%, fmb) were chosen from the results of the drying experiment (3.1.1.1) for seeds harvested in 2003. The results of 'sub-imbibed' storage at 16°C for 5 weeks at these lowered water contents are compared with those of freshly harvested seeds (i.e. hydrated storage) stored under the same conditions for the same duration.

The axis and cotyledonary water contents of freshly harvested, undried, seeds were 1.82 ± 0.11 and 0.53 ± 0.06 $g.g^{-1}$, respectively (Figures 25A and 25B). 'Mild' partial desiccation (11 h) lowered the water content of the axes and cotyledons to 1.3 ± 0.18 and 0.31 ± 0.09 $g.g^{-1}$, respectively and to 1.04 ± 0.1 and 0.21 ± 0.08 $g.g^{-1}$, respectively for more severe drying (19 h) (Figures 25A and 25B). Axis water contents were thus being reduced by around 28 and 42%, respectively for 'mild' and 'severe' drying. Mean axis and the cotyledonary water contents remained unchanged during subsequent storage of freshly harvested, undried, seeds (i.e. hydrated storage) and for both 'sub-imbibed' storage regimes (Figures 25A and 25B).

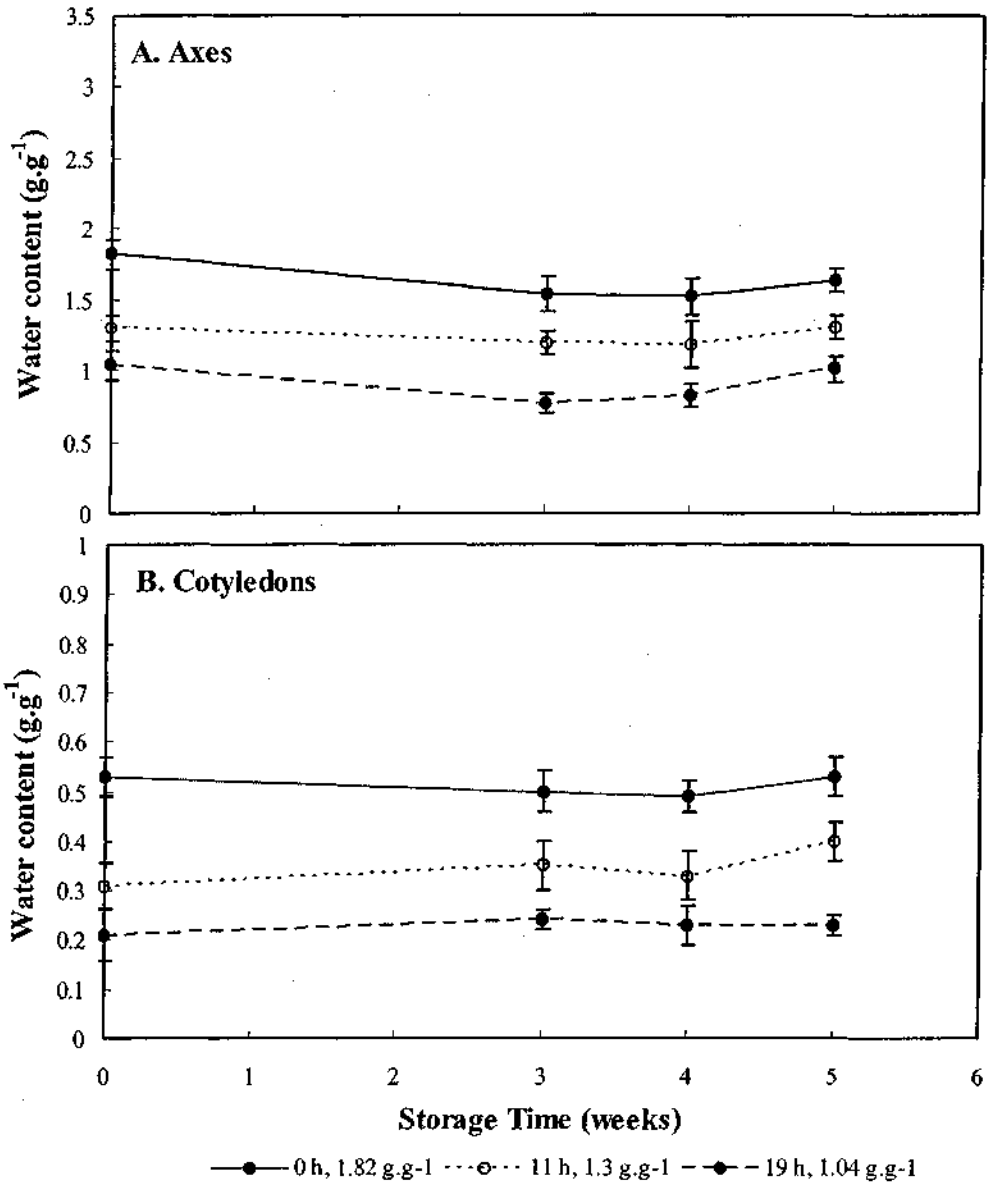


Figure 25: Axis (A) and cotyledonary (B) water contents of *T. dregeana* seeds, harvested in 2003, following hydrated (solid line, solid symbols) and 'sub-imbibed' storage, after both 'mild' (11 h) (dashed line, open symbols) and 'severe' (19 h) (dashed line, solid symbols) partial drying, for 5 weeks at 16°C (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.2.1.2.2 Germination

The germination totality of freshly harvested (undried) seeds was 100% and seed viability was unaffected by desiccation to axis water contents of 1.3 ± 0.18 and 1.04 ± 0.1 $g.g^{-1}$, respectively, as all the seeds germinated when assessed prior to being placed into storage (Figures 26A-C). However, seed vigour was adversely affected by further desiccation, even when seeds were assessed immediately after drying. While 72% of fresh (undried) seeds had germinated within 9 days, seeds dried to 1.3 ± 0.18 and 1.04 ± 0.1 $g.g^{-1}$ mean axis water contents achieved similar levels of germination only after 13 and 16 days, respectively (Figures 26A-C).

While all the seeds at the original water content had retained viability following 3 weeks hydrated storage, the germination totality of *T. dregeana* seeds following 'sub-imbibed' storage for the same duration in both cases, had decreased by 16 and 24%, respectively (Figures 26A-C). Storage for longer than 3 weeks adversely affected seed viability in all cases. Viability was completely lost following 5 weeks 'sub-imbibed' storage at mean axis water content of 1.04 ± 0.1 $g.g^{-1}$ (Figure 26C), while only 12 and 8% of the seeds germinated following hydrated and 'sub-imbibed' storage (at an axis water content of 1.3 ± 0.18 $g.g^{-1}$), respectively, for the same duration (Figures 26A and 26B).

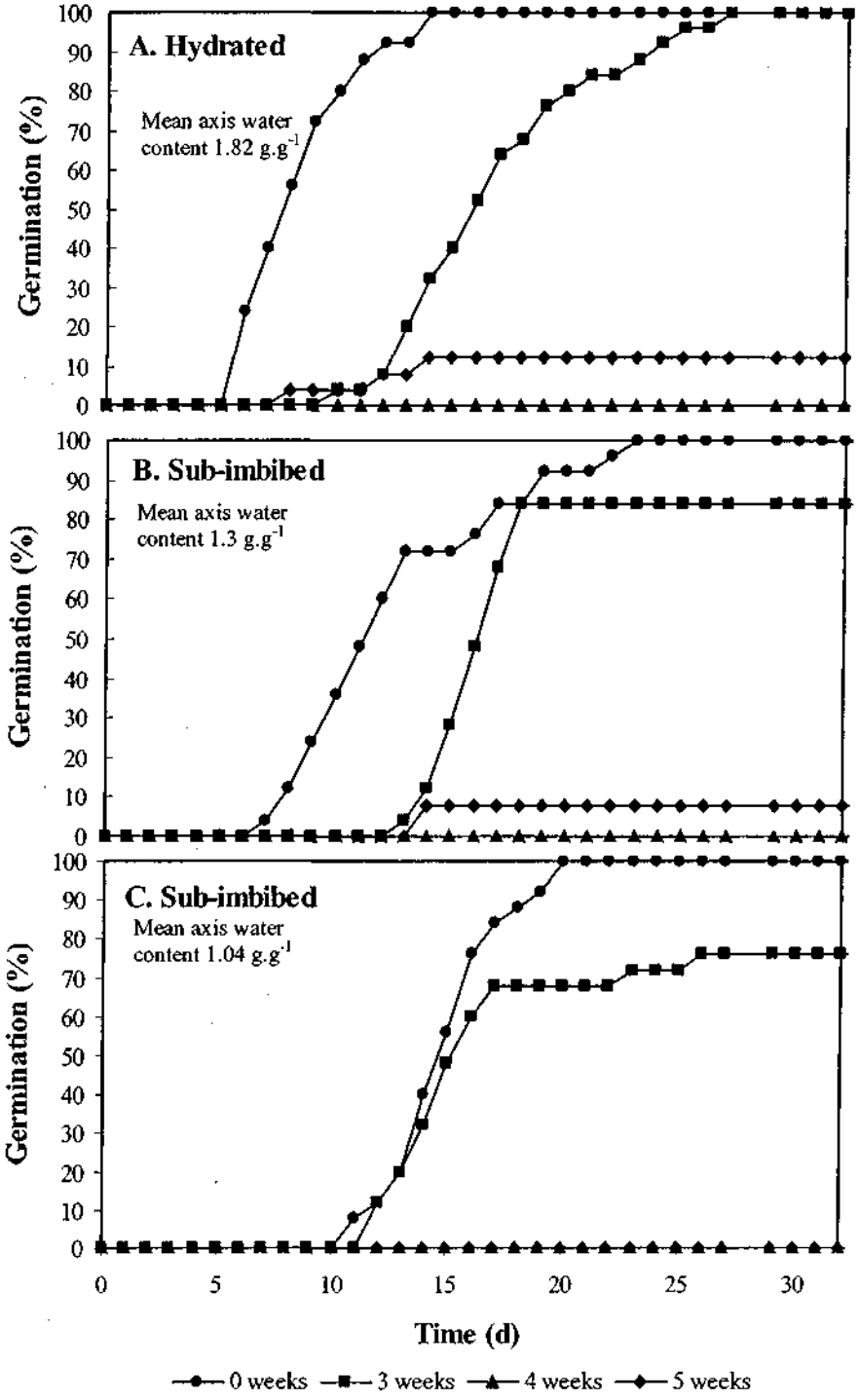


Figure 26: The effect of hydrated (A) and 'sub-imbibed' (B) and (C) storage over 5 weeks at 16°C, on vigour and viability of *T. dregeana* seeds, from the 2003 harvest. Axis water content declined from 1.82 $g.g^{-1}$ to 1.3 (B) and 1.04 $g.g^{-1}$ (C) for seeds dried for 11 and 19 h, respectively (n = 25 seeds).

3.2.1.2.3 Electrolyte leakage

Mean electrolyte leakage, which was determined for the axes and the cotyledons of *T. dregeana* separately, was considerably higher from the axis tissues than from the cotyledons, when assessed prior to storage, in all cases (Figures 27A and 27B). In addition, partial desiccation for 11 and 19 h to water contents of 1.3 ± 0.18 and 1.04 ± 0.1 $g.g^{-1}$, respectively, increased the amount of electrolyte leaked from both the axes and cotyledons, when compared with the leakage of electrolytes from the component tissues of freshly harvested, undried seeds at the start of the storage experiments (Figures 27A and 27B). While there was little or no change in the extent of leakage from the axes and cotyledons of seeds following 4 weeks 'sub-imbibed' storage at a axis water content of 1.3 ± 0.18 $g.g^{-1}$, the leakage of electrolytes increased following both hydrated storage and 'sub-imbibed' storage at axis water content of 1.04 ± 0.1 $g.g^{-1}$ when assessed after the same storage period (Figures 27A and 27B). When assessed after 5 weeks, however, the mean electrolyte leakage had inexplicably decreased to a certain extent in some cases (Figure 27).

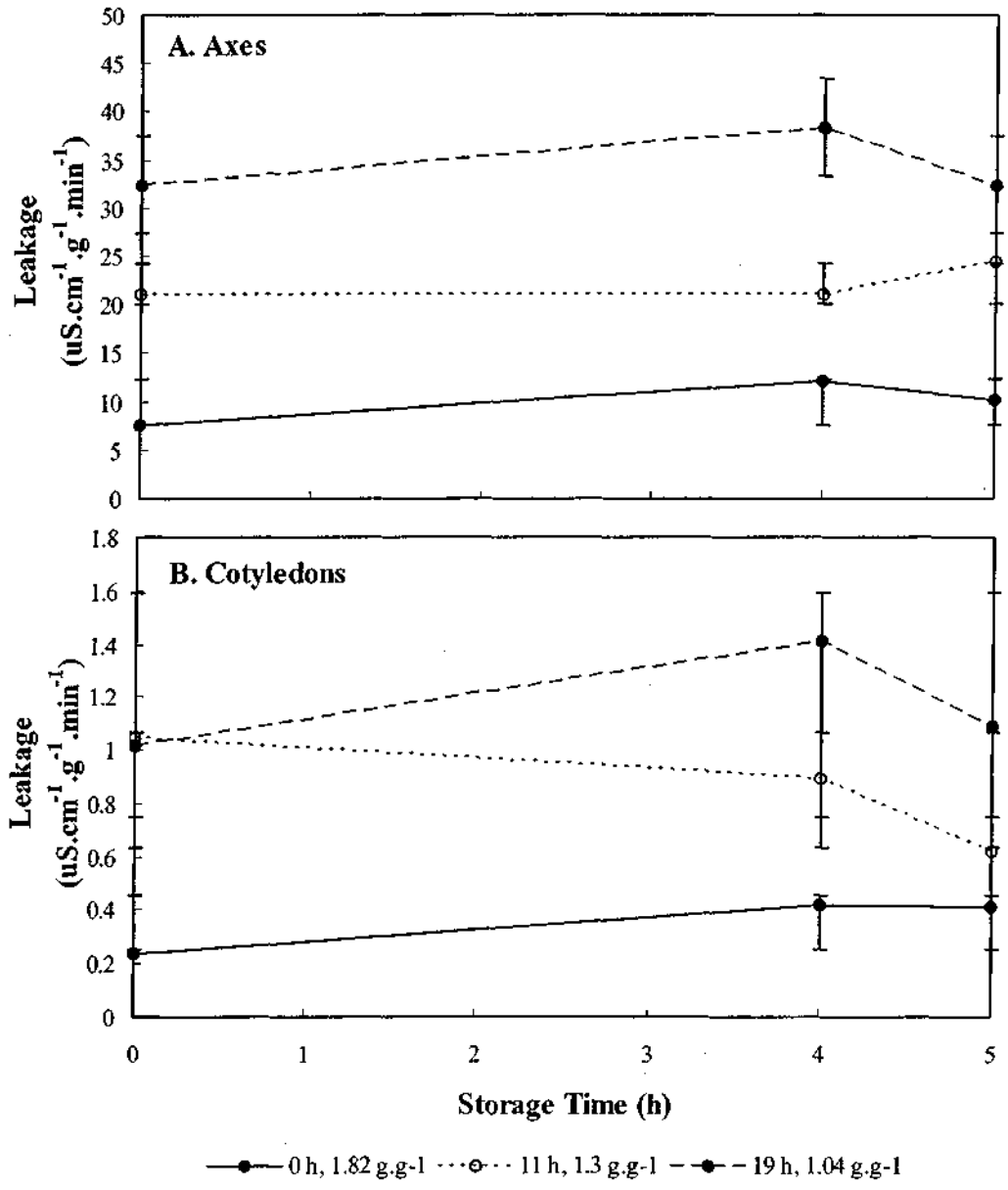


Figure 27: The leakage of electrolytes from axes (A) and cotyledons (B) of *T. dregeana* seeds, harvested in 2003, following hydrated (solid line, solid symbols) and 'sub-imbibed' storage, at an axis water content of 1.3 $g.g^{-1}$ (dashed line, open symbols) an axis water content of 1.04 $g.g^{-1}$ (dashed line, solid symbols), for 5 weeks at 16°C (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.2.2 *Trichilia emetica*

Trichilia emetica seeds were harvested over two consecutive years; *viz.* 2002 and 2003. Freshly harvested, undried seeds and seeds that had been partially dried for 6 h (i.e. hydrated storage and 'sub-imbibed' storage, respectively) were stored for 3 weeks at 6, 16 and 25°C.

The results of hydrated and 'sub-imbibed' storage are presented separately for the two seed lots.

3.2.2.1 *Trichilia emetica* seeds harvested in 2002

3.2.2.1.1 Water Content

Prior to storage the axis (0.77 ± 0.08 $g.g^{-1}$) and cotyledonary (0.13 ± 0.02 $g.g^{-1}$) water contents of the seeds which had been partially dried were considerably lower than those of the freshly harvested, undried seeds (1.5 ± 0.25 and 0.79 ± 0.43 $g.g^{-1}$, axes and cotyledons respectively) (Figures 28A and 28B). The seeds were stored for a maximum of 3 weeks, during which there was some fluctuation in the water content of both axes and the cotyledons of the seeds maintained in both the hydrated and partially dried states at all three temperatures (Figures 28A and 28B). However, after 3 weeks 'sub-imbibed' storage, there had been little change in the mean cotyledonary water content of the seeds stored at any of the three temperatures (Figure 28B). In contrast, there was a decrease in mean cotyledonary water content during hydrated storage (0.79 ± 0.43 $g.g^{-1}$ prior to storage and 0.24 ± 0.13 - 0.44 ± 0.11 $g.g^{-1}$ after storage) (Figure 28B). Generally the mean water content of the axes decreased (after an initial increase) during storage, the extent of the decrease being least for seeds stored at 6°C, whether hydrated or 'sub-imbibed' (Figure 28A).

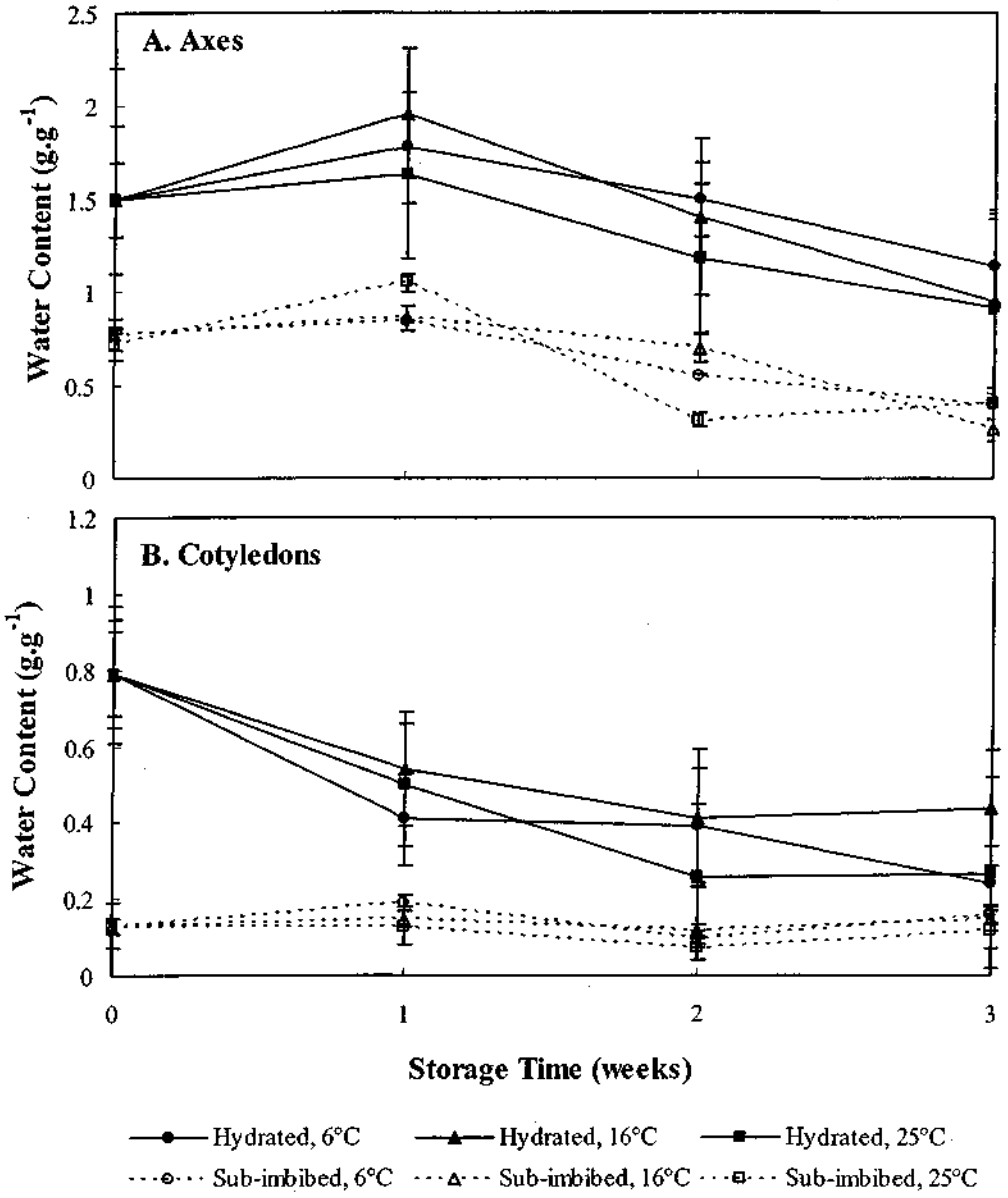


Figure 28: Axis (A) and cotyledonary (B) water contents of *T. emetica* seeds, harvested in 2002, following hydrated (solid lines, solid symbols) or 'sub-imbibed' storage (dashed lines, open symbols) for 3 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.2.2.1.2 Germination

Germination of freshly harvested *T. emetica* seeds was rapid, as radicle emergence was initiated by some seeds after 1 day, and all of the seeds germinated within 3 days (Figure 29). Unstored, partially dried seeds germinated at a slightly slower rate but all of the seeds had germinated after 6 days (Figure 29).

The undried (hydrated) seeds stored at 16 and 25°C achieved 100% germination totality throughout the storage period; however, storage reduced the rate of germination, when compared with freshly harvested seeds (the seeds which had been stored reached 100% germination only after 8 days compared with 3 days for the freshly harvested seeds) (Figures 29B and 29C). However, the vigour and viability of seeds during 'sub-imbibed' storage at the same temperatures was considerably reduced, particularly in those seeds stored at 25°C. Viability was lost completely after 3 weeks storage at 25°C, and, during storage, only 30 and 5% of the seeds germinated after 1 and 2 weeks respectively (Figure 29C). The 'sub-imbibed' seeds stored at 16°C maintained higher viability than those stored at 25°C, with 50 and 20% of the seed lot germinating after 2 and 3 weeks, respectively (Figure 29B). Generally, viability retention for both 'sub-imbibed' and the hydrated seeds was better when storage at 16 than 25°C (Figures 29B and 29C), which could be ascribed to fungal proliferation at the higher temperature

Seeds of *T. emetica* showed marked chilling sensitivity and storage at 6°C dramatically reduced their storability (Figure 29A). Deleterious effect of lower temperatures appeared to be exacerbated when seeds were partially dried; none of the 'sub-imbibed' seeds germinating following storage, while approximately 10% of the hydrated seeds germinated after 1 week storage (Figure 29A). However, the apparent differences in seed responses associated with the hydration levels were too small to be considered significant.

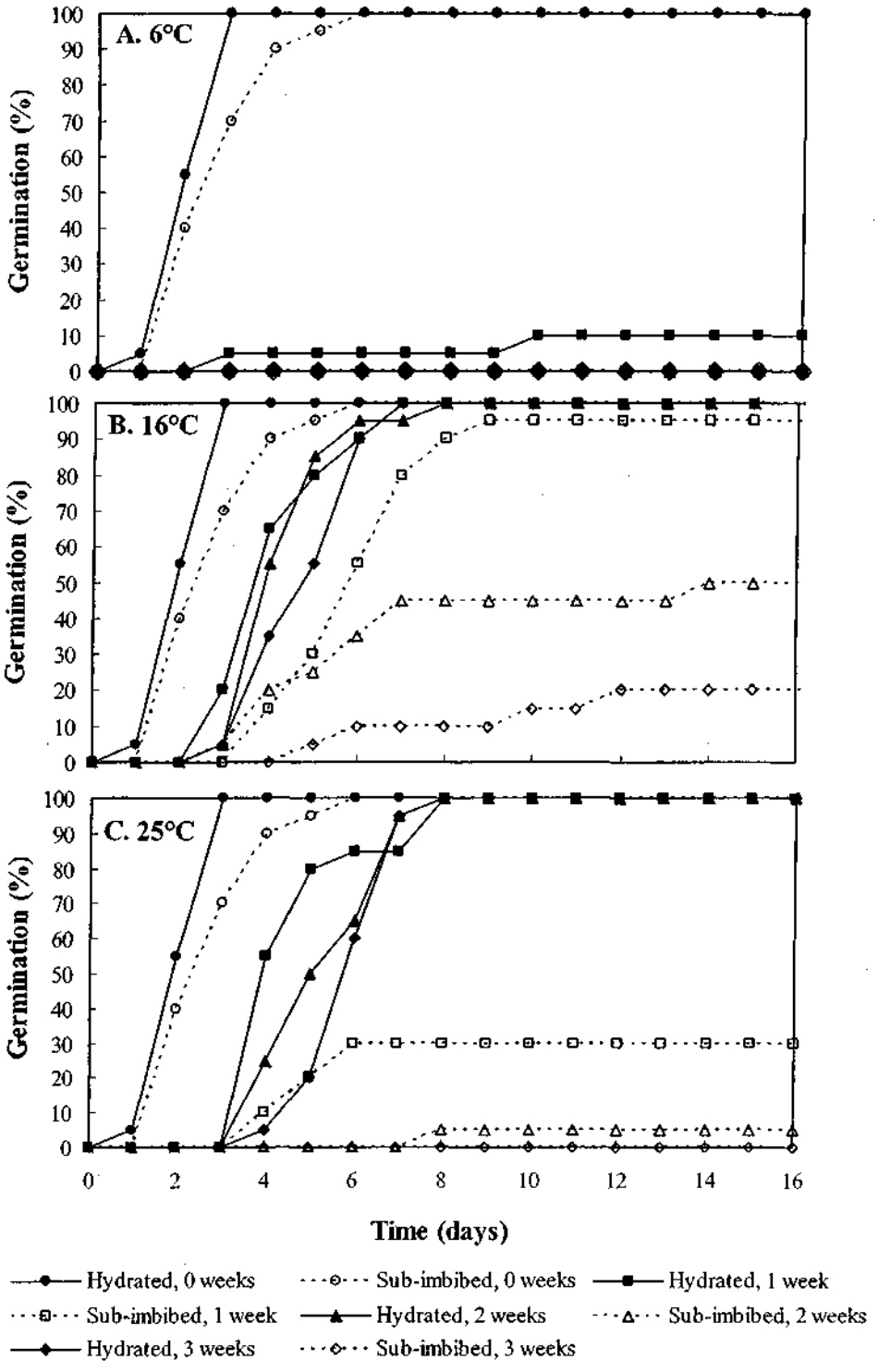


Figure 29: The effect of hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 3 weeks at 6 (A), 16 (B) or 25°C (C) on the viability and vigour of *T. emetica* seeds, harvested in 2002 (n = 25 seeds).

3.2.2.1.3 Respiration

The freshly harvested seeds had a considerably higher rate of respiration (1.13 ± 0.08 nmol $CO_2.g^{-1}$ $dm.min^{-1}$) than the partially dried seeds (0.68 ± 0.07 nmol $CO_2.g^{-1}$ dm. min^{-1}) (Figure 30), when assessed prior to storage. Seed respiration declined progressively during both hydrated and 'sub-imbibed' storage, at all three temperatures, when assessed over the 3 week storage period (Figure 30).

The seeds stored at 25°C had the lowest levels of respiration (both undried and 'sub-imbed' condition) when assessed after 3 weeks storage (Figure 30). Considering their viability loss, the apparently higher respiratory rate of hydrated seeds stored at 6°C might be attributed to proliferation of associated fungi which was indicated by visual inspection.

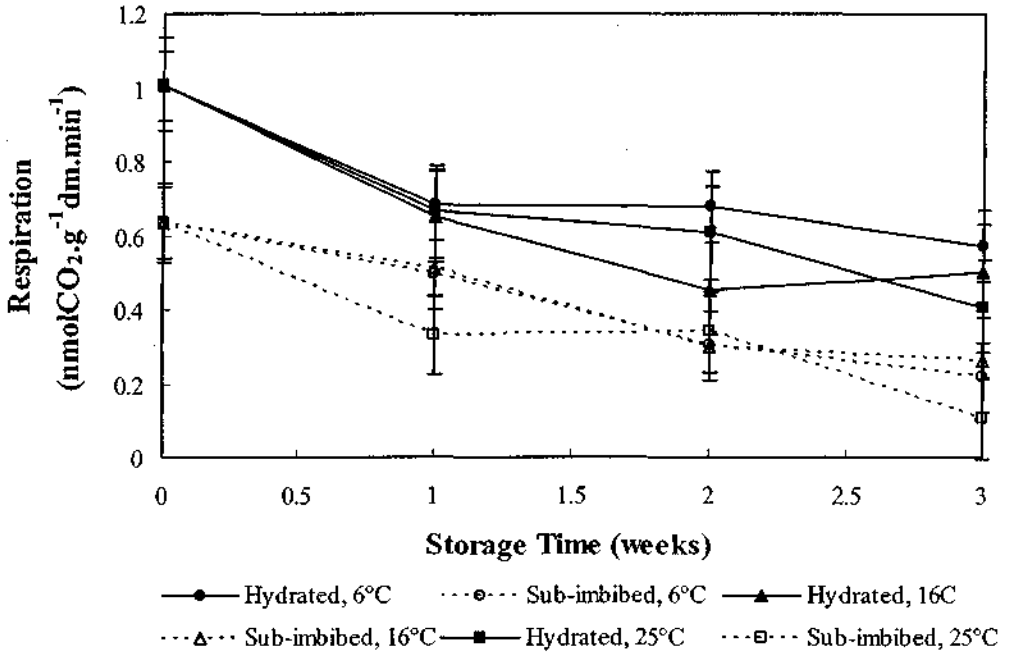


Figure 30: The rate of respiration, determined by measuring the rate of CO_2 evolution, of whole *T. emetica* seeds harvested in 2002, following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 3 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.2.2.1.4 Electrolyte Leakage

Electrolyte leakage was determined before storage and after 3 weeks hydrated and 'sub-imbibed' storage. Leakage was measured separately for cotyledons and axes. Prior to storage the mean electrolyte leakage from the cotyledons of the freshly harvested, undried seeds (0.53 $\mu S.cm^{-1}.g^{-1}.min^{-1}$) was much lower than the mean leakage from those of the 'sub-imbibed' seeds (2.36 $\mu S.cm^{-1}.g^{-1}.min^{-1}$). Prior to storage, the mean electrolyte leakage from the axes was similar regardless of whether these were from fresh, undried seeds or 'sub-imbibed' seeds (Table 1; [note that these results are presented in tabular form, as only two datum points were taken per storage condition]).

Mean electrolyte leakage from the cotyledons from the hydrated seeds did not increase markedly following hydrated storage at 25°C for 3 weeks, when compared with the seeds sampled after 0 weeks storage (Table 1). However, the mean leakage of electrolytes from the cotyledons increased substantially from 0.53 to 1.16 $\mu S.cm^{-1}.g^{-1}.min^{-1}$ and 3.13 $\mu S.cm^{-1}.g^{-1}.min^{-1}$, following hydrated storage at 16 and 6°C, respectively, for the same duration (Table 1). The leachate from axes of the seeds, whether hydrated and 'sub-imbibed', stored at 6°C showed more than two-fold increase in conductivity after 3 weeks storage, the difference being greater than for those from seeds stored at 16°C and especially at 25°C if 'sub-imbibed' (Table 1). While leakage of axes from hydrated seeds increased after the 3-week storage period at 16°C, the axes from the hydrated seeds stored at 25°C showed a decrease in mean electrolyte leakage after 3 weeks in storage (Table 1).

Table 1: The leakage of electrolytes from axes and cotyledons of *T. emetica* seeds, harvested in 2002, prior to and following hydrated or 'sub-imbibed' storage for 3 weeks at 6, 16 or 25°C (n=10 seeds)

	Leakage ($\mu S.cm^{-1}.g^{-1}.min^{-1}$)	
Axes	**No Storage**	**3 weeks storage**
Hydrated, 6°C	34.16	72.18
Sub-imbibed, 6°C	32.13	70.35
Hydrated, 16°C	34.16	39.62
Sub-imbibed, 16°C	32.13	60.98
Hydrated, 25°C	34.16	22.82
Sub-imbibed, 25°C	32.13	41.34
Cotyledons		
Hydrated, 6°C	0.53	3.13
Sub-imbibed, 6°C	2.36	3.58
Hydrated, 16°C	0.53	1.16
Sub-imbibed, 16°C	2.36	2.75
Hydrated, 25°C	0.53	0.63
Sub-imbibed, 25°C	2.36	3.5

3.2.2.2 *Trichilia emetica* seeds harvested in 2003

3.2.2.2.1 Water Content

Figure 31 shows the mean water contents of the cotyledons and the axes from freshly harvested, undried seeds and partially dried *T. emetica* seeds harvested in 2003 prior to, and following, 1, 2 and 3 weeks of storage at 6, 16 and 25°C.

The axes from the hydrated seeds essentially maintained the original water content for 2 weeks in storage. However, by week 3, seeds stored at 6°C showed an increase in mean axis water content, while axes from those stored at 16 and 25°C showed a decrease in water content (Figure 31A). The 'sub-imbibed' seeds, although at a considerably lower mean axis water content, showed a similar pattern of change (Figure 31A).

At the outset, the cotyledons from the 'sub-imbibed' seeds were at a considerably lower water content (mean 0.3 ± 0.06 $g.g^{-1}$) than those from the undried seeds (mean 0.49 ±

0.06 $g.g^{-1}$) (Figure 31B). The cotyledons from the 'sub-imbibed' seeds showed a general decrease in mean water content during storage, with those from seeds stored at 6°C having the highest water content following storage (Figure 31B). The cotyledons from the hydrated seeds stored at 16 and 25°C did not show any real change in mean water content during and following storage compared with that prior to storage, while the cotyledons of the hydrated seeds stored at 6°C showed an increase in water content after 3 weeks of storage (Figure 31B).

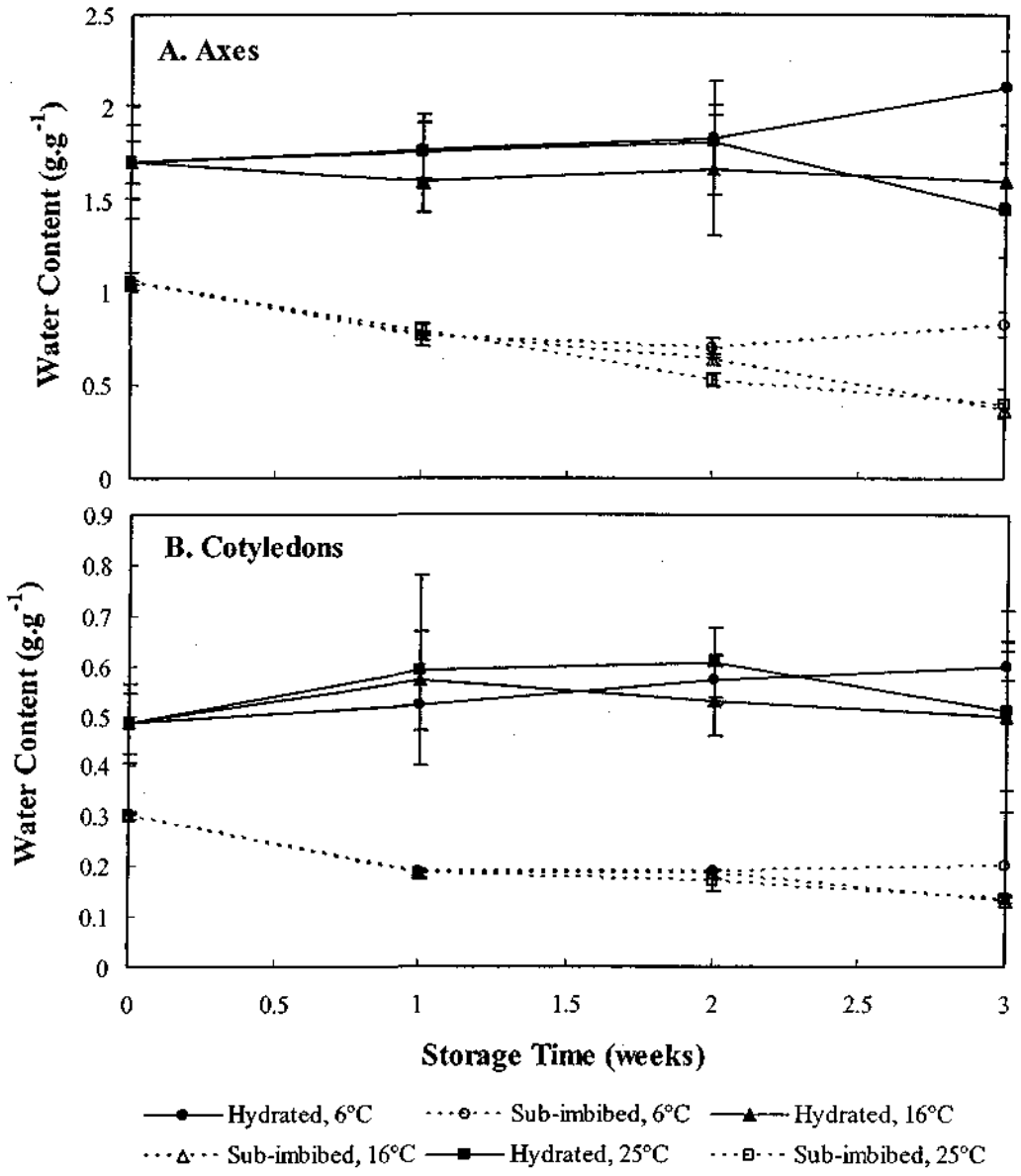


Figure 31: Axis (A) and cotyledonary (B) water contents of *T. emetica* seeds, harvested in 2003, following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 3 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean. Where the bar is so small as to obscure the datum point, it has been omitted.)

3.2.2.2.2 Germination

Germination of freshly harvested *T. emetica* seeds collected in 2003 was slow, relative to the 2002 harvest, as 100% germination took 8 days (Figure 32) to achieve when assessed prior to storage, compared with 3 days (Figure 29) for the latter seeds. Partial desiccation of the seeds (prior to storage) not only reduced the germination rate of these seeds, but also may have had an effect on germinability, as 100% germination was not achieved. However in view of the relatively small sample size (n=25) this cannot be said with certainty.

The hydrated seeds maintained higher viability than the 'sub-imbibed' seeds over the 3 week storage period. At 16 and 25°C, following 3 weeks storage the percentage germination of the hydrated seeds was 84 and 76%, respectively, while that of the 'sub-imbibed' seeds was 0% regardless of storage temperature (Figure 32B and 32C). Storage at 6°C was deleterious to seed survival for both hydrated and 'sub-imbibed' storage (Figure 32A), with survival recorded as 4 and 0%, respectively.

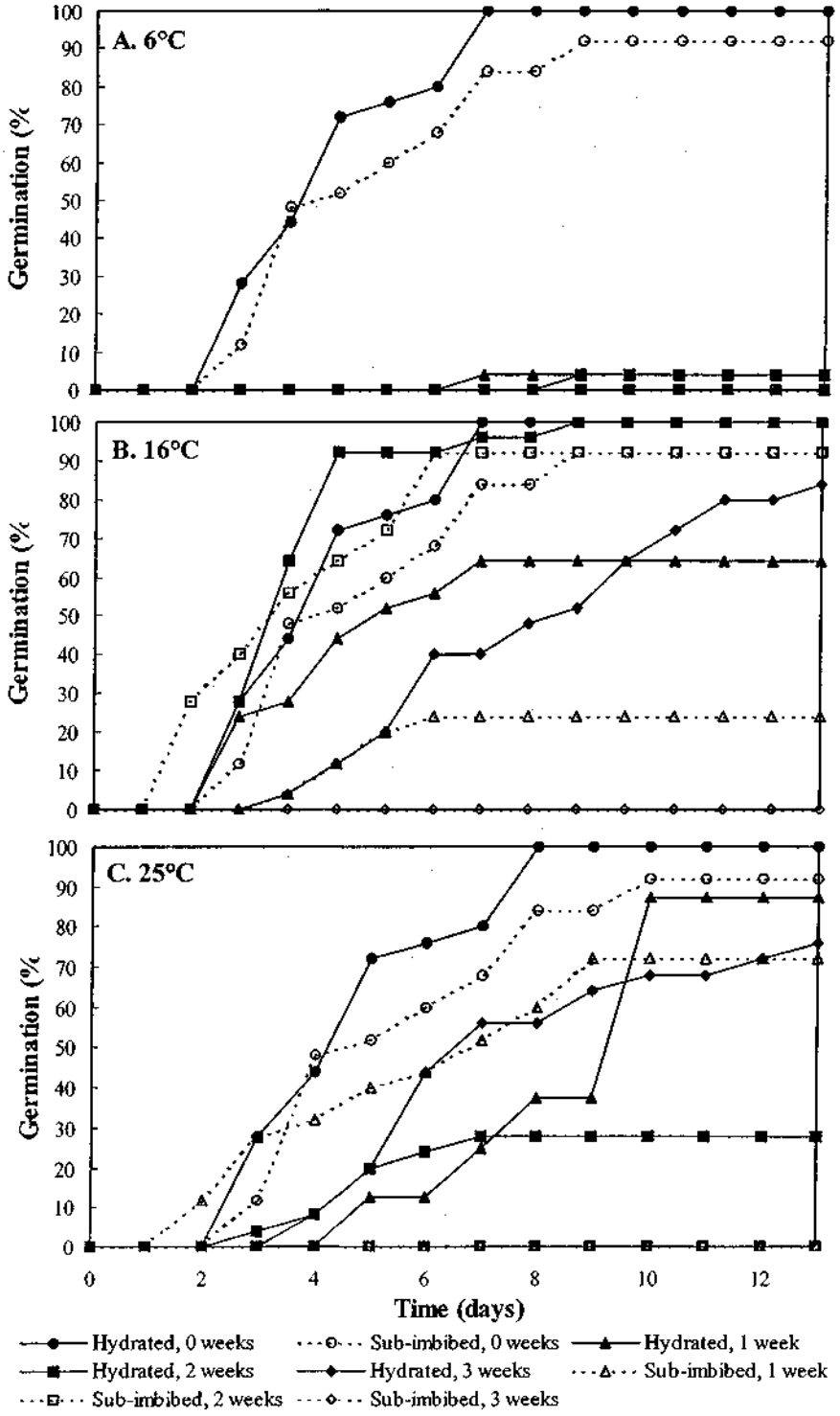


Figure 32: The effect of hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 3 weeks at 6 (A), 16 (B) or 25°C (C) on the viability and vigour of *T. emetica* seeds, harvested in 2003 (n = 25 seeds).

3.2.3 *Podocarpus henkelii*

3.2.3.1 Water Content

The seeds of *P. henkelii* were shed at relatively high water content (53%, fmb.). Embryo water content was 1.67 ± 0.13 $g.g^{-1}$, while that of the gametophyte was 0.99 ± 0.14 $g.g^{-1}$ (Figure 33A and 33B). Partial desiccation reduced the water contents of the embryos and gametophytes by only 5 and 10% to 1.59 ± 0.23 and 0.89 ± 0.15 $g.g^{-1}$, respectively. The embryos from the seeds stored at 6 and 16°C fluctuated slightly in water content during storage; however, after 4 weeks there was no overall change (Figure 33A). The embryos from the hydrated seeds stored at 25°C increased more than two-fold in water content between 4 weeks and the end of the storage trial at 11 weeks (Figure 33A), but this did not occur for hydrated material at the other storage temperatures, or for the embryos from the 'sub-imbibed' seeds at any of the storage temperatures. During storage the gametophyte tissue from both the hydrated and the 'sub-imbibed' seeds did not undergo any marked change in water content over 11 weeks (Figure 33B).

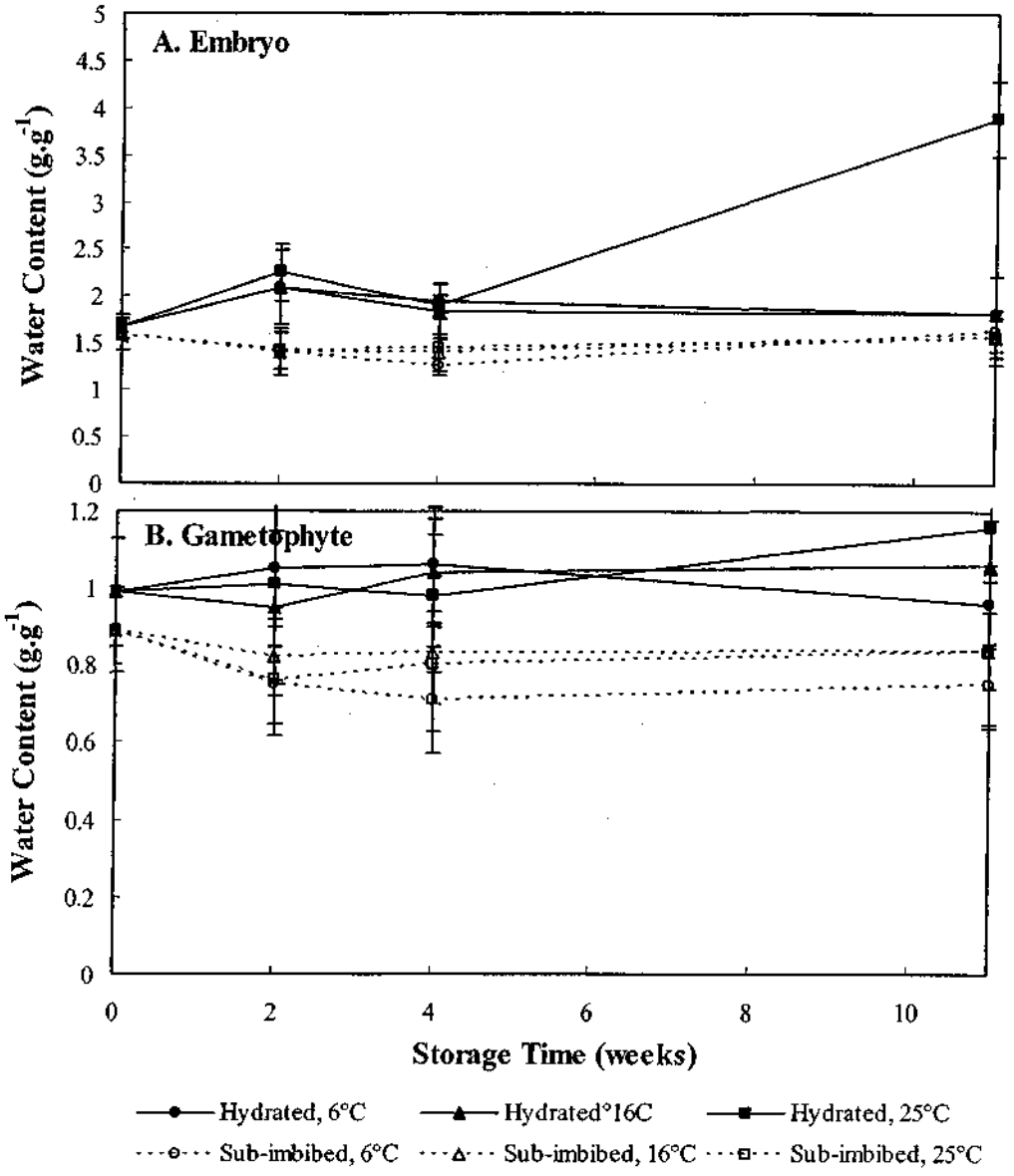


Figure 33: Embryo (A) and mega gametophyte (B) water contents of *P. henkelii* seeds following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 11 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean. (Where the bar is so small as to obscure the shape of the datum point, it has been omitted).

3.2.3.2 Germination

Radicle emergence was first observed 7 days after newly shed hydrated seeds were placed to germinate, and 100% germination was recorded within 21 days (Figure 34). The germination response of partially dried *P. henkelii* seeds, when assessed prior to storage, was essentially similar to that at shedding (Figure 34). While the viability of *P. henkelii* seeds was unaffected following hydrated storage for 11 weeks at 6 and 16°C, the rate at which the seeds germinated was enhanced after 2, 4 and 11 weeks, when compared with the seeds at shedding (Figure 34A and 34B). Following storage at 6 and 16°C the hydrated seeds initiated germination earlier (after 5 days) and 100% had germinated after 13 days (Figure 34A and 34B). In contrast, however, hydrated seeds stored at 25°C had lost viability completely after 11 weeks in storage (Figure 34C). Vigour was not (initially) impaired following 'sub-imbibed' storage at 6 and 16°C, although germination totality was reduced as only 60 – 80% of the seeds germinated (Figure 34A and 34B).

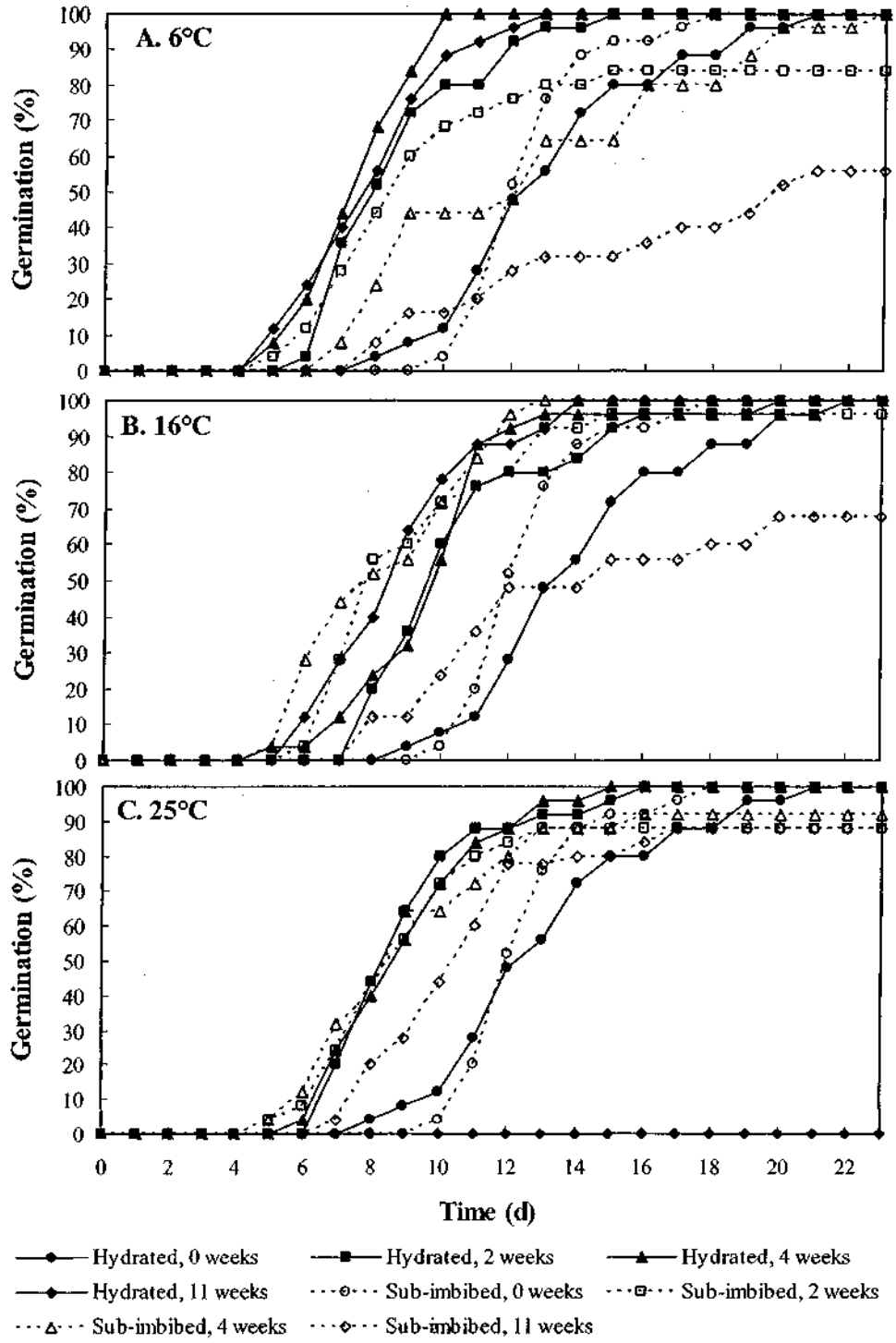


Figure 34: The effect of hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 11 weeks at 6 (A), 16 (B) and 25°C (C) on the viability and vigour of *P. henkelii* seeds (n = 25 seeds).

3.2.3.3 Shoot Production

The percentage shoot production following hydrated and 'sub-imbibed' storage at 6, 16 and 25°C was recorded to determine the effects of storage under these conditions on the number of seedlings produced. With the exception of the 25°C/hydrated seed regime after 11 weeks 'sub-imbibed' storage adversely affected seedling production, when compared with the percentage of seeds that produced shoots following hydrated storage for the same durations and storage temperatures (Figure 35). Following 'sub-imbibed' storage at 16 and 6°C for 11 weeks only 4 and 8% (respectively) of the seeds produced normal shoots, while 72 and 84% of the seeds which were stored at the same temperatures in the fully hydrated condition, produced shoots. Storage at 25°C was deleterious as the percentage of seeds that ultimately produced shoots was low regardless of whether the seeds were stored at the harvest water content or after partial drying (Figure 35).

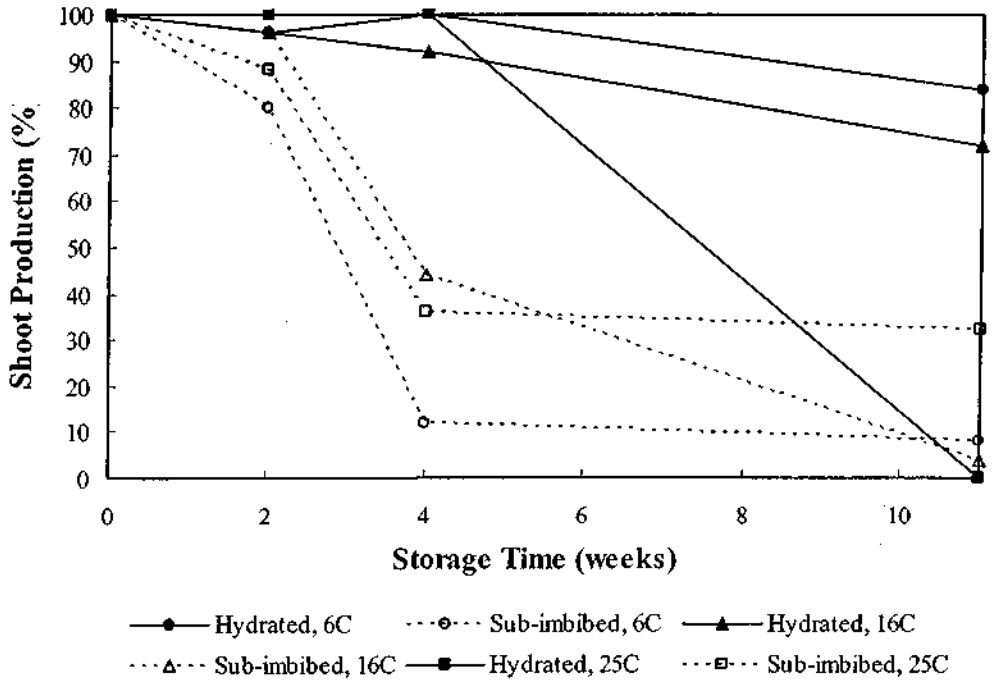


Figure 35: The effect of hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) of *P. henkelii* seeds for 11 weeks at 6, 16 or 25°C on shoot production (n = 25 seeds).

3.2.3.4 Respiration

Prior to storage, the embryos from the 'sub-imbibed' seeds respired at a much lower rate (4.02 ± 0.34 nmol $CO_2.g^{-1}$ dm. min^{-1}) than those from hydrated seeds (11.30 ± 0.5 nmol $CO_2.g^{-1}$ dm. min^{-1}) (Figure 36). Following 2 weeks in hydrated storage, mean embryo respiration declined; in contrast, embryo respiration increased following 'sub-imbibed' storage at 16 and 25°C for the same period (Figure 36). When assessed after 11 weeks storage, however, there was no difference among the mean respiration levels of the embryos taken from seeds stored at the harvest water content and those which were partially dried before storage. Furthermore storage temperature did not effect respiratory rate (Figure 36).

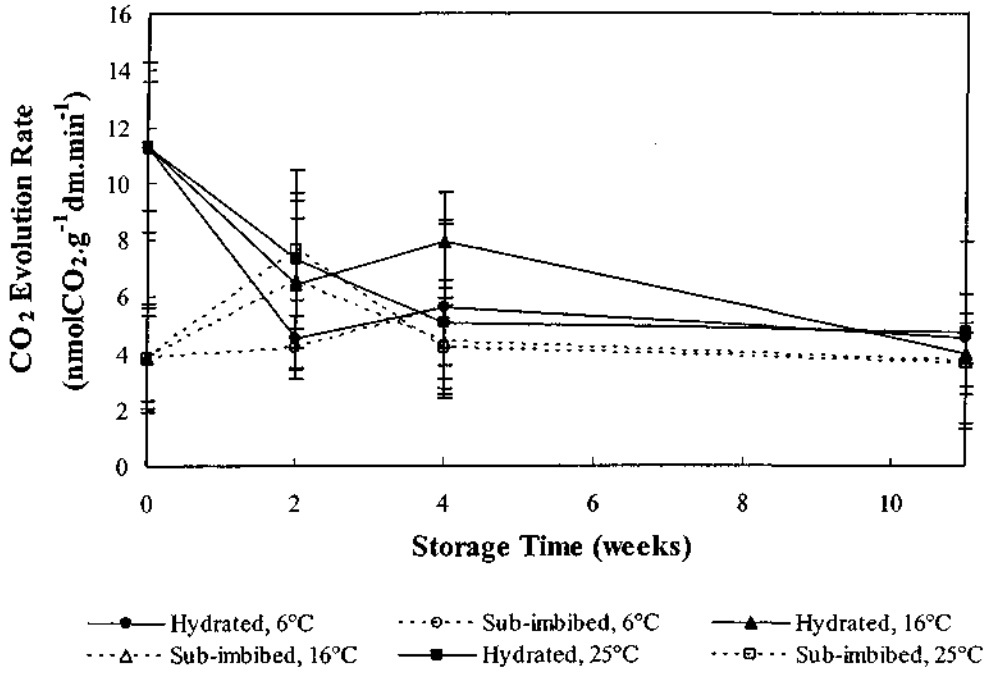


Figure 36: The rate of respiration, determined by measuring the rate of CO_2 evolution, of *P. henkelii* embryos following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 11 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation of the mean.

3.2.3.5 Electrolyte Leakage

Electrolyte leakage was determined before storage and after 2 and 11 weeks hydrated and 'sub-imbibed' storage. Leakage was measured on individual embryos.

Prior to storage the electrolyte leakage from the embryos of the freshly harvested, undried seeds (mean 5.0 $\mu S.cm^{-1}.g^{-1}.min^{-1}$) was lower than that from the embryos of the 'sub-imbibed' seeds (mean 7.5 $\mu S.cm^{-1}.g^{-1}.min^{-1}$) (Figure 37). Mean electrolyte leakage from the embryos from the undried and 'sub-imbibed' seeds increased slightly following storage at 6 and 16°C and from the 'sub-imbibed' seeds stored at 25°C for 10 weeks, when compared with the seeds sampled prior to storage (Figure 37). In sharp contrast, however, the mean leakage of electrolytes from the embryos of the seeds following 11 weeks hydrated storage at 25°C increased more than five-fold (from 5.0 $\mu S.cm^{-1}.g^{-1}.min^{-1}$ to 26.93 $\mu S.cm^{-1}.g^{-1}.min^{-1}$) (Figure 37).

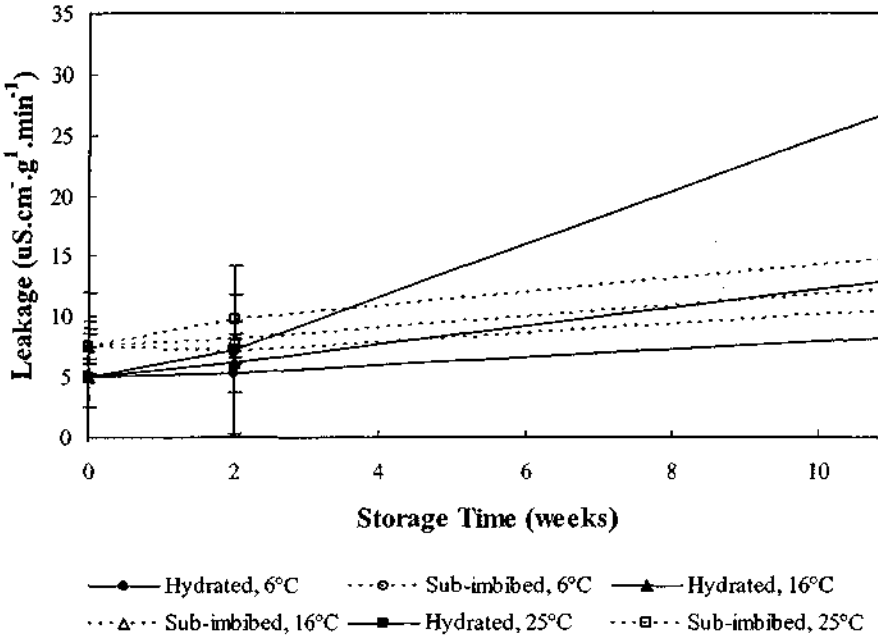


Figure 37: The leakage of electrolytes from embryos of *P. henkelii* seeds following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 11 weeks at 6, 16 or 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean. (Where bars are so small as to obscure the shape of the datum point, it has been omitted)

3.2.4 *Syzygium cuminii*

3.2.4.1 Water Content

The seeds of *S. cuminii* were not stored at 6°C because previous studies found the seeds to be damaged during storage at low temperatures (Mittal *et al.*, 1999). The seeds harvested in 2001 were stored for 6 weeks at 16 and 25°C (Figure 38). When assessed at the start of the storage experiment, neither the mean axis water content (Figure 38A) nor that of the cotyledons (Figure 38B) of those seeds that had been partially dried, in preparation for 'sub-imbibed' storage, was substantially lower than that of freshly harvested, undried seeds. The mean water content of the axes varied, to some extent for the hydrated seeds but particularly following 'sub-imbibed' storage for 6 weeks the axes of seeds stored at both temperatures showed a relatively marked decrease in mean water content that was already apparent earlier (Figure 38A). Axes from seeds stored in the fully hydrated state showed a gradual increase in water content (Figure 38A).

During storage, there was a decline in the mean water content of the cotyledons of seeds maintained in the partially dried state at both temperatures (Figure 38B). The cotyledonary water content (i.e. 0.55 ± 0.05 $g.g^{-1}$) of the seeds after 6 weeks 'sub-imbibed' storage at both temperatures was somewhat, lower than in these seeds at the start of the experiment (Figure 38B), a decline from 0.68 ± 0.03 $g.g^{-1}$ to 0.55 ± 0.05 $g.g^{-1}$. In contrast, there was little or no change in the mean cotyledonary water content during hydrated storage, which, after 6 weeks storage at both temperatures, was similar to that of freshly harvested seeds (Figure 38B).

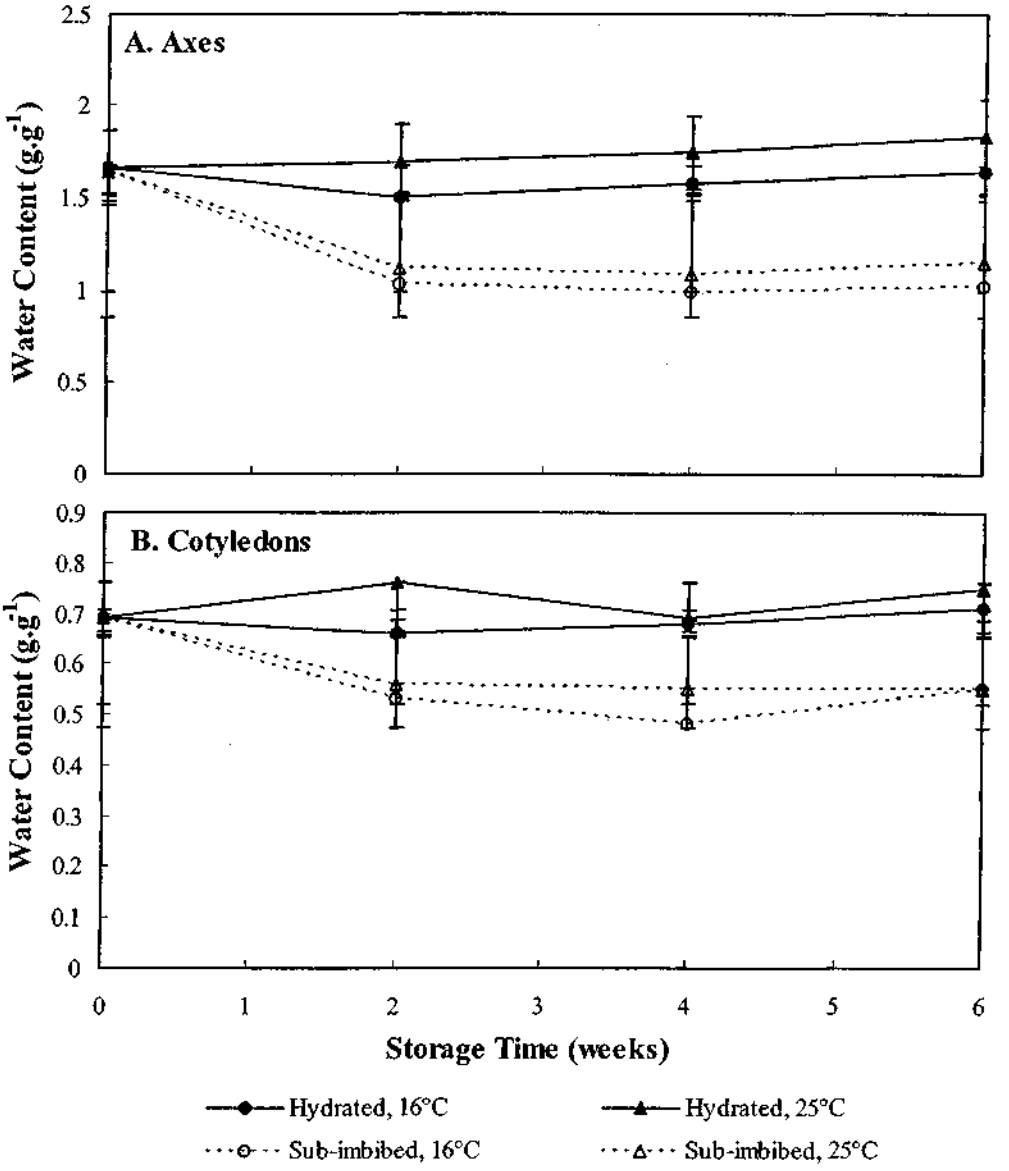


Figure 38: Axis (A) and cotyledonary (B) water contents of *S. cuminii* seeds following hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 6 weeks at 16 and 25°C (n = 10 seeds). Bars represent the standard deviation above and below the mean.

3.2.4.2 Germination

Germination of newly shed, hydrated seeds was rapid, as radicle emergence was initiated 4 days after the seeds were placed to germinate, germination totality was 100% within 9 days (Figure 39). The germination response of partially dried *S. cuminii* seeds, when assessed prior to storage, was similar to that of the seeds at shedding (Figure 39). The viability of *S. cuminii* seeds was unaffected following hydrated storage for 2 weeks at 16 and 25°C, as was the rate at which the seeds germinated, when compared with the rate achieved by newly shed seeds (Figure 39A and 39B). Further hydrated storage at 16°C for 4 and 6 weeks resulted in decreases in germination totality to 60 and 40% respectively, and additionally germination rate was affected, radicle emergence being initiated after 7-10 days (Figure 39A). 'Sub-imbibed' storage at 16 and 25°C resulted in a reduction in germination rate and totality (Figures 39A and 39B). After 6 weeks 'sub-imbibed' storage the seeds lost viability completely, regardless of the storage temperature (Figures 39A and 39B).

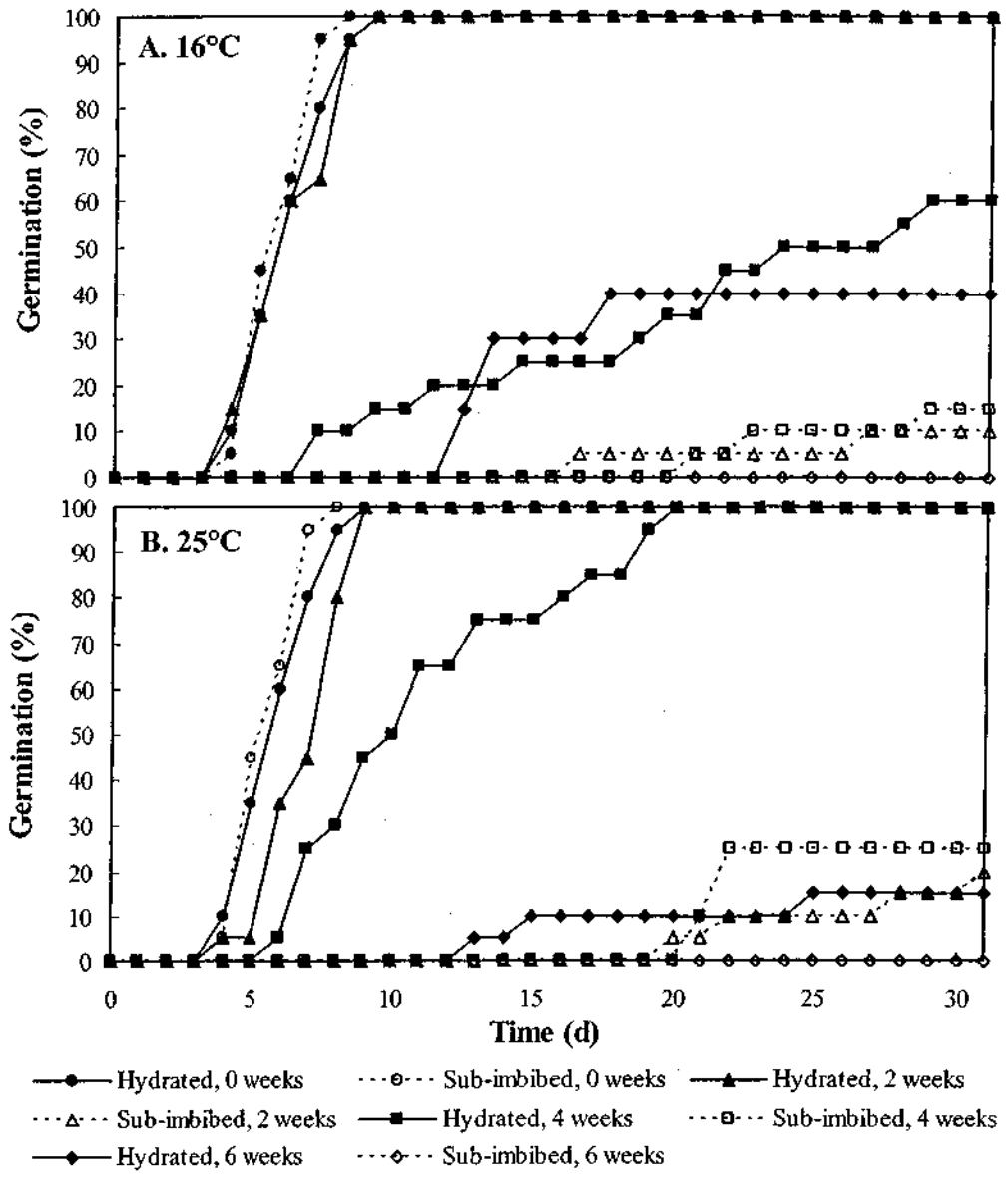


Figure 39: The effect of hydrated (solid line, solid symbols) or 'sub-imbibed' storage (dashed line, open symbols) for 6 weeks at 16 (A) and 25°C (B) on the vigour and viability of *S. cuminii* seeds (n = 25 seeds).

3.3 Ultrastructural Observations

Only the *T. emetica* seeds were processed for microscopy. Fully hydrated and 'sub-imbibed' seeds were removed for processing before storage and after storage (3 weeks) at all three temperatures. In all cases, observations were made on root cells of, or near, the meristem.

3.3.1 Newly harvested, fully hydrated seeds

The ultrastructural condition of the root cells of, or near the meristem of fully hydrated freshly harvested seeds suggested moderate intracellular activity. Well developed Golgi bodies were present although not in large numbers (Figure 40.3). Short profiles of ER were abundant in many cells (Figure 40.3 and 40.4). The cells contained many mitochondria. Most showing little development of inner membranes and non-uniformly dense matrices (Figure 40.2 and 40.4). The nuclear profile was spherical and the nucleolus was prominent, with some condensed chromatin in contact with the nuclear envelope (Figure 40.1). The cell walls were thin with an intact plasmalemma (Figures 40.1 and 40.3). Polysomes were evident in the cytoplasm (Figure 40.3). The plastids showed some internal membrane development, and some contained a small amount of starch (Figure 40.1 and 40.2). The general condition of the cells was good.

3.3.2 Partially dried, unstored seeds

The ultrastructural condition at this stage suggested that the cells maintained their intracellular integrity, and, in fact had apparently undergone a measure of stimulation. This was indicated by polysome formation (Figure 41.2) and abundant mitochondria. The mitochondrial matrices were not uniform, however, and internal membrane development was sparse (Figure 41.1 and 41.2). There had been considerable starch accumulation in some of the plastids (Figure 41.1). The nuclear profile had become slightly irregular, and the envelope was indented, perhaps as a consequence of the organelles crowded in the perinuclear vicinity (Figure 41.1). Chromatin was associated with the inner membrane of

the envelope and in the nuclear matrix, and was distinctly condensed (Figure 41.1). There was no apparent increase in the degree of vacuolation or in the size of the vacuoles (Figure 41.1). The rough ER profiles were longer, apparently having proliferated in response to drying, although some profiles were unusually branched (Figure 41.3). Organelles were abundant but there was some evidence that the spatial distribution had changed, leading to their accumulation around nucleus (Figure 41.1). The axes of these seeds showed evidence interpreted as increased intracellular activity (compared with the newly harvested, fully hydrated seeds) indicated by more condensed chromatin (Figure 41.1), and a proliferation of organelles (Figure 41.1) and ER (Figure 41.3), but the cells also exhibited some signs of abnormality.

3.3.3 Fully hydrated seeds stored at 6, 16 or 25°C for 3 weeks

3.3.3.1 Material stored at 6°C

Seeds stored at 6°C had all lost viability. The cells from the fully hydrated seeds stored for 3 weeks showed intracellular deterioration which included marked vacuolation to the extent that the vacuoles occupied a large volume of the cell (Figure 42.1 and 42.4). Many vacuoles showed an increase in content, containing loosely diffuse material (42.1 and 42.4). There had been proliferation of ER and in many cells the membranes were organised into concentric rings, which usually surrounded other structures (Figure 42.1, 42.2 and 42.4). The mitochondria lacked cristae and the matrices were largely electron translucent (Figure 42.2 and 42.3). The nuclear profiles appeared normal in most cells and chromatin had remained associated with the envelope. However, the nucleoli appeared diminished compared with those in the unstored material (Figure 42.4). Golgi bodies had persisted, but the cisternae were distended and many associated vesicles could be seen, some of which, however, may have resulted form cisternal degradation (Figure 42.3). Many small vesicles were present between the plasmalemma and the cell wall (Figure 42.2 and 42.3). Plastids were present and did contain some starch (Figure 42.1).

3.3.3.2 Material stored at 16°C

Seeds stored in the hydrated condition at this temperature had all remained viable. There was no extensive vacuolation although it was evident that vacuolation had taken place (Figure 43.1 and 43.3). As these Figures illustrate, many of these vacuoles appeared elongated, and may have formed directly from modified ER cisternae (Figure 43.3). The mitochondria were relatively abundant, but there was little crista development (Figure 43.2 and 43.3). The ER profiles were abundant and stacked but many showed marked distension (Figure 43.2), perhaps indicative of incipient vacuolation. The plasmalemma was closely associated with the cell wall in some places, but in others there was a relatively marked measure of plasmalemma withdrawal (Figure 43.2 and 43.3). The nuclear profile was not spherical but the nuclear envelope was intact (Figure 43.1 and 43.3).

3.3.3.3 Material stored at 25°C

Seed viability was 100% after 3 weeks, and the intracellular situation reflected enhanced metabolic activity. The many mitochondria had evenly dense matrices with well developed cristae, although occasional mitochondria were largely electron transparent (Figures 44.1 and 44.3). The well-developed mitochondria had elongated considerably, and some showed evidence of division (Figure 44.3). The plasmalemma was irregular, possibly as a consequence of addition by fusion, of Golgi-derived vesicles (Figure 44.2). The nuclear profile was slightly irregular, the chromatin was condensed and not evenly visible throughout the nucleus; however there was no abnormal clumping. The nucleolus was prominent, with less dense internal regions in some instances (Figure 44.1). Golgi bodies appeared closely stacked with associated vessicles (Figure 44.2). Polysomes abounded in the cytomatrix (Figure 44.2).

3.3.4 Partially dried seeds ('sub-imbibed' seeds) stored at 6, 16 and 25°C for 3 weeks

3.3.4.1 Material stored at 6°C

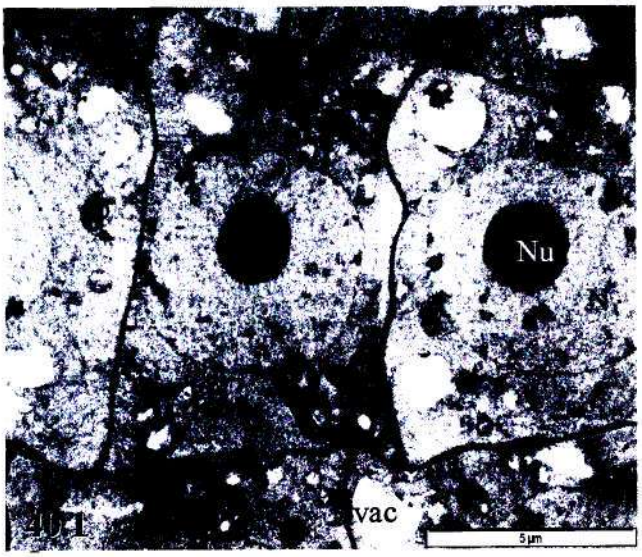
These cells showed extensive degradation and the organelles were largely unrecognisable (Figure 45.1). Some cells were characterised by extensive vacuolation (Figure 45.2) and localised withdrawal of the plasmalemma from the cell (Figure 45.2). The plasmalemma was discontinous and damaged (Figure 45.2). Many vacuoles had formed abnormally close aggregates (Figure 45.2). In many cells the nucleus maintained a spherical shape, but the nucleolus and chromatin were indistinct (Figure 45.1).

3.3.4.2 Material stored at 16°C

A small proportion – 20% - of these seeds had retained viability However, material observed microscopically appeared to represent non-viable specimens only. The situation in these axes was similar to that observed in the 'sub-imbibed' seeds stored at 6°C. The cells were characterised by extensive vacuolation and indications of abnormal aggregation of these compartments (Figure 46.1). Localised, and extensive withdrawal of the plasmalemma from the cell wall was evident as well as damage of this vital boundary membrane (Figure 46.1 and 46.2). Remnants of ER were visible in some areas of the cytomatrix, while other organelles were largely unidentifiable (Figure 46.2). The nuclei were opaque and the nucleoli were indistinguishable (Figure 46.2).

3.3.4.3 Material stored at 25°C

All the seeds stored at 25°C in the 'sub-imbibed' condition had lost viability after 3 weeks. The cells showed marked deterioration (Figures 47.1 and 47.2). The cytomatrix appeared compacted, perhaps partly the consequence of severe plasmalemma withdrawal and derangement (Figure 47.1 and 47.2). Most organelles could not be resolved but there were remnants of extensive ER formations in areas (Figure 47.2). This affords evidence of earlier endomembrane activity, and odd vesicles had persisted (Figure 47.2).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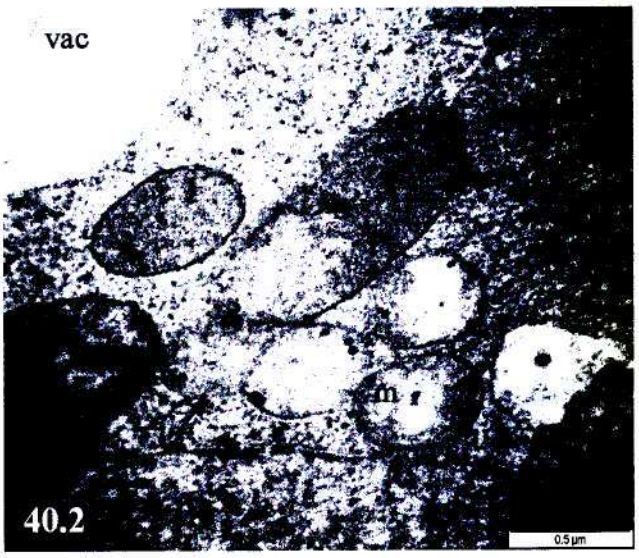
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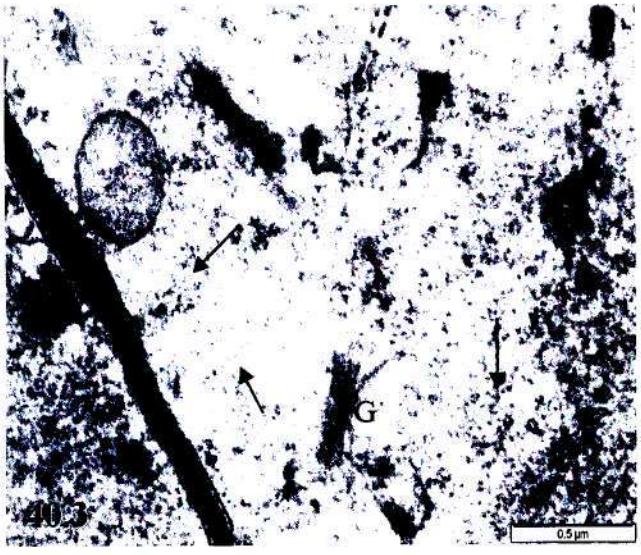
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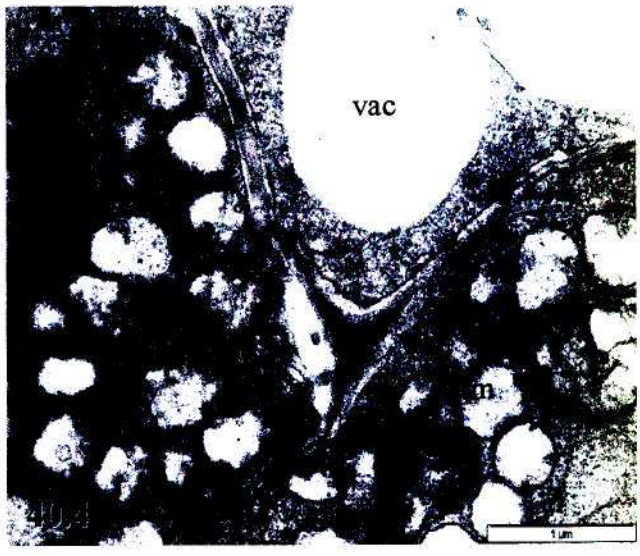
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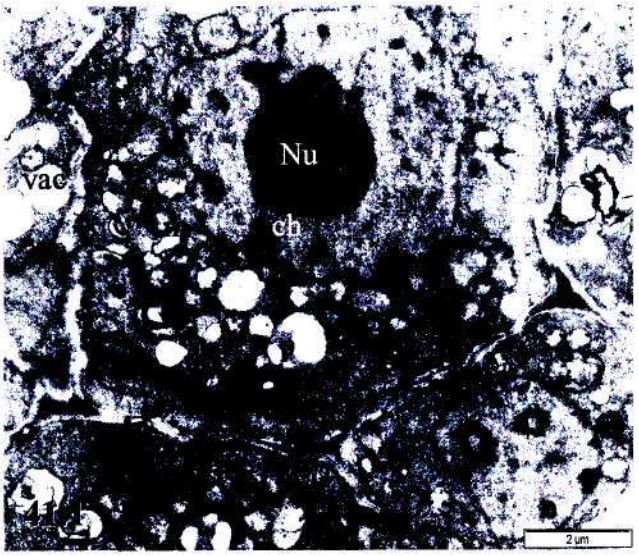
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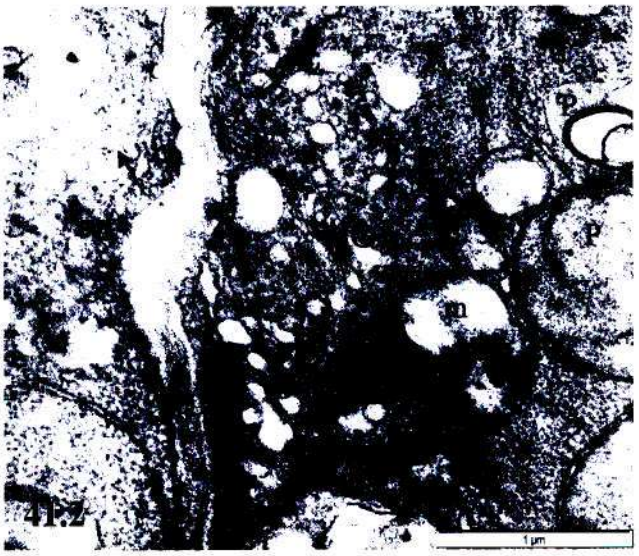
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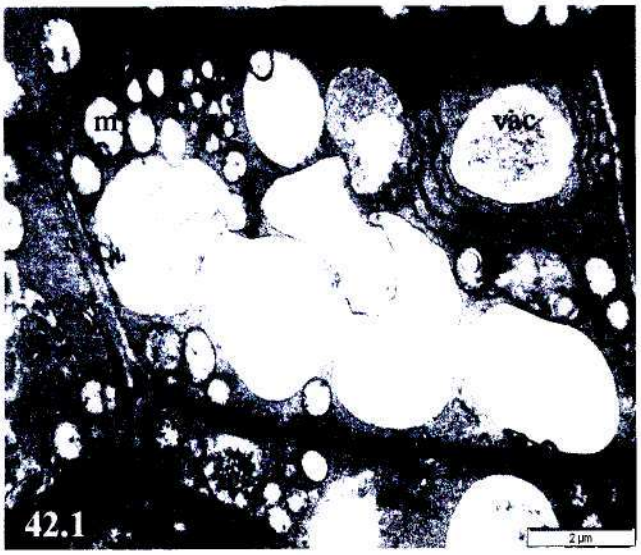
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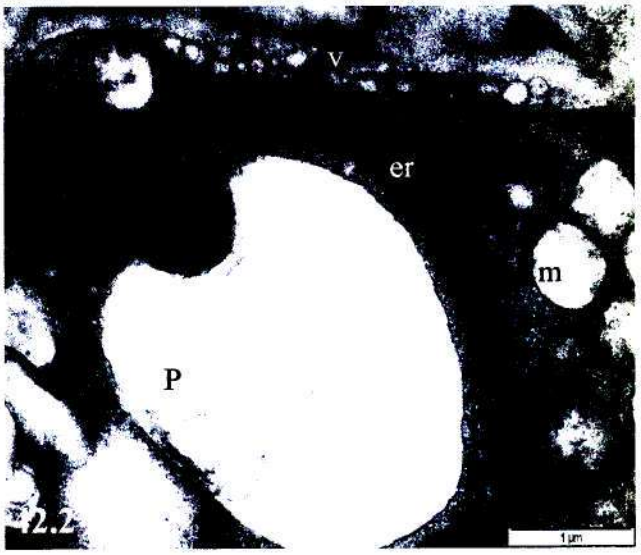
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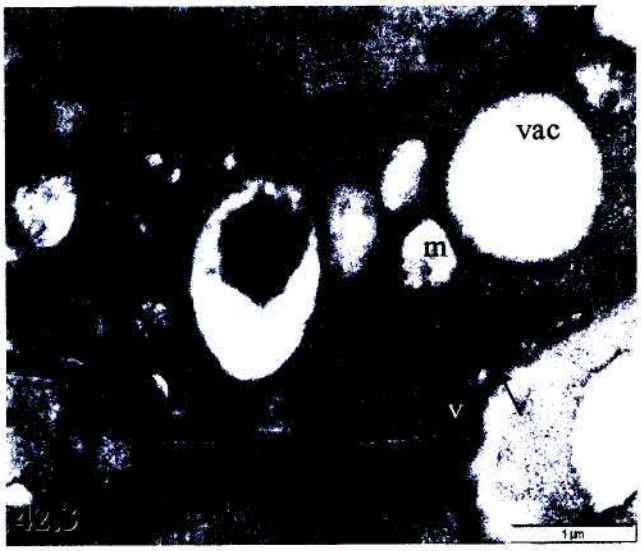
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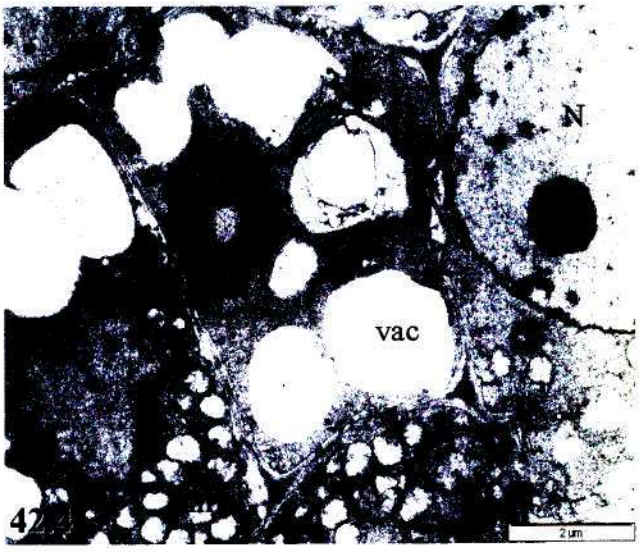
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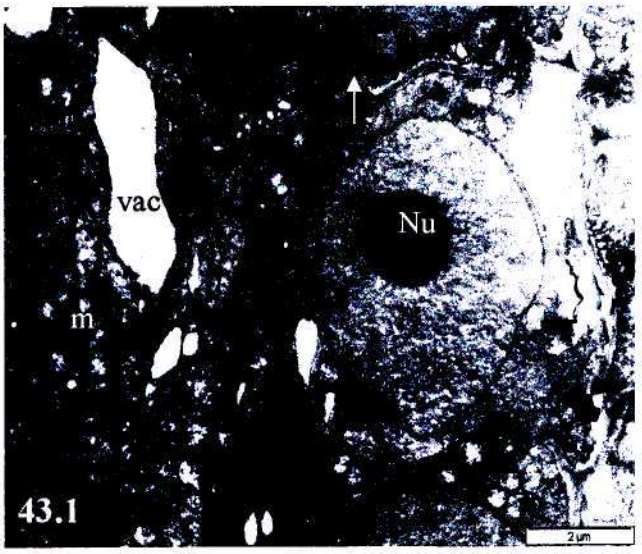
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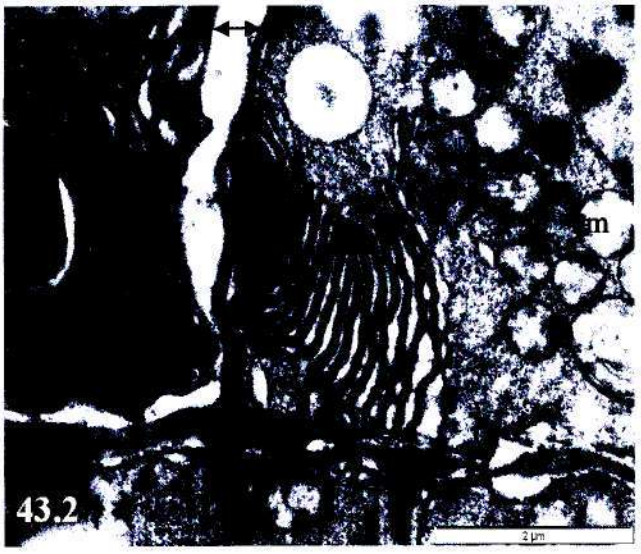
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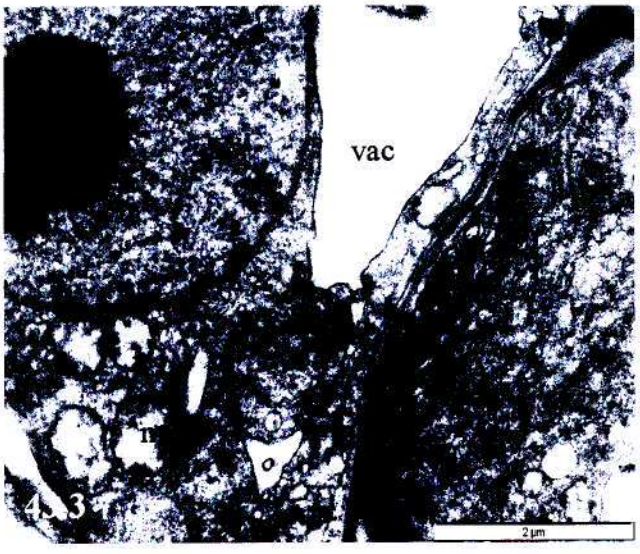
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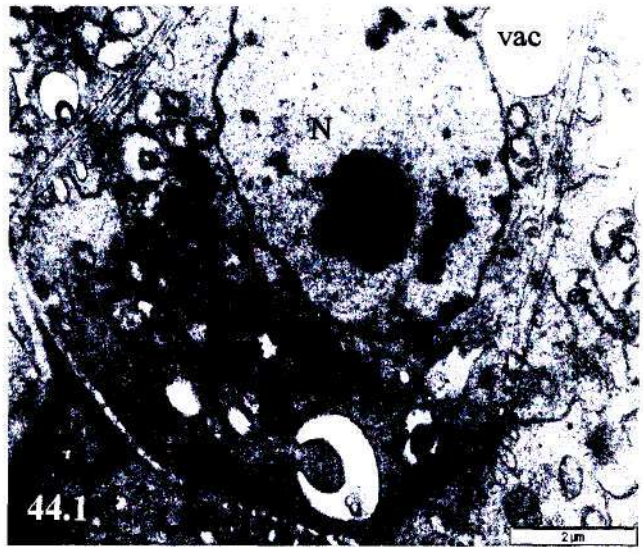
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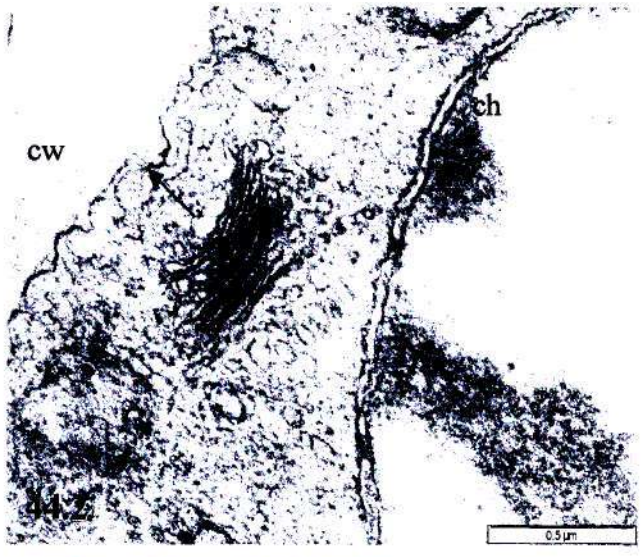
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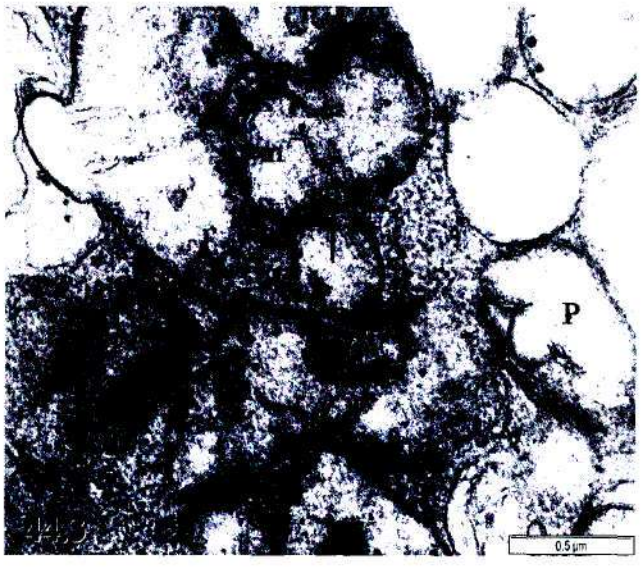
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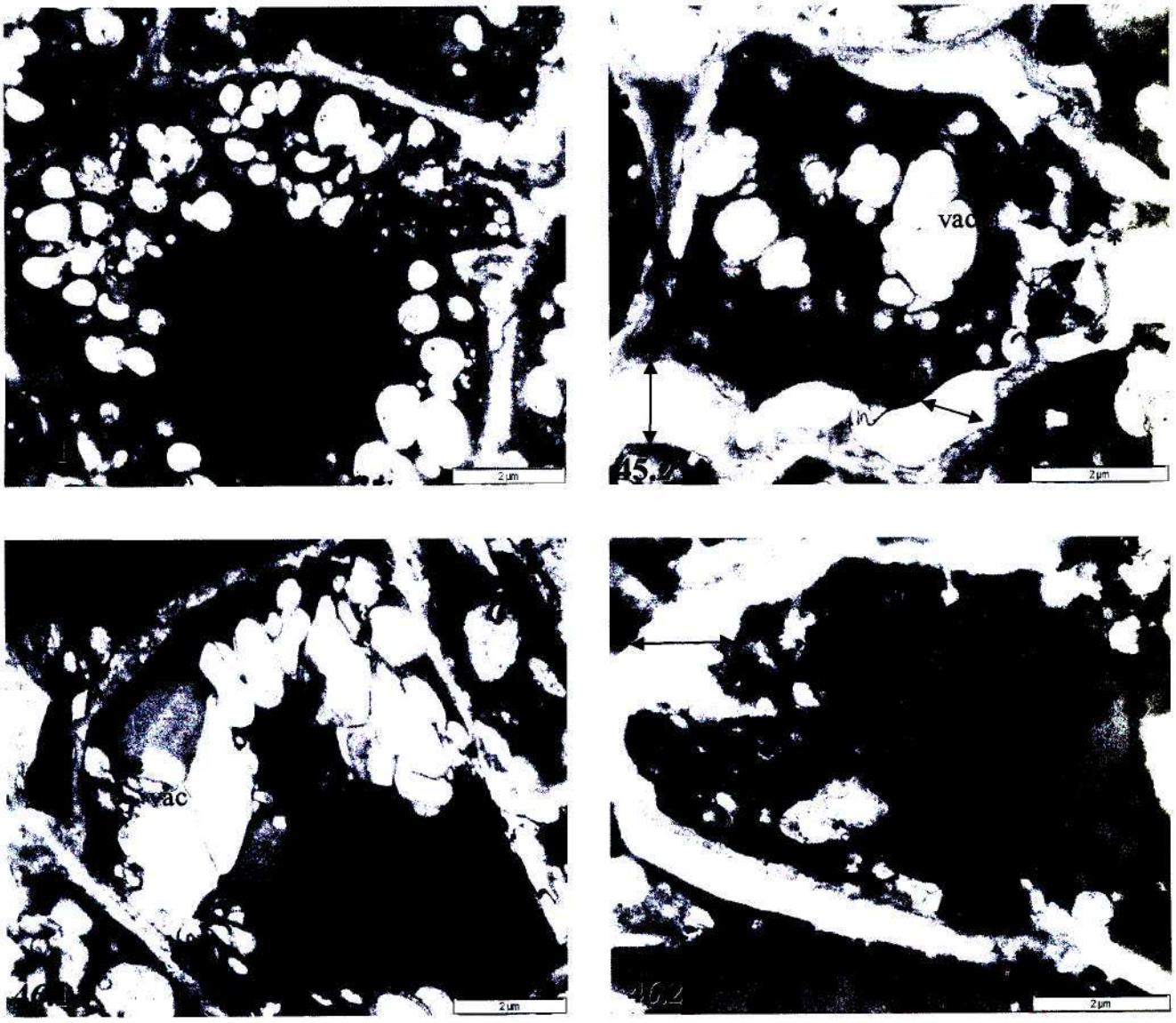
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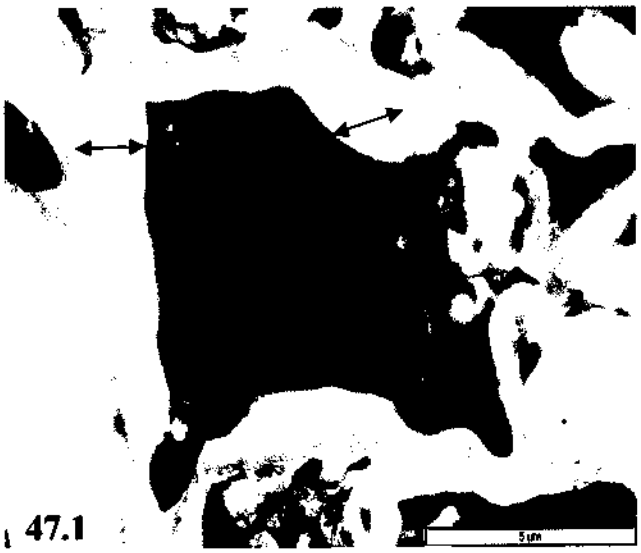

47.1

47.2

4. DISCUSSION

4.1 Initial observations

The seeds of all the species used in the current study had water contents typical of recalcitrant seeds, i.e. shedding water content was high (water content of axes of recalcitrant seeds generally ranges from 0.43 - 4.0 $g.g^{-1}$ [Berjak and Pammenter, 1999]) and the water content of the axis was higher than that of the cotyledons. There was variation in the axes water content among the four species, *T. dregeana* axes had the highest water content at shedding while the axes from the *S. cuminii* seeds had considerably lower water contents. This observation, that there is variation in shedding water content (axes and storage tissues) among species, is consistent with the concept of a continuum of post-harvest seed behaviour even within the recalcitrant category, which is favoured by Berjak and Pammenter (1999). Shedding water content might suggest that *T. dregeana* seeds are the most desiccation sensitive of the species studied. However, shedding water content alone is not a defining feature; rather, it must be seen in the context of the response of seeds to desiccation and storage (Berjak and Pammenter, 2001b).

Investigations by Choinski (1990) provided evidence that *T. dregeana* seeds should be classified as recalcitrant. In the present study, the mean water content of the 2001 harvest of *T. dregeana* seeds was 0.89 ± 0.12 $g.g^{-1}$ and the 2003 harvest was 0.72 ± 0.08 $g.g^{-1}$; however, the water content of the axes (2.92 ± 0.16 $g.g^{-1}$ and 2.81 ± 0.2 $g.g^{-1}$, respectively) was similar regardless of the harvest. The seeds of *T. emetica* were described as recalcitrant by Maghembe and Msanga (1988); the seeds are shed at a slightly lower water content than the *T. dregeana* seeds. The shedding water content was found to be 0.59 ± 0.18 $g.g^{-1}$ and there was no substantial difference in seed water content between the 2002 and 2003 harvests (Figure 8). Axis water content of the *T. dregeana* seeds was presently found to be slightly higher than the that reported by Goveia *et al* (2004), showing the variation in water content among seeds collected at different sites within the same geographical location, and during different seasons.

The seeds of *P. henkelii* have the characteristics typical of recalcitrant seeds (Farrant *et al.* (1989). Palmer and Pitman (1972) reported *P. henkelii* seeds to be shed at a

water content of 62% (wmb). The seeds used in the current experiments were found to be shed at a lower water content of 1.13 ± 0.17 $g.g^{-1}$ (53% [wmb]). The water content of the embryo was 1.8 g g^{-1} and the gametophyte was 1.0 g g^{-1} (Figure 14).

The water content of fresh *S. cuminii* seeds used in this study 43% fmb (axes 1.66 ± 0.31 $g.g^{-1}$ and cotyledons 0.69 ± 0.03 $g.g^{-1}$, [Figure 20]), was consistent with what had been previously reported (Rawat and Nautiyal, 1997; Mittal *et al.*, 1999 and Ouedrago *et al.*, 1999).

Maghembe and Msanga (1988) showed that both the germination rate and totality of *T. emetica* seeds is greatly enhanced by the removal or scarification of the aril and the seed coat. This was found to be more effective in promoting germination than the other pre-sowing treatments that were tested. Choinski (1990) confirmed that removal of the aril also enhances germination for *T. dregeana* seeds. In the present study, therefore and the aril and testa were removed, so that the seeds exhibited no constraints to rapid germination. It was also noted that there was considerable fungal infestation of the aril, which decreased survival and germination. In the current investigation, fresh seeds of *T. dregeana* and *T. emetica* germinated easily and without visible fungal proliferation, after removal of the aril and seed coat, and 100% germination was achieved within 15 days for *T. dregeana* seeds (Figure 7) and 4-5 days for *T. emetica* seeds (Figure 10). In all experiments performed *T. emetica* seeds were found to germinate faster than *T. dregeana* seeds.

Removal of the epicuticular wax, the epidermis or the entire structure, called the epimatium, from *P. henkelii* seeds leads to rapid water uptake and germination because the epimatium constitutes a barrier to germination by restricting water uptake (Noel and van Staden, 1975). Thus in these experiments a $1mm^3$ portion of the epimatium was removed in the area where radicle protrusion would occur. In other recalcitrant species seed coverings have also been shown to affect germination. For example, in *Avicennia marina*, retention of the pericarp, which is a reservoir of the germination inhibitor abscisic acid, prevents germination unless conditions are sufficiently wet and conducive to both the sloughing of the structure and seedling establishment (Farrant *et al.*, 1993).

The quality and maturity of the seeds can vary between harvests. Presently, however, there was no difference in germination rate between fresh *T. dregeana* seeds collected in 2001 and 2003. However, Berjak *et al.* (2004) found for this species that there was a difference in quality, of the seeds collected in different years, correlated with a marked difference in germination rate and totality. A similar observation was made here for *T. emetica* seeds, which showed differing germination rates in 2002 and 2003, the seeds collected in 2003 germinating more rapidly than those collected in 2002 (Figure 10).

Interseasonal and intersite differences in seed lot response to desiccation have been recorded in some recalcitrant species (Tompsett and Pritchard, 1993; Finch-Savage and Blake, 1994; reviewed by: Berjak and Pammenter, 2004). Berjak (1996) found that for the ostensibly mature seeds of *Camellia sinensis*, embryonic axes water content varied from 2.0 ± 0.3 to 4.4 ± 2.4 $g.g^{-1}$ for harvests made in different years. Choinski (1990) found the variation in germination was greater than 40% between seeds from two different sites in Harare, Zimbabwe. It was reported that there were differences in a variety of traits among seed lots of *Euterpe edulis* from one season to the next (Martins *et al.*, 2000). Negash (2003) reported that the germination of *Podocarpus falcatus* seeds harvested from the same tree over three consecutive seasons was significantly different. Additionally, that author reported that seed provenances from central, south-eastern, southern and western Ethiopia showed significant differences in germination totality. Daws *et al.* (2004) found that there was a relationship between the heat sum during development and seed size, germination rate, water relations and desiccation sensitivity. This suggests that environmental conditions during development can affect a range of seed traits that determine different degrees of recalcitrance within different species. Furthermore, this may provide an explanation for the variability between seed lots, as was recorded in the current experiments.

For seeds of any one species there is also intraseasonal variation. Certain intraseasonal effects, the cause(s) which remain largely unknown, impose degrees of variability upon seeds of individual species. For most species, seed-to-seed variability in the axis water content within any single harvest is significant (Berjak and Pammenter, 1997). Chien and Lin (1997) reported that the later (within a season) seeds of *Machilus*

kusanoi were harvested, the greater was the degree of deterioration on dehydration. It is important to note that the seeds of the 'Drying and Storage' experiments were collected approximately two weeks apart in the current study which may explain differences in viability.

Germination of *P. henkelii* seeds occurred within 17 days with 50% of the seed lot germinating within 11 days (Figure 16). Dodd *et al.* (1989) reported that 6 days after the start of germination, 10% of the seeds had germinated, which is consistent with the results obtained in this study. However, Palmer and Pitman (1972) reported germination to be slow and sporadic when seeds were incubated on a moist substrate. Noel and Van Staden (1975) pointed out that the absence of maturation drying does not preclude dormancy since, in a physiological sense, *P. henkelii* must be regarded as having coat imposed dormancy. Alternatively it could be a matter of embryo immaturity. Woodenberg, pers. Comm.[5] found that the embryos of cycad seeds may need a post-shedding development period to grow to full size and therefore be poised for germination. This may possibly be the case with *P.* henkelii seeds.

The fresh seeds of *S. cuminii* reached 100% germination after 9 days, with 50% of the seed lot germinating within 5-6 days (Figure 22). This result is similar to that recorded by Mbuya *et al.* (1994) who reported 100% germination to occur within 1-2 weeks. In contrast, other workers have described the germination of *S. cuminii* seeds to be poor and delayed. Mittal *et al.* (1999) reported that the seeds reached 97% germination only after 4 weeks. Ellis *et al.* (1985) suggested that delayed germination may be a consequence of seed dormancy. However, Mittal *et al.* (1999) proposed that it is more likely that *S. cuminii* seeds are not physiologically mature when harvested from the ripe fruits. This suggestion is in keeping with the results from *P. henkelii* seeds used in these experiments and the work on cycads by Woodenberg, pers. Comm. Likewise the axes of *T. dregeana* are also considered to be immature at harvest (Goveia *et al.*, 2004). Finch-Savage (1992) and Finch-Savage and Blake (1994) have suggested that seed recalcitrance is a consequence of premature termination of development, which is supported by the observations of Daws *et al.* (2004), relating environmental conditions to seed development status at shedding.

[5] Woodenberg, W. School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa.

Mittal *et al* (1999) showed that *S. cuminii* seeds stored at 16°C for 20 weeks germinated much faster than the newly shed seeds, although viability was slightly compromised to some extent as only 88% of the seeds germinated. According to those authors, this decline in viability was probably due to seed-associated mycoflora, a problem typical of this species. It must be noted that the *S. cuminii* seeds used for this study were extracted from mature fruits, and then transported from Tanzania. As recalcitrant seeds are metabolic, and will initiate germanitive metabolism at the shedding water content (e.g. Berjak and Pammenter, 2004), this may have allowed the seeds time to mature further before the current assessments were undertaken, hence germination was not delayed.

4.2 Drying

The seeds of *T. dregeana, T. emetica* and *S. cuminii* lost water rapidly during the early stages of dehydration following which the rate of water loss subsequently declined (Figures 5, 8 and 20, respectively). This is consistent with the observations in most studies dealing with desiccation of whole seeds or excised embryonic axes of non-orthodox species (e.g. Farrant *et al.*, 1989; Pammenter *et al.*, 1991; Ntuli *et al.*, 1997). In contrast, the water loss from *P. henkelii* seeds was slower and more gradual (Figure 14). In addition, the rate of dehydration was similar between the seed component tissues (Figure 14), whereas the water loss by the axes of *T. dregeana*, *T. emetica* and *S. cuminii* seeds was greater than that of the corresponding cotyledons (Figures 5, 8 and 15).

Choinski (1990) found that once *T. dregeana* seeds were dried from a water content of 35%, fmb (0.54 $g.g^{-1}$) to water contents below 30% (fmb), (0.42 $g.g^{-1}$) viability began to decline. In these experiments, this observation was confirmed. Seeds from the 2001 harvest, which were dried for 48 h (to an axis water content of 1.18 ± 0.28 $g.g^{-1}$) showed a dramatic decrease in viability (Figure 7) and vigour (Figure 7) when compared with the seeds dried for 19 h (to an axis water content of 1.54 ± 0.32 $g.g^{-1}$). In this experiment the seeds did show some decline in viability when dried to axis water contents below 2.16 ± 0.30 $g.g^{-1}$ (6 h drying), which could have been due to fungal infection associated with the seeds. In contrast, *T. dregeana* seeds harvested in

2003 all maintained 100% germination despite a slight decline in vigour when dried from an axis water content of 2.81 ± 0.18 $g.g^{-1}$ to 1.04 ± 0.10 $g.g^{-1}$ (30%) (Figure 6 and 7). Viability was completely lost when the seeds were dried to an axis water content below 1.04 ± 0.10 $g.g^{-1}$. The seeds from the 2003 harvest appeared to be less desiccation sensitive than the seeds from the 2001 harvest, as they maintained viability to lower axis water contents (Figure 6).

The seeds from the 2003 harvest lost water more slowly than the seeds collected in 2001 (Figure 5). After both batches of seeds were dried for 48 under the same conditions, the seeds from the 2001 harvest showed 50% germination while those from the 2003 harvest only reached 5% germination (Figure 7); however, it is important to note that the seeds from the 2001 harvest maintained a higher axis water content after the 48 h drying period. Nevertheless after 72 h drying, when similar axis water contents had been attained, the seeds from the 2001 harvest still exhibited 30% germination while the seeds collected in 2003 lost viability completely (Figure 7). This could be a factor the quality of the seeds collected in 2001. It was noted at the start of these experiments that those seeds were of exceptionally good quality. The quality of recalcitrant seeds at the outset does affect the desiccation and storage response, as has been reported for *T. dregeana* (Drew *et al.*, 2000; Berjak *et al.*, 2004).

The 2003 harvest of seeds of *T. emetica* showed precipitous loss in viability when dried below an axis water content of 1.09 ± 0.15 $g.g^{-1}$. The seeds from both harvests, initially dried at similar rates; probably as a consequence, seeds from the 2003 harvest showed a greater decline in viability after drying to an axis water content 0.8 ± 0.06 $g.g^{-1}$) (Figures 9 and 10), indicating that these seeds had accumulated more damage (loosely, were more desiccation sensitive) during the slower water loss.

The seeds collected during the 2003 season appeared to be less mature than the seeds from the 2002 harvest, the freshly harvested seeds, judging from initial germination rate (which was slower for the 2003 harvest). This indicates that the seeds from the 2003 batch had not reached full maturity. Furthermore the seeds of the 2002 harvest which were dried to axis water contents down to 1.09 $g.g^{-1}$ showed a stimulation in germination rate, not exhibited by seeds harvested in 2003. This too might be

interpreted as an indication of the more advanced developmental status of the 2002 harvest.

Kioko (2002) found that the embryonic axes of *T. dregeana* did not dehydrate as fast or to the same extent as those of *Warburgia salutaris*. Drew *et al.* (2000) also reported that *T. dregeana* axes are resistant to desiccation. The embryonic axes did not dehydrate to the same extent as the axes of comparable size from other species e.g. tea (Berjak, *et al.*, 1993). This resistance to water loss may be due to a thick cuticle and other intrinsic tissue characteristics. This would be advantageous in preventing a damaging degree of water loss especially in the field, particularly as the seeds are liberated from the seeds on shedding, and lack a hard testa. In several recalcitrant species the pericarp has been shown to lower the rate of water loss (e.g. Pammenter *et al.*, 1998).

Kioko (2002) found that slowly dried seeds of *T. dregeana* and *T. emetica* lost the ability to germinate at axis water content of 0.55 g g^{-1} (attained after 6 days) and 0.42 g g^{-1} (attained after 3 days), respectively. In this experiment *T. dregeana* seeds showed dramatic decline in viability at an axes water content of 0.85 ± 0.21 g g^{-1} (attained after 3 days) (Figures 6 and 7) and *T. emetica* seeds at an axes water content of 0.35 ± 0.11 g g^{-1} (attained after 2 days) (Figures 9 and 10), again highlighting the intrinsic variability of seed responses within species as discussed by Berjak and Pammenter (2004). The embryonic axes of *T. emetica* are considerably smaller than those of *T. dregeana* and dry more quickly, probably as consequence of the greater surface area to volume ratio (Kioko, 2002). This difference in the rates of water loss was apparent in these experiments where *T. emetica* whole seeds dried down more quickly, especially during the early stages of dehydration.

The work done by Kioko (2002) demonstrated that embryonic axes of *T. dregeana* which were flash dried remained viable to considerably lower water contents. The excised axes reached constant water contents after 1 to 3.5 h flash drying (depending on the species), in contrast to which, the axes which were slowly dried within the seeds took a few days to approach similar water contents. Kioko (2002) found that axes of *T. dregeana* which were flash dried remained viable to a water content of 0.16 g g^{-1} and axes of *T. emetica* remained viable to water content of 0.26 g g^{-1}, although

emetica axes are more sensitive to the extremes of dehydration than the axes of *T. dregeana* (Kioko, 2002). The freshly harvested seeds of *T. emetica* and *T. dregeana* (with aril and testa removed) showed high germination capacity, but the rate of germination was markedly different. The seeds of *T. emetica* reached 100% germination after 4 and 7 days (2002 and 2003 harvest respectively) (Figure 10), while *T. dregeana* seeds, sown under the same conditions, reached full germination after only 15 days (for seeds from both harvests [Figure 7]). It has been suggested that metabolic activity which leads to germination affects recalcitrance in terms of desiccation sensitivity (Pammenter and Berjak, 2000), and this implies that *T. emetica* seeds should be relatively more recalcitrant. Alternatively, it is possible that the *T. dregeana* seeds are less mature at harvest/shedding, therefore take a longer time to germinate than the *T. emetica* seeds (Goveia et *al*, 2004 and see storage results). It has been suggested that, prior to the onset of germination, the less mature recalcitrant seeds are the more desiccation sensitive they are (Finch-Savage, 1992), which has been confirmed by Daws *et al.* (2004, 2005) for *Aesculus hippocastanum and Acer pseudoplatanus,* respectively. The present evidence suggests that the seeds of the two *Trichilia* species were indeed at different stages of development.

In this study the *T. dregeana* (2003 harvest), *T. emetica* (both harvests), *P. henkelii* and *S. cuminii* seeds showed a stimulation of germination rate following limited drying (which was non-injurious) (Figures 7, 10, 16 and 22). Further drying (below a relatively high water content) resulted in a reduction in vigour and viability. Kioko (2002) pointed out that stimulation of germination in this way is not restricted to whole seeds, as that author observed this effect in mildly flash dried embryonic axes of *Trichilia* spp. This phenomenon has been observed in other recalcitrant species e.g. *Ekebergia capensis* (Pammenter *et al.*, 1998) and *Camellia sinensis* (Diegel, 1991) and may be a characteristic typical of recalcitrant seeds (Pammenter and Berjak, 1999). Pammenter *et al.* (1998) have noted that the initial application of what will be an ultimately lethal stress on recalcitrant seeds, is generally stimulatory. The negative effects are manifested only with time and/or below particular water contents, and thus drying may initially promote of increased metabolic activity.

This has implications for the seeds during storage. If they are mildly dehydrated before storage, apparently germinative metabolism will proceed faster and therefore

these seeds will reach the point where exogenous water (Berjak, *et al.* 1989) is required sooner than the seeds which are stored in the fully hydrated condition. The mechanisms underlying the stimulatory response are not well understood and require further investigation. However, it is suggested that, in the field, established seedlings are more resilient than seeds. Thus the stimulatory response may be viewed as a survival mechanism. However, stimulation of germination is not invariable, the seeds of *T. dregeana* from the 2001 harvest did not exhibit this after mild drying. This may relate to the developmental status of the axes when the seeds are shed, which remains to be elucidated.

A notable point concerning *P. henkelii* seeds is that seed fall occurs in early winter and is followed by a short dry season. Dodd and van Staden (1981) reported that the epimatium is not efficient in controlling or preventing water loss. Therefore it may be possible that these seeds are adapted to the loss of some water, which may not have a deleterious effect, as this phenomenon may occur naturally. If this is the case it could be that the seeds exhibit one or more of the mechanisms that contribute to desiccation tolerance (reviewed by Pammenter and Berjak, 1999). Farrant *et al.* (1989) showed that embryo cells of *P. henkelii* contain considerable insoluble storage reserves, and are not markedly vacuolated. These are both features that should facilitate resilience to a degree of water loss (Farrant *et al.*, 1997). King and Roberts (1980) suggested that 'sub-imbibed' storage may offer a solution to the problem of germination occurring during storage. Nevertheless, storage of *P. henkelii* seeds in this way resulted in a measurable decline in viability (see Storage Experiment).

In the case of *P. henkelii* seeds, the drying experiment showed that water appeared to be withdrawn evenly from the embryo and the gametophyte tissues in the seeds during desiccation in silica gel (Figure 14). Drying whole seeds over 22h had no effect on embryo water content, nor did it not adversely affect the ability of the seeds to germinate when compared with the newly shed (undried) seeds (Figure 15). However, the seeds which had been subjected to 22 h in silica gel showed increased vigour, and germination appeared to be stimulated, occurring more rapidly (Figure 16). Drying of the seeds for longer did result in a reducation of embryo water content, and decreased capacity for germination (Figure 16) and, viability decreased precipitously with decrease in embryo water content (Figure 15). It was on this basis that a whole seed

water content of 50% (drying time 22 h, embryo water content 1.8 ± 0.17 $g.g^{-1}$) was chosen for the main experiment, as it was desirable that the viability of the seeds was not seriously affected by drying. As seen for the *Trichilia* spp. seeds, radicle emergence can occur at water contents where the production of normal seedlings no longer occurs (Figure 15). Even after very slight water loss from the embryo, production of normal seedlings from these seeds declined when compared with the control seeds.

The results of the desiccation trials confirmed that fresh seeds of *S. cuminii* are desiccation sensitive, losing viability at a whole seed water content of 35% (reached after 65 h drying, when mean axis water content had declined to 1.04 $g.g^{-1}$ from 1.66$g.g^{-1}$) (Figure 22). A 'critical water content' of ~ 50% (1 $g.g^{-1}$) was previously reported for seeds of this species (Ouedraogo *et al.*, 1999; Srimathi *et al.*, 2001). However as cautioned by Pammenter and co-workers (1998), it is not possible to determine a 'critical water content' for viability loss of recalcitrant seeds without consideration of the drying rate and temperature. Viability decreased dramatically with decline in axis water content, and normal seedling production was lower than germination assessed by radicle protrusion, at the same axes water content (Figure 21).

It would appear that *P. henkelii* and *S. cuminii* seeds are relatively more desiccation sensitive than *T. dregeana* and *T. emetica*, both losing viability at higher axis water contents. However, it must be noted that *P. henkelii* and *S. cuminii* seeds took longer than those of the *Trichilia* spp. to lose water, therefore loss of viability may be a consequence of time at partially hydrated levels.

Loss of membrane integrity is an indication of desiccation damage in seeds, and can be expressed in terms of the rate and extent of electrolyte leakage from the tissues, as electrolytes will leak from damaged tissues faster than when undamaged. This method has been used successfully for a number of desiccation sensitive seed tissues to assess desiccation damage as it gives a reliable assessment of damage that correlates well with viability characteristics (Vertucci and Leopold, 1987; Vertucci 1989; Pammenter *et al.*, 1991; Berjak *et al.*, 1992; Li and Sun, 1999; Wesley-Smith *et al.*, 2001). However a slight change in membrane permeability does not necessarily result in loss

of viability. Furthermore, death of the embryo/axis would be expected to occur only when a critical proportion of the cells exhibit loss of membrane integrity. In these experiments electrolyte leakage was measured only for *T. emetica* (2002 harvest) seeds (measurements were taken for cotyledons and axes separately) and *P. henkelii* seeds (measurements were taken for embryos only). For the *T. emetica* seeds initial drying of both the axes and the cotyledons for up to 4h resulted in a decrease in the rate of electrolyte leakage from the tissues but further drying resulted in a substantial increase in the rate and quantity of electrolyte leakage (Figure 13). This corresponds with the stimulation of germination which was apparent after initial drying. The seeds that showed increased vigour, appeared to lose fewer electrolytes. A marked increase in electrolyte leakage was noted after the seeds were dried to an axis water content of 1.18 ± 0.11 g g^{-1}, after 6 h drying (Figure13); however, seed viability remained high after this drying period (Figure10). The leakage data for the embryos of the *P. henkelii* seeds also showed a decrease in electrolyte leakage after 150 min of drying. Germination/stimulation increased only after drying the seeds to an embryo water content of 0.92 ± 0.14 $g.g^{-1}$, after which the seeds lost viability completely (Figure 16). The embryos of *P. henkelii* generally showed much lower absolute leakage than the axes of *T. emetica*. Experiments on *Ekebergia capensis* seeds did not show a similar trend: there was a gradual increase in the rate of leakage with decreasing water content (Pammenter *et al.*, 1998). Fresh, mature seeds of *Landolphia kirkii* showed a constant level of leakage until axis water content was reduced to 0.4 $g.g^{-1}$, with a steep incline occurring thereafter (Berjak *et al.*, 1992). The work by Wesley-Smith *et al.* (2001) showed an increase in electrolyte leakage, of axes from *Artocarpus heterophyllus* seeds, with a decrease in water content, with a steep increase between 0.4 $g.g^{-1}$ and 0.2 $g.g^{-1}$. It is important to note that in all the studies reported above, the axes were dried rapidly. In contrast, when *A. heterophyllus* axes were dried slowly, Wesley-Smith *et al.* (2001) reported an increase in leakage at a water content of 0.8 $g.g^{-1}$. The data strongly indicate that the extent and rate of electrolyte leakage from recalcitrant material subjected to dehydration (or any) stress, is meaningful only in the context of the rate of application, the duration, and the severity of the stress.

The rate of CO_2 evolution can be a useful indicator of the course of metabolic changes during respiration (Franks and Drake, 2003). It has been noted that oxidative activity in orthodox seeds increases with increasing water content (Vertucci and Leopold,

1984; Dahal *et al.*, 1996) and decreases with decreasing water content (Leprince *et al*, 1999). Collectively, recalcitrant seeds exhibit metabolism that continues-often unabated during development and germination (Farrant *et al.*, 1989, 1997; Salmen-Espindola *et al.*, 1994; Berjak and Pammenter, 1997). A decrease in respiration, with decreasing water content, contributes to greater longevities in dry seeds (those seeds which can tolerate substantial water loss) and greater tolerance to low water contents in rapidly dried, desiccation sensitive axes (Walters *et al.*, 2001). Those authors suggested that the lower respiratory axes in dried pea may be associated with greater longevity and tolerance to slow drying when compared with tea axes. Leprince *et al.* (1999) reported that respiration, in drying cotyledons of *Castanea sativa* remained, unabated until a critical water content corresponding to the loss of membrane integrity was reached. In recalcitrant seeds of *Araucaria angustifolia* O_2 uptake rates remained unabated until the death of the embryo (Salmen-Espindola *et al.*, 1994). Oxidative activity still occurred at water contents where viability was irreparably lost (Salmen-Espindola *et al.*, 1994; Walters *et al.*, 2001). Franks and Drake (2003) reported a 10-fold increase in CO_2 evolution in the recalcitrant seeds of *Idiospermum australiense* during drying. Changes in respiration rate, assessed by CO_2 evolution during desiccation of recalcitrant seeds have been attributed to 'uncontrolled metabolism' (Vertucci and Farrant, 1995; Leprince *et al.*, 1999; Pammenter and Berjak, 1999; Walters *et al.*, 2001). Metabolic imbalance appears to begin early in the desiccation process, with the products of this imbalance leading to irreparable damage (Salmen-Espindola *et al.*, 1994; Leprince *et al.*, 1999; Buitink *et al.*, 2000).

The respiration measurements on the whole seeds of *T. emetica* showed that there was a stimulation of respiratory events after drying (Figure12); this probably accompanied the increase in vigour during germination. Respiration declined during further drying, but apparently continued even when the seeds were dried to water contents where viability was completely lost (Figure 12). Respiration in the embryos of *P. henkelii* seeds decreased significantly, after even the shortest drying period (Figure 18) and decreased slightly with further drying. Nevertheless, apparent respiration, assessed by CO_2 evolution rate, continued, suggesting that these embryos also remained metabolically active throughout drying, even once germination capacity had been lost. It is also possible that CO_2 producing degradative reactions occurred in these seeds,

which were no longer viable. The levels of CO2 measured would therefore not be an indication of respiration in these seeds.

Podocarpus henkelii seeds maintained considerably higher levels of respiratory activity (Figure 18) during drying than the seeds of T. emetica (Figure 12). This could be an indication that the P. henkelii seeds are more desiccation sensitive. It has been suggested that desiccation sensitivity in recalcitrant seeds is intimately associated with their persistent high metabolic activity (Vertucci and Farrant, 1995; Berjak and Pammenter, 1997). However, embryos of *P. henkelii* are far larger than axes of *T. emetica*, thus it appears feasible to suggest that the more protected, inner tissue of the *P. henkelii* embryos remained more hydrated-and metabolically active-than overall water contents suggest. However, a critical cell volume (and presumably, type) would necessarily remain uncompromised, for embryo viability to be retained. It would appear that this threshold of damaged cells was exceeded, resulting in viability loss.

The relationship between oxidative activity and axis survival suggests that the mechanisms for damage in axes containing more than 0.5 g g^{-1} may be similar in desiccation tolerant and sensitive axes (Walters *et al.*, 2001). In contrast, at lower water contents, desiccation tolerant and sensitive axes are different, the damage in sensitive types being the consequence of removal of structure–associated water, which is not tolerated (Pammenter *et* al., 1993). This is described as desiccation damage *sensu stricto* (Pammenter *et al.*, 1998; Walters *et al.*, 2001).

4.3 Storage

The water contents chosen for the storage of seeds in the 'sub-imbibed' condition were determined by the results of the drying experiments. The seeds were dried to the lowest water content that appeared not to adversely affect germination capacity, when compared with the control seeds. It is essential that the viability of the seeds is not compromised by drying, as subsequent viability loss could be due to the deleterious effects of drying and not necessarily as a consequence of storage in the 'sub-imbibed' condition. However, it should be noted that the seeds took a number of days (depending on the species) to reach the target water contents currently used. During

this time it is likely that a measure of metabolism related damage would have occurred (Pammenter *et al.*, 1998) but not of the severity to compromise germinability, if the seeds were planted out immediately.

In terms of longevity, previous information indicated that the seeds of *T. dregeana* would last in storage at ambient temperatures for only three to four weeks (Pammenter, pers. comm.[6]), and would not germinate if exposed to temperatures of 15°C or below (Choinski, 1990). However, the results obtained by Berjak *et al.* (2004) were contrary to those observations, as total germinability of *T. dregeana* seeds remained largely unchanged during a 5 month storage period in the hydrated condition at 16°C. The present study confirmed that storage below 15°C is lethal to most of the seeds whether fully hydrated or not. After the 5 month storage period it was found that most of the seeds stored at the shedding water content under conditions precluding dehydration, had protruded radicles, which is a manifestation of the ongoing germinative processes during storage (Berjak *et al.*, 2004). The phenomenon of germination during storage, initially recorded for its nuisance value (e.g. Chin and Roberts, 1980), was originally described in *Avicennia marina* by Pammenter *et al.* (1994) who emphasised that this manifestation of metabolic activity was, in fact, the major underlying cause of seed recalcitrance (Berjak *et al.*, 1989). The phenomenon of germination of hydrated seeds during storage was observed during the current study in all the species studied.

Drew *et al.* (2000) found that the seeds of *T. dregeana* retained viability for 16 to 21 days (during storage at 16 and 25°C), much shorter periods than those recorded by Berjak *et al.* (2004). In the present study, seeds harvested in 2001 retained viability better during storage for longer periods than the seeds collected from the same site during 2003. The seeds collected during 2001 maintained 100% viability during storage at 16 and 25°C for 22 weeks (Figure 24), while the 2003 harvest maintained 100% viability for only 3 weeks (Figure 26). The widely divergent survival periods could be attributed to the variability among recalcitrant seeds of the same species in characteristics that influence longevity, such as maturity at harvest and relative desiccation sensitivity, time elapsed between shedding and collection and whether or not infecting micro-organisms persist and are active during the storage period. This

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variability occurs among different harvests within a species, and from seed-to-seed within the same harvest (Berjak *et al.*, 1989; Finch-Savage and Blake, 1994). The present experiments provide evidence for the post-harvest maturation of hydrated seeds during storage (see Figure 24, seeds stored at 6 and 16°C), those stored for 8 weeks germinated faster than the fresh seeds. Goveia *et al.* (2004) showed that *T. dregeana* seeds are under-developed at harvest; and continued development during storage for 8 weeks in the current investigation, poised the *T. dregeana* seeds for germination.

Recalcitrant seeds of tropical/sub-tropical provenance invariably have associated fungal inoculum (Sutherland *et* al., 2002). Contamination is one of the major causes of seed deterioration during storage, particularly because the conditions used for storage of recalcitrant seeds (which are usually warm and always humid), favour fungal proliferation (Mycock and Berjak, 1990; Berjak, 1996). The surface application of fungicides is effective only in preventing the spread of infection but does not destroy the mycoflora within the seed tissues. A reduction in storage temperature has been successful in extending the storage lifespan of chilling-tolerant temperate recalcitrant seeds, by curtailing fungal proliferation (Suszka and Tylkowski, 1980), but Kioko (2002) found that this practice could not be applied to *T. dregeana* seeds. The seeds stored at 6°C during the current experiment (2001 harvest) confirmed this. After 22 weeks in storage the seeds showed a significant decline in viability, which was not observed in the seeds stored at 16 or 25°C (Figure 26). Interestingly though, the seeds stored at 6°C show increased vigour after initial storage periods compared with those seeds stored at 16 and 25°C. This may be explained in the same terms as the intermediate effects of mild dehydration stress, discussed above. Tompsett (1987) found that chilling damage may occur only after a certain period in storage for *Dipterocarpus longan* seeds, which were affected by low temperatures only after 5 weeks in storage. Presently the seeds stored at 16°C retained the highest viability and vigour throughout storage (Figure 26), therefore storage temperatures of 6 and 25°C were excluded from experiments performed on the 2003 harvest. Despite storage at 6°C the seeds were found to be contaminated by fungal mycelium. It has been reported for fully hydrated (Calistru *et al.*, 2000) and 'sub-imbibed' (Drew *et al.*, 2000) recalcitrant seeds that fungal degradation is accelerated by extrinsic or intrinsic factors that favour debilitation of the seeds.

Trichilia emetica seeds are also chilling sensitive. Kioko *et al.* (2006) found that seeds stored at 6°C showed extensive ultrastructural damage, which was accompanied by total loss of viability after 20 days. Storage at 25°C resulted in seeds maintaining 100% viability during the same period. Presently fungi proliferated on, and in, seeds stored at 6°C within 3 weeks, while the seeds stored at 16 and 25°C did not show any discernible fungal proliferation. All the seeds that were used for the storage experiments were from a common batch, supporting the proposal for recalcitrant seeds by Berjak (1996), which suggests that vigorous individuals stored under favourable conditions have mechanisms to counteract micro-organism proliferation, at least in the short term (e.g. Calistru *et al.*, 2002) There has been evidence to suggest that the seeds of *S. cuminii* are chilling sensitive. Mittal *et al.* (1999) demonstrated that the vigour of *S. cuminii* seeds stored at 5°C for 20 weeks was greatly reduced when compared with that of similar seeds stored at 16°C although 100% viability was maintained in both cases. Therefore the storage experiments on *S. cuminii* were presently carried out at only at 16 and 25°C.

Pritchard *et al.* (1995) demonstrated for *Aruacari hunsteinii* seeds that a reduction in storage temperature was effective in reducing metabolic rate and thus extending storage lifespan and suggested that this might generally be the case, provided the seeds are not chilling sensitive. Extension of storage lifespan at lowered temperatures (as well as reducing fungal proliferation) is likely to occur because the rate at which germinative metabolism occurs would be markedly reduced. It is possible that the metabolic rate in these seeds is decreased sufficiently to prevent the requirement for additional water being manifested during storage, and the associated degradative changes occurring as rapidly as seeds stored at higher temperatures. The seeds of *P. henkelii* were demonstrated not to be chilling sensitive, as storage at 6°C did not result in decreased viability of the fully hydrated seeds (during storage for up to 11 weeks) (Figure 35). However it did not extend storage lifespan, when compared with the seeds stored at 16°C. Seeds stored at 6°C showed the greatest vigour at each sampling, while seeds stored at 25°C germinated at the slowest rate (Figure 34 and 35). It is important to note that *P. henkelii* seeds have a temperate provenance as opposed to the tropical/sub-tropical provenance for the other species studied.

The *T. dregeana* seeds ('sub-imbibed' and fully hydrated) from the 2001 harvest maintained 100% viability during the storage periods used, with the only decline in viability being observed in those seeds stored at 6°C for 22 weeks. However, the 'sub-imbibed' seeds showed diminished vigour than fully hydrated seeds stored at the same temperature for the same time period (Figure 24). This indicates that the 'sub-imbibed' seeds may have lost viability sooner than the hydrated seeds had the storage period been extended. The seeds collected during 2003 were dried to two different water contents for 'sub-imbibed' storage, but stored at 16°C only. The fully-hydrated seeds maintained the highest viability and vigour compared with 'sub-imbibed' seeds stored for the same time periods (Figure 26). Drying the seeds to a mean axis water content of 1.04 $g.g^{-1}$ proved to be more deleterious during storage than drying to a mean water content of 1.3 $g.g^{-1}$, indicating that it is not only lowering the water content, but the extent to which it is lowered, that has deleterious effects (Figure 26).

The axes and the cotyledons from the *T. dregeana* seeds (2001 harvest) exhibited fluctuations in water content during storage (Figure 23). After 22 weeks in storage the water content of the cotyledons from the 'sub-imbibed' seeds was lower than that of the cotyledons from fully hydrated seeds; however, there were no significant differences in the water contents of the axes after 22 weeks, despite differences between the hydrated and 'sub-imbibed' material, when the seeds were placed into storage (Figure 23). The axes and cotyledons from the 2003 harvest did not show the same fluctuations during storage, those from the 'sub-imbibed' seeds remaining at lower water contents throughout the storage period (Figure 25). It should be noted that the seeds (axes and cotyledons) from the 2003 harvest were at a considerably lower water content than the seeds from the 2001 harvest (Figures 23 and 25), confirming the variable nature of recalcitrant seeds within species.

The situation of *T. emetica* seeds in storage resembled that of *T. dregeana* but the former were much shorter-lived, and, when hydrated, germinated considerably faster during storage. The water contents of the axes and the cotyledons (from the seeds of the 2002 harvest) showed fluctuations during storage, the fully hydrated seeds showed a substantial net water loss (Figure 28). The axes and cotyledons from the seeds collected during 2003, showed smaller fluctuations during storage and less net water loss (Figure 31). Water content fluctuation during storage is a great challenge to the

successful storage of recalcitrant seeds, because elevation might accelerate germination and loss could contribute to dehydration-associated, metabolism-linked damage.

It is evident from the germination data that 'sub-imbibed' storage not only confers no advantage over storage in the fully hydrated state, but rather is deleterious to seed survival during storage (Figures 29 and 32). Those seeds stored in the 'sub-imbibed' state have greater decreased vigour and viability after the same storage periods than the fully hydrated seeds.

After 2, 4 and 11 weeks of storage, hydrated seeds maintained 100% viability and germinated more vigorously than newly shed, unstored seeds (Figure 34). This is manifested by apparently increased vigour after storage, and occurred at all temperatures, the only exception being the fully hydrated seeds stored at 25°C for 11 weeks.

The germination results for *P. henkelii* suggest that when assessed immediately after drying, prior to 'sub-imbibed' storage, partially dried seeds germinated more vigorously than the newly shed seeds (Figure 34), which is consistent with the observations made during the drying experiment (Figure 16). Removal of a small proportion of water appears to stimulate germinative metabolism. Fully hydrated seeds, however, whether stored at 6 or 16°C maintained viability and vigour better than the 'sub-imbibed' seeds, stored at the same temperatures for the same durations (Figure 34). King and Roberts (1980) suggested that a loss of viability could be caused by the difficulty in maintaining constant water content during storage. Those authors reported that fluctuations in water content occurred even in conditions where relative humidity was controlled. This was evident in the present experiments, both the axes and the cotyledons showing changes in water content during the storage period (Figure 33). However, it is suggested that this may be a result of the redistribution of water within the tissues rather than a net water loss from the seed. Nevertheless, the problem may be far more complicated.

Firstly, there is the possibility that the 'sub-imbibed' seeds accumulated metabolism-related damage during drying. Furthermore during 'sub-imbibed' storage, seed longevity may be further negatively affected as a result of unbalanced metabolism

under mild, but prolonged conditions of water stress (Pammenter *et al.*, 1998). Unbalanced metabolism has been suggested by those authors to include uncontrolled free radical formation.

The *P. henkelii* seeds stored at 25°C showed the same response as the seeds stored at 6 and 16°C after 2 and 4 weeks storage; however, after 11 weeks the fully hydrated seeds lost viability completely while the 'sub-imbibed' seeds maintained 85% viability (Figure 34). This surprising trend that at 25°C 'sub-imbibed' storage appeared to be advantageous, may have an explanation in terms of an extraneous factor. It is likely that the storage lifespan of the fully hydrated seeds was curtailed due to fungal proliferation, which was noted after 4 weeks (despite measures taken to counteract this). No similar visible manifestation of fungal proliferation was apparent on the 'sub-imbibed' seeds at 25°C. Therefore 'sub-imbibed' storage might not facilitate longevity extension, if fungal proliferation is prevented in the fully hydrated seeds. *Podocarpus henkelii* is a temperate species, also a possible factor in accounting for the adverse effects of sustained 25°C storage. Although water requiring, because recalcitrant, high water content and high temperature prior to germination could conjecturally be equated with accelerated ageing.

During dehydration, to achieve the 'sub-imbibed' state, it is possible that surface drying of the seeds was accompanied by the death of the peripherally-located fungal inocula. During storage at 25°C, such seeds would have been at a considerable advantage over those undried seeds, where surviving elements of the mycoflora could, and would, have proliferated. During storage at 6 and 16°C, fungal metabolism may have been reduced. However, had the storage period exceeded the 11 weeks presently used, fungal proliferation probably would have increased on, and ultimately in, the undried seeds even at 6°C.

The fully hydrated seeds stored at 25°C may have been adversely affected by fungi after 4 weeks in storage because of increased susceptibility caused by the stress imposed by warm wet-storage conditions (Calistru *et al.*, 2000). Recalcitrant seeds harbour a range of fungi, even when newly harvested (Mycock and Berjak, 1990). However, the fungal infection may only be manifested after some storage time, as was the case in the present study. Additionally the storage of hydrated seeds at 25°C provides conditions which are optimal for fungal proliferation. Calistru *et al.* (2000)

suggest that it is not possible to discriminate with certainty among the deteriorative sub-cellular events caused by inherent deterioration or the effect caused by fungus.

The results from the shoot production experiments suggest that 'sub-imbibed' storage (at 6 and 16°C) greatly reduced the ability of embryos of *P. henkelii* to produce shoots when compared with the fully hydrated seeds stored at the same temperatures (Figure 35). As emphasised by Kioko (2002), germination can occur at water contents where the capacity for shoot development is lost or never acquired. Thus seeds may produce a radicle but be unable to produce shoots; in this instance the objective of seed storage would not be achieved. The seeds stored at 25°C show the same trend up to 4 weeks storage, but after 11 weeks all the fully hydrated seeds were completely dead (Figure 35), while a small percentage of the 'sub-imbibed' seeds produced shoots (see above).

It has been repeatedly suggested that 'sub-imbibed' storage might be successful because the seeds would not proceed with germination during storage, but that vital metabolism would continue (King and Roberts, 1980). However, at intermediate water contents respiration occurs (Vertucci and Leopold, 1984), but metabolism becomes dysfunctional and can lead to severe damage because activated oxygen species are produced as by-products (e.g. Leprince *et al.*, 1999, 2000).

During the storage period, there were changes in water content of the axes and the cotyledons of *S. cuminii* seeds, especially in the 'sub-imbibed' situation. The 'sub-imbibed' seeds showed a general decline in water content (Figure 38). Axes and cotyledons from the freshly harvested seeds stored hydrated showed only a slight increase in water content during the 6 weeks of storage (Figure 38).

It was previously reported that *S. cuminii* seeds maintained at 25-30°C lost viability within 2-3 weeks (Rawat and Nautiyal, 1997; Srimathi *et al.*, 1999) and seeds maintained at 16°C retained their initial germinability following 20 weeks of storage. However, the results obtained from the present experiments showed that *S. cuminii* seeds only retained viability for 4 weeks at 16°C and 6 weeks at 25°C (Figure 39). This is not consistent with those previous findings which indicate that storage is extended, at least at the lower temperature of 16°C. Total storage time reported (Rawat and Nautiyal, 1997; Srimathi *et al.*, 1999) was far longer than that presently obtained, highlighting the variability of different harvests, and seeds collected from

widely differing locations. While storage of the seeds of this species at temperatures lower than 16°C is not presented here, there is some evidence to suggest that they are chilling sensitive. Mittal *et al.* (1999) demonstrated that the vigour of *S. cuminii* seeds was greatly reduced, despite viability being 100% after storage at 5°C, when compared with that of similar seeds stored at 16°C.

'Sub-imbibed' storage was found to be ineffective in extending storage lifespan; of *S. cuminii* seeds in fact, longevity was actually curtailed (Figure 39). The viability of the 'sub-imbibed' seeds declined from 100% to 10 and 20% following storage for only 2 weeks at 16 and 25°C, respectively, while the hydrated seeds stored for that period retained 100% viability at both storage temperatures (Figure 39). It is clear that storage lifespan of *S. cuminii* seeds is actually diminished by initial partial drying.

Prior to storage, the 'sub-imbibed' seeds of *P. henkelii* and *T. emetica* showed much lower respiration than the fully hydrated seeds, but during storage the fully hydrated *P. henkelii* seeds showed a general respiratory decline (Figure 36) and, after 10 weeks there was no difference in rate of CO_2 evolution among the seeds stored at different temperatures. All the fully hydrated *P. henkelii* seeds maintained 100% viability after 10 weeks in storage, except those stored at 25°C (Figure 34). The 'sub-imbibed' seeds showed an increase in respiration after 2 weeks in storage, and respiration subsequently declined after further storage (Figure 36). After the 10 week storage period the respiration rate of the hydrated and 'sub-imbibed' seeds is the same (Figure 36). After 2 weeks in storage the 'sub-imbibed' seeds begin to show a decline in viability at all temperatures (Figure 34).

Thus, it is evident that although respiration occurs in seeds that have been partially dehydrated, this metabolism may be deranged and potentially damaging to the cells. The *T. emetica* seeds also showed a general decline in respiration during the storage period, however respiration persisted (Figure 30). The seeds stored at 25°C had the lowest respiration after storage. Additionally, although requiring meticulously-controlled investigations, contribution of the seed-associated mycelia to the CO_2 evolution, remains a very real possibility.

The electrolyte leakage data for *P. henkelii* seeds indicates that there was an increase in leakage with time in storage for the fully hydrated and the 'sub-imbibed' seeds at

all three temperatures (Figure 37). The fully hydrated seeds stored at 25°C showed a dramatic increase in electrolyte leakage after 4 weeks of storage (Figure 38), coinciding with the total decline in viability of these seeds. This decline in viability was thought to be a result of severe proliferation. Structural barriers such as cell walls and membranes are degraded by fungi, and this would lead to a substantial increase in the amount and rate of electrolyte leakage from the cells. This would only occur if membrane damage occurs (which is likely) because the wall is a sieve under all conditions. The *T. emetica* seeds showed an increase in leakage with increasing storage duration (Table 1). The 'sub-imbibed' seeds of *T. dregeana* show much higher leakage than the fully hydrated seeds (stored at 16°C), which is an indication that more tissue damage, particularly to membranes, occurred in the 'sub-imbibed' seeds. The total leakage of these seeds did not change markedly over the storage period (Figure 27).

4.4 Ultrastructure

The ultrastructural examination showed the axes from freshly harvested seeds to be in a state consistent with active metabolism (Figures 40.1 - 40.2), as would be expected of mature recalcitrant seeds, especially those which entrain germination rapidly (Berjak *et al.*, 1992, 1993; Farrant *et al.*, 1992, 1993). These seeds did not show any intra-cellular abnormality (Figures 40.1 - 40.4). The seeds which had been partially dried retained 100% viability and showed evidence of an increase in intracellular activity (Figures 41.1-41.3), which is consistent with the increased vigour presently observed for *T. emetica* seeds after short periods of drying. Pammenter *et al.* (1998) have noted that then initial application of an ultimately lethal stress on recalcitrant seeds is generally stimulatory both physiologically and ultrastructurally; the negative effects are manifested only with time.

Studies on several different species have shown that intracellular and metabolic enhancement occur in the early post-shedding stages, culminating in events such as cell division and the onset of substantial vacuolation, which are indicative of ongoing germination (Berjak *et al.*, 1984, 1989; Farrant *et al.*, 1985, 1989; Pammenter *et al.*, 1994). In the absence of provision of an external water supply, intra-cellular degradation occurs thereafter (Farrant *et al.*, 1989) and ultimately viability is lost, despite there having been no significant dehydration (King and Roberts, 1980; Fu *et al.*, 1990; Xia *et al.*, 1992; Pritchard *et al.*, 1996). Initiation of metabolic events in the partially dried seeds was typical of events of early germination. In these experiments the partially dried seeds (without storage) showed mitochondrial changes indicative of an increase in activity, manifested as increased respiratory activity; the sustained presence of cytoplasmic and membrane-bound polysomes, implying ongoing protein synthesis; and the appearance of Golgi bodies, indicating increased endomembrane and sub-cellular activity, in general (Figure 41.1-41.3).

Ultrastructural observations confirmed that the seeds of *T. emetica* are damaged by chilling. The seeds stored at 6°C, (under both hydrated and 'sub-imbibed' conditions)

showed extensive deterioration and damage of the cells (Figures 42.1, 42.2, 45.1 and 45.2). It was notable that there was little discernible contamination of hydrated seeds stored at 16 or 25°C, while those stored at 6°C were overrun by fungi after two weeks. As all the seeds were from a common batch, this strongly suggests that vigorous, metabolically-active individuals stored under favourable conditions, have mechanisms to counteract micro-organism proliferation (at least in the short term), as proposed for recalcitrant seeds by Berjak (1996), and confirmed by Calistru *et al.* (2000).

Kioko (2002) found that seeds of *T.* emetica stored at 6°C showed signs of deterioration by day 10. Those cells had electron translucent mitochondria and plastids with no discernible internal structure, even though cellular membranes such as the plasmalemma and nuclear envelope appeared undamaged. By day 20 of storage the cells exhibited extensive ultrastructural derangement. The plasmalemma had receded from the cell wall and was discontinuous in many places, and organelles were extensively degraded (Kioko, 2002). However it must be noted that such ultrastructural examination cannot discern inherent deteriorative changes in axes, from those mediated by fungal activity.

Berjak *et al.* (1992) suggested that, while vacuolation is normally associated with the onset of germination, accelerated and abnormal vacuolation might be the consequence of an applied stress on hydrated metabolically active seeds. This was observed in the seeds stored at 6°C (hydrated and partially dried) and the partially dried seeds stored at 16 and 25°C (Figures 42.1, 45.2, 46.1 and 47.1). The vacuoles were often arranged in abnormally close aggregations (Figure 45.2 and 46.1) Many vacuoles contained material, suggestive of autophagy of cellular constituents (Figures 42.1 and 42.2). Autophagy is a process that occurs in normal cells whereby damaged components are removed, by the vacuoles (which are major lytic compartments) and hydrolysed to produce and release the constituent molecules. These materials are then recycled. (Kim and Klionsky, 2000; Klionsky and Emr, 2000). It essentially serves as a mechanism to re-establish cell homoeostasis. Autophagy of cellular reserves was reported to occur in plant cells as a mechanism to generate substrates for respiration (during sucrose starvation) (Journet *et al.*, 1986; Aubert *et al.*, 1996; Moriyasu and Oshumi, 1996). Berjak *et al.* (1989, 1990)

suggested that this process can be triggered almost immediately by signals other than reduced respiratory substrate. Autophagy appears to be almost invariably increased in cells which have undergone stress, which is probably in response to an increased proportion of damaged components that have to be removed as part of the suite of repair mechanisms. Alternatively, autophagy provides a mechanism for the removal of structures not immediately essential to survival or those structures damaged by drying (Berjak *et al.*, 1989).

Membranes are particularly susceptible to damage during slow drying. This is expected on the basis that the degradative processes are aqueous- based and oxidative in nature (Hendry, 1993). Membranes are commonly cited as the primary site of desiccation injury (e.g. Senaratna and McKersie, 1986). This was strongly suggested in the current experiments as plasmalemma withdrawal and damage was observed in the seeds which were partially dried prior to storage (Figure 45.2, 46.2 and 47.1). The fully hydrated seeds stored at 6°C showed unusual collections of vesicles between the plasmalemma and the cell wall (Figure 42.2 and 42.3), which are suggested to be visible manifestations of chilling-induced plasmalemma injury. Similar vesicles were seen in chilling sensitive neem seeds and interpreted in this way (Berjak *et al.*, 1995). The fully hydrated seeds stored at 16°C showed some plasmalemma separation from the cell wall in places (Figure 43.2), while those stored at 25°C showed some plasmalemma irregularities (Figure 44.2). In the latter case, these irregularities are suggested to result from active incorporation of exocytotic, Golgi-derived vesicles.

Chemical (e.g. oxidative stress; Walters *et al.*, 2001) rather than physical (i.e. structural damage is inferred from the observations that membrane abnormalities were most frequent in slowly-dried axes. The water contents investigated here are greater than those reported to induce structural damage to membranes (-12 Mpa; Vertucci and Farrant, 1995), and are therefore more likely to support lesions caused by dysfunctional metabolism (Vertucci, 1993; Salmen-Espindola *et al*, 1994; Leprince *et al.*, 1999, 2000; reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak, 1999).

The ER in axes dried slowly within the seeds often proliferated and became rearranged into concentric rings, a phenomenon previously associated with cessation of growth in drought stressed mustard seeds (Bergfield and Schopfer, 1984), and with inhibition of protein synthesis in heat-shocked pollen tubes (Kandasamy and Kristen, 1989). This was observed in the fully hydrated axes of seeds stored at 6°C (Figure 42.1, 42.2 and 42.4), where the concentric rings of ER were usually surrounding another organelle or structure. Proliferation of ER suggests an onset of metabolic stress and that ER may play a pivotal role during dehydration (e.g. Bergfield and Schopfer, 1984). Abundance of ER may be indicative that some compensatory activity (to do with autotive repair) repair is taking place. The fully hydrated seeds stored at 6°C showed a proliferation of ER and many of the profiles extended cisternae. The phenomenon is a common response to stress. ER proliferation and concentric formations are the early stages of intensified vacuolation by cytolysome formation, which serves to remove damaged organelles and ground cytomatrical material efficiently from the cells.

Kioko (2002) observed that the fully hydrated *T. emetica* seeds stored at 16 and 25°C retained full germinability with cells showing apparent absence of ultrastructural damage. Ongoing metabolism at these temperatures was indicated by numerous cristae in the mitochondria, Golgi bodies, profiles of endoplasmic reticulum, and a cytomatrix with abundant non-membrane bound polysomes, and many plastids. By 60 days in storage at 16 and 25°C, ultrastructural damage was evident in many cells. The cells had undulating cell walls with a peculiar wavy appearance (Kioko, 2002). This may be a further indication of prolonged mild water stress, which the seeds are suggested to have suffered as germination proceeded during storage. In the current experiment the fully hydrated seeds stored for 3 weeks at 16 and 25°C showed similar characteristics to those observed by Kioko (2002) except that a measure of plasmalemma withdrawal was evident after storage at 16°C (Figures 43.2).

Presently the partially dried seeds stored at 16 and 25°C displayed ultrastructural abnormalities after 3 weeks in storage (e.g. Figure 47.1). Less severe indications of stress were poorly-formed cristae in mitochondria, and damaged plasmalemma and irregular,

often folded. However, in many cases the cells were characterised by total devastation. Significantly, fungal contamination was evident in only a few seeds at each sampling interval. However, the deteriorating ultrastructural state, coupled with germination of the seeds during storage, indicated that the seeds of *T. emetica* do not have a useful storage lifespan of longer than 2 months (Kioko, 2002). It is evident from the ultrastructural observations that 'sub-imbibed' storage does not enhance storage lifespan of the seeds as these seeds showed more damage than the fully hydrated seeds.

4.5 Concluding Comments

The results of the present study indicated that 'sub-imbibed' storage is not a feasible option for the four recalcitrant species studied. The present results indicated that storage in the fully hydrated condition was advantageous over 'sub-imbibed' storage. Removal of water did not enhance survival (in any of the species) during storage, in fact viability was actually compromised when the seeds were stored in this way. This confirms that these specific recalcitrant seeds cannot withstand removal of even small amounts of water before storage; however, they may withstand removal of small amounts of water if the seeds are planted immediately following drying. In this instance, the seeds even showed increased vigour. It is possible to conclude that the deleterious effect of removing water are manifested during storage but water removal beyond a certain point will result in loss of viability regardless of whether the seeds are planted immediately or not.

This study gives conclusive evidence that 'sub-imbibed' storage is not a feasible option for the species studied, even in the short-term.

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