

## Optimising Conditions for Neem (*Azadirachta indica*) Seed Longevity



**Moctar Sacandé**

CNSF, Ouagadougou, Burkina Faso (Current address: Seed Conservation Department, Royal Botanic Gardens, Kew, Wakehurst Place, Haywards Heath, West Sussex RH17 6TN, UK)

**Folkert A. Hoekstra**

Dept. Plant Sciences, Wageningen University, Laboratory of Plant Physiology, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands



### Summary

Our recent investigations have gone a long way towards explaining the complex storage behaviour of neem seeds. We have shown that physiological maturity occurs before morphological maturity, but high quality seeds are obtained from yellow mature fruits. Hydrated seeds (37% MC) are sensitive to chilling temperatures and dried seeds (6% MC) become particularly sensitive to imbibitional stress and often die during rehydration. Both these sensitivities to chilling and imbibition were associated with the high transition temperature ( $T_m$ ) of 10°C for neem seed membranes. The construction of phase-state diagrams revealed that at 20°C and 75% RH, neem seed tissues are out of the glassy state; under these conditions seeds died within 6 months. At 32% RH, seeds are within the glassy state and high viability was maintained for at least two years. Seed longevity is improved at temperatures between 20 and -20°C, and sensitivity to chilling/subzero temperatures is reduced when seeds are further dehydrated. We built a model that describes the optimum conditions for storage of neem seeds based on these studies. The observation that seeds can survive desiccation to c. 6% MC (0.05 g H<sub>2</sub>O g<sup>-1</sup>dw), freezing at -20°C and careful rehydration, suggests that seeds from mature yellow fruits are orthodox in behaviour. This may be used to develop protocols to extend the longevity of similar tropical seeds

### Introduction

Seeds can lose their viability during dehydration, during rehydration and gradually over the course of storage. Viability is generally determined by placing the seeds under standard conditions of moisture and temperature to encourage germination and growth. Although this is the only obvious means of testing for viability, standard germination tests do not reveal how, where and when damage occurs in seeds. Seeds lose viability due to physical, chemical and metabolic processes occurring during dehydration, storage (ageing), and/or rehydration that can damage both the cytoplasm and cell membranes (Priestley, 1986; Bewley and Black, 1994). Deterioration of membranes is a primary indicator of cell death (Priestley, 1986; Hoekstra and Golovina, 1999).

Neem, *Azadirachta indica*, is an important pan-tropical species with a multitude of uses and is in high demand by farmers in many developing countries. However, neem seeds are reputed to be difficult to handle and store for extended periods of time. The seeds have been variously described as desiccation-tolerant and -intolerant, and also as moderately desiccation-tolerant but chilling-sensitive (Hong *et al.*, 1998; Hong and Ellis, 1998; Sacandé *et al.*, 1998). Such complex behaviour makes neem seeds interesting plant material to study. These investigations aimed to further understand the biology of germination and storability.

We studied seed developmental maturity and its effect on seed quality. Because sensitivity to chilling and imbibitional stress is pronounced in most tropical species, we carried out detailed investigations during incubation and rehydration of neem seeds at a range of temperatures. A study of seed-water relations was undertaken to understand the role and the properties of water in neem seed using differential scanning calorimetry. Based on the phase-state diagrams for neem seed tissues and our previous storage data, a model depicting optimum conditions for the storage longevity of neem seeds was built. In this paper, the loss of viability is reviewed in relation to the properties of membranes and melting transition temperatures of neem seeds.

## Material and Methods

### 1. Seed Development and Germinability

To accurately estimate developmental stages and the physiological maturity of seeds, morphological characteristics and germinability of neem seeds from Burkina Faso were monitored over the whole production period on selected trees at weekly intervals. The disintegration of the corolla, a recognisable event resulting from pollination, was used as a marker of zero time of fruit and embryo development.

Germinability of excised embryos and whole seeds from fresh fruits at various stages of development was also tested. Seeds were incubated on moist filter paper, in a cabinet at a constant optimum temperature of 25°C for germination. All germination data were statistically analysed using the  $\chi^2$ -test on the binomial proportions, with the level of significance at  $P \leq 0.05$ .

### 2. Chilling Sensitivity and Imbibitional Stress

Many plants of tropical origin suffer injury when they are kept under chilling temperatures for some time. To study the effects of chilling temperatures on neem seed survival, highly viable (96% germinated seeds) fresh seeds (37% moisture content on a fresh weight basis) were incubated at a range of constant temperatures from 2.5 to 30°C, for their germination capacity.

Neem seeds are non-dormant, but soaking them in water for a few hours before sowing helps to improve the germination rate and capacity. However, during imbibition (rehydration), dry seeds leak intracellular solutes into the surrounding medium, leading to death if the leakage persists. A more detailed study was therefore conducted to explore possible imbibitional damage. Seeds were soaked in tap-water for 4 h at different temperatures from 2 to 35°C and thereafter transferred to 25°C for germination, as described above.

### 3. State/Phase Diagrams of Neem Seeds

A differential scanning calorimeter (Pyris 1 DSC, Perkin-Elmer, Norwalk, CT, USA) was used to determine the melting transitions and changes in heat capacity of mature neem seeds. Axes and cotyledons with different water contents were cooled to  $-100^{\circ}\text{C}$  and scans were recorded from  $-100^{\circ}\text{C}$  to  $120^{\circ}\text{C}$  at the same rate of  $10^{\circ}\text{C min}^{-1}$ . The enthalpy ( $\Delta H$ ) of the melting (first order) transitions of water and second order (glass) transitions were assessed. The midpoint of the temperature range over which the change in specific heat occurred was used to determine the glass-to-liquid transition temperature ( $T_g$ ).

### 4. Moisture Content Determination

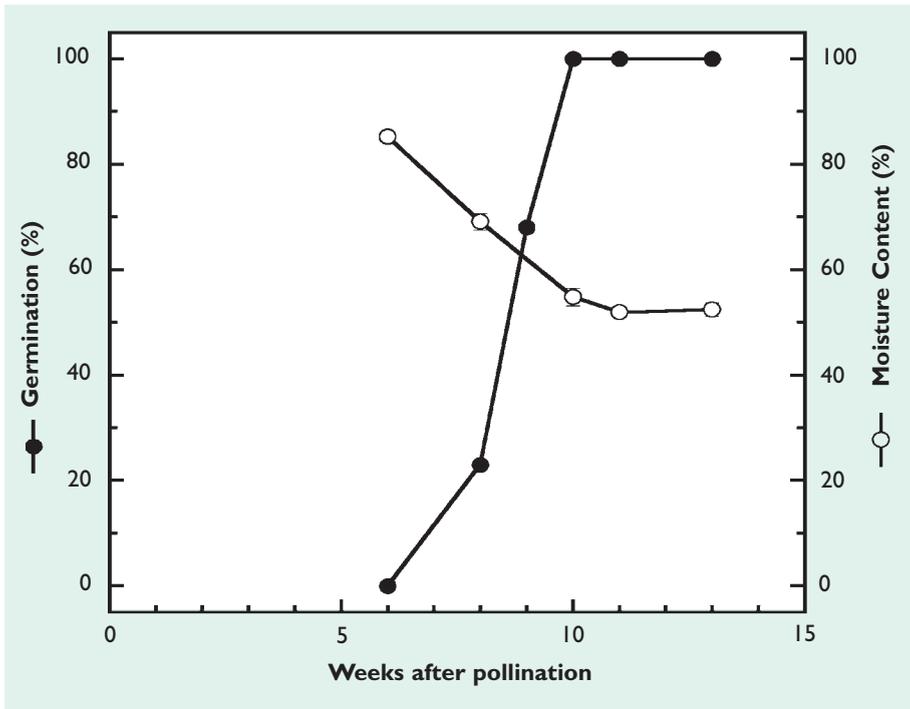
Seed moisture contents (MC) were determined gravimetrically by oven-drying at  $103^{\circ}\text{C}$  for 17 h. Unless otherwise indicated, moisture contents are expressed as a percentage of dry weight.

## Results and Discussion

### 1. Characterisation of Seed Development and Germinability

Neem flowers withered in the second week after opening, after which fruits started to develop. Embryos could be isolated from the immature fruit and measured when they had reached a length of approximately 1–2 mm, which occurred after 4 weeks of development. The fruit had then grown from its initial length of 2 mm to approximately 13 mm, which is already close to its maximum length (data not shown). Thus, the developmental stage of the seed is not easily derived from the fruit length. The minimum developmental period to yellowing of the initially green fruits was approximately 12–13 weeks. From 13–14 weeks onwards, brown fruits were also observed, so that all stages of fruit development were occurring on the same branch.

Abnormal seedlings with defective hypocotyl extension were obtained at intermediate times (after 6 weeks) of development, when embryos were half the length of the fruits. Embryos and whole seeds acquired full germinative capacity with 100% normal seedlings at 10 weeks (Figure 38.1), coincident with the cessation of embryo elongation when the fruits were still green. Thus, physiological maturity (germination competence) occurs before morphological maturity, but high storage-quality seeds are only obtained from mature, yellow fruits. The moisture content decreased from 85% at six weeks, to a stable level of approximately 51% (fresh weight) after 10 weeks. This level was reached simultaneously with the acquisition of the maximum germination capacity. However, when seeds of all ages were dried to the same MC of 8% and stored at  $-20^{\circ}\text{C}$  over a year, seeds from yellow fruits performed better than those from the green or brown (data not shown).

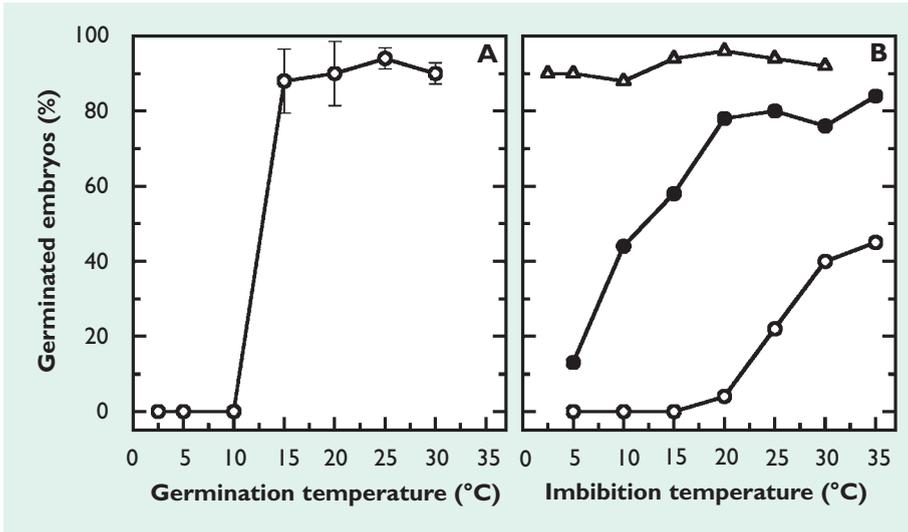


**Figure 38.1** Relationship between moisture content and the acquisition of germination capacity during development of neem seeds. Samples of fresh seeds were oven-dried for moisture determinations and incubated for germination (ISTA, 1993). Standard error values never exceed symbol size.

## 2. Chilling Sensitivity and Imbibitional Stress

When seeds fail to tolerate dehydration, it may be necessary to cool them to low temperatures (above 0°C) to optimally reduce metabolic activity in order to prolong storage longevity. However, neem seeds were chilling sensitive (Figure 38.2) and died within a few months (Sacandé *et al.*, 1998). No seeds germinated at  $\leq 10^{\circ}\text{C}$ , but maximum germination occurred when seeds were incubated at  $\geq 15^{\circ}\text{C}$ , indicating that the threshold temperature for germination lies between 10 and 15°C (Figure 38.2A). Final germination percentages obtained at temperatures ranging from 15 to 30°C did not differ significantly from each other.

At ambient temperatures, the viability of neem seeds dried to 8% MC was reduced (data not shown). From similar results of comparable experiments (Chaisurisri *et al.*, 1986; Maithani *et al.*, 1989; Ezumah, 1986), it was concluded that neem seeds display recalcitrant storage behaviour. Recalcitrant seeds from temperate climates are usually more tolerant to low temperatures than those



**Figure 38.2** Fresh neem seeds from Ouagadougou, Burkina Faso, were incubated at constant temperatures from 2.5 to 30°C for estimates of their germination capacity (A). The effect of imbibitional temperature (for 4 hours) on the germinability at 25°C was also studied (B). The tested seeds were freshly harvested (37% MC), previously dried (to approx. 6% MC) for 6 weeks, or stored for 2 years at 6% MC. All germination data are significantly different ( $P \leq 0.05$ ) when they differ by 25% or more ( $\chi^2$ -test). Figure 38.2A from Sacandé (2000).

from tropical climates. For example, *Quercus* spp. can germinate after several months at 2°C (Pritchard and Manger, 1990), whereas the seeds of many tropical species suffer chilling injury or may be killed even at sub-ambient temperatures in the range of 10 to 15°C (King and Roberts, 1979).

Chilling injury in many plants of tropical origin has been partly attributed to a conformational transition in cell membranes from the liquid crystalline to the gel phase (Lyons *et al.*, 1979). Such a transition is often followed by lateral phase separation, i.e., the sorting of membrane components according to their molecular species (Platt-Aloia and Thomson, 1987; Sharom *et al.*, 1994). The *in situ* phase transition temperature ( $T_m$ ) as measured in intact tomato hypocotyls was around 10–15°C (Raison and Orr, 1986; Crowe *et al.*, 1989b). In contrast, the *in situ*  $T_m$ s of plants from temperate zones are usually around or below 0°C (Crowe *et al.*, 1989b). The  $T_m$  of 10°C of membranes in the root tips of neem seedlings confirmed that neem has the typically high  $T_m$  of a plant from the tropics (Sacandé *et al.*, 2001). This may be due to the relatively high degree of saturation of the esterified acyl chains of the phospholipids (reviewed in Hoekstra and Golovina, 1999; Sacandé *et al.*, 2000). Hence, temperatures below  $T_m$  lead to gel phase formation and lateral phase separation, followed by extensive leakage during imbibition.

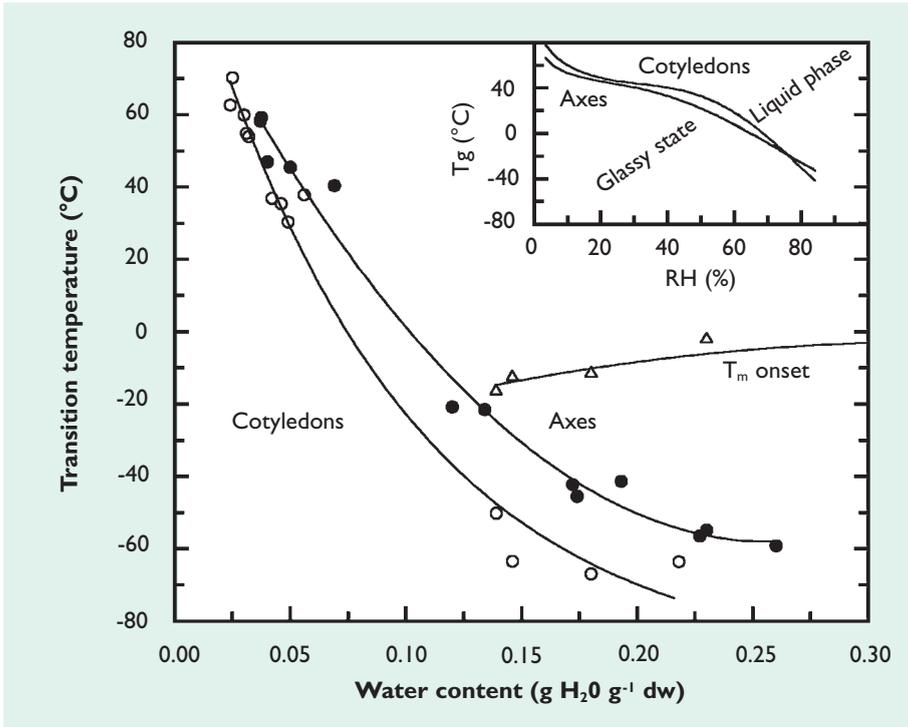
Neem seeds dried further to 6% MC lost their chilling sensitivity (data not shown), but became sensitive to damage on imbibition (Figure 38.2B). Germination percentages improved with increasing temperature of soaking for seeds dried to 6% MC ( $0.05 \text{ g H}_2\text{O g}^{-1}\text{dw}$ ) reaching an optimum at between 20–35°C for non-aged and 30–35°C for aged seeds, there being no germination at 40°C. The fresh sample (37% MC) was not sensitive to 4 h of cold soaking, but further incubation resulted in permanent damage at temperatures below 10°C (Figure 38.2A).

Imbibitional leakage has been extensively studied in pollens and model lipid systems and has been attributed to plasma membranes being in the gel phase at imbibition (Crowe *et al.*, 1989a). The rigidity of the plasma membrane at imbibition is the critical factor in whether the organ(ism) survives rehydration or not (Hoekstra *et al.*, 1999). Increasing the elasticity of these membranes prior to imbibition by preheating or prehydration from the vapour phase can alleviate the stress (Hoekstra and Golovina, 1999) and prevents rupture during imbibition. The sensitivity to imbibitional stress also increased with seed ageing (Figure 38.2B). It was necessary to soak seeds at higher temperatures to compensate for the increased imbibitional stress. Because of this extreme sensitivity, dry seeds were probably often killed during imbibition rather than during storage.

Although phase changes are reversible upon return to above- $T_m$  temperatures, extended periods of time under low temperature conditions may have drastic consequences (Figure 38.2A; Sacandé *et al.*, 2001). The increases during drying of the membrane's  $T_m$ , even exceeding ambient temperature, may not harm viability *per se*, as many organ(ism)s can be stored successfully in the deep-freezer at -20°C, a condition that favours the existence of gel phase in the membranes. However, at rehydration, extensive leakage may occur which leads to the death of the seed.

### 3. Predictive Storage Model

The study of the properties of water in embryos by differential scanning calorimetry allowed detection of the presence of cytoplasmic glasses. Figure 38.3 shows the temperature at which the midpoint of the glass transition occurred, shifting to lower values with increasing water contents. Such a shift with water content is indicative of transitions caused by the melting of glass. A glass is an extremely viscous liquid with solid state-like properties, but retains the disorder and physical properties of the liquid phase (Franks *et al.*, 1991). Glasses have been shown to occur in seeds and pollen (Buitink *et al.*, 1998). The transition temperatures of the axes were higher than those of the cotyledons, due to the difference in lipid content between these tissues (Sacandé *et al.*, 2000). Between approximately -20°C and +30°C,  $T_g$  was undetectable because of interference with the lipid melting. The relationship



**Figure 38.3** State/phase diagrams for mature neem seed axes and cotyledons derived from the mid-temperatures of glass transitions. The tissues are in the glassy state below the curves and in the liquid phase above the curves. The phase diagram of water in the cotyledons is also presented by  $T_m$  onset. The relationship of  $T_g$  versus the relative humidity (RH) at 20°C is shown in the inset (data from Sacandé *et al.*, 2000, with permission of Oxford University Press).

of  $T_g$  versus the relative humidity (RH) at 20°C that is shown in the inset, resulted from calculations that combined the state diagrams with the fitted sorption curves (not shown). The slower molecular mobility in the glass has been linked to a slower rate of ageing, rendering chemical reactions improbable, which is considered to substantially enhance long-term stability. By contrast, the non-glassy, liquid phase allows an accelerated physical and chemical deterioration of seeds (Sun and Leopold, 1993), probably as a result of an increased molecular mobility (Buitink *et al.*, 1998).

To find out how neem seeds can best be stored, we combined our previous experimental data concerning longevity (Sacandé *et al.*, 1998) and the presence of a glassy state under conditions of different MCs and temperatures in a diagram (Figure 38.4). The diagram allows us to speculate about the possible significance of water relations with respect to long-term

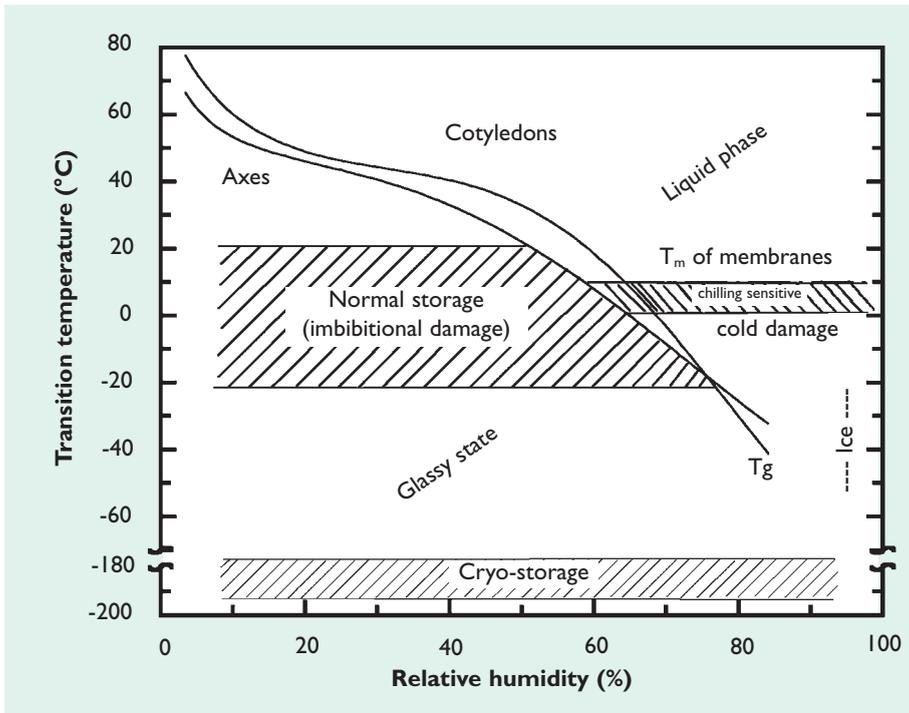


Figure 38.4

Diagram depicting optimum conditions for storage of mature neem seed. The curves indicate  $T_g$  in cotyledons and axes of neem seeds equilibrated at different RHs at 20°C. Above the curves tissues are in liquid phase; below the curves they are in the glassy state. The hatched areas show storage conditions between 20 and -20°C which gave good results over two years (from Sacandé *et al.*, 1998) and cryogenic storage at liquid nitrogen temperatures. Conditions at which seeds are chilling- (0–10°C) or cold- (sub-zero °C) sensitive, or experience ice formation, are also indicated. The  $T_m$  of hydrated membranes is shown at approx. 10°C. Reproduced from Sacandé (2000).

storage and chilling/subzero sensitivity of neem and other tropical, putative 'intermediate' seeds (Figure 38.4). At 20°C and 75% RH, for example, both the cotyledons and the axes are expected to be out of the glassy state. Under these conditions, seed longevity is limited to a maximum of 6 months (Sacandé *et al.*, 1998). The sensitivity to chilling and subzero temperatures is lost in seeds that are stored at 53% RH (embryo MCs  $\leq 0.048$  g H<sub>2</sub>O g<sup>-1</sup> dw). Extension of storage longevity beyond 2 years is possible when seed tissues are in the glassy state, i.e.,  $10\% \leq \text{RH} \leq 53\%$  and storage temperatures are between 20 and -20°C. The low amount of water at these RH's brings the seeds below the glass transition temperature, but keeps them above the region at which the structural stability of cells is compromised. The critical water content below which the seeds will not germinate was difficult to determine, because such dry seeds are very sensitive to imbibitional stress (Figure 38.2B). Therefore, the box of effective storage is left open at the low moisture side in the diagram. Other storage studies also give indications that dehydration alleviates chilling stress and that neem seeds (Hong and Ellis, 1998; Tompsett and Kemp, 1996) can be successfully stored in the glassy state. Moreover, successful cryogenic storage at liquid nitrogen temperatures has been reported for relatively dry neem seeds (Berjak and Dumet, 1996) in contrast to seeds at higher MC, which do not tolerate cryogenic storage (Chaudhury and Chandel, 1991). Damaging ice crystals might have formed in the latter seeds. Chilling sensitivity in neem seed is observed above 60% RH, whilst below this RH, the region of storage temperature and RH where seeds experience imbibitional stress is also illustrated. Even with high moisture tissues, liquid nitrogen storage may be successful when freezing rates are fast enough to avoid intercellular ice formation (Vertucci, 1989).

## Conclusions

These investigations offer new insights into the biology of neem seeds, with membrane properties as a key factor in their survival. Careful manipulations provided a 'window' for neem seed lots to maintain viability whilst sufficiently dehydrated and in the glassy state.

Understanding the role played by water in seeds has resulted in the development of efficient methods for improved handling and storage longevity of such material. Although the ability to survive rehydration is a part of desiccation tolerance, the fact that careful rehydration at high temperatures can considerably improve germination percentages is evidence that neem seeds can withstand severe dehydration without loss of viability. This therefore allows us to consider them as desiccation tolerant and orthodox in their storage behaviour.

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