Seed Science Research (2004) **14**, 205–217

DOI: 10.1079/SSR2004170

Ageing increases the sensitivity of neem (*Azadirachta indica*) seeds to imbibitional stress

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Abstract

Imbibitional stress was imposed on neem (*Azadirachta indica*) seeds by letting them soak for 1 h in water at unfavourable, low temperatures before further incubation at 30°C. Sensitivity to low imbibition temperatures increased with a decrease in seed moisture content (MC). To investigate a possible involvement of seed age in the extent of imbibitional damage, initially high-quality seed lots that differed in storage history (10 weeks versus 10 months) were examined at 4 and 7% MC (fresh weight basis). After 10 months of storage, the 7% MC seeds had become sensitive to imbibitional stress. Further drying (1 week) to 4% MC affected aged seeds more than non-aged seeds. Barrier properties of cellular membranes in axes excised after 1 d of rehydration were estimated using a spin-probe technique. The proportion of cells with intact membranes increased with increasing imbibition temperature. For each temperature tested, there were more cells with leaky membranes after 10 months than after 10 weeks of dry storage. Localization of embryo cells displaying loss of turgor and abnormal cellular structure was accomplished using cryo-planing, followed by cryo-scanning electron microscopy. Inspection of the cryo-planed surfaces confirmed that imbibitional damage was temperature dependent, occurring at the periphery. Ageing increased the number of imbibitionally damaged, peripheral cell layers. Germination was estimated to fail when less than 70% of axis cells were alive. We conclude that ageing increases the sensitivity to imbibitional stress. Both the fast ageing and the sensitivity to imbibitional stress might explain the apparent controversies about neem seed desiccation tolerance and storage behaviour.

Keywords: *Azadirachta indica*, cryo-planing, cryo-scanning electron microscopy, electron spin resonance, germination, imbibition, membrane integrity, seed viability, temperature

Introduction

According to their storage behaviour, seeds are classified into three categories, namely orthodox, intermediate and recalcitrant (Hong *et al.*, 1996). Approximately 90% of plant species have seeds that fall into the first category. The remaining 10% exhibit problems with the maintenance of seed viability (Hong *et al.*, 1996), which would concern approximately 25,000 plant species, worldwide. Nearly 75% of these plant species have seeds that are characterized by recalcitrant storage behaviour, with a large proportion originating from the tropics. Recalcitrance is defined as the inability of seeds to withstand drying below 96–98% relative humidity at 20°C (Hong *et al.*, 1996). An additional problem with these seeds is that they are often chilling sensitive, which further limits the possibilities of keeping them alive in storage. Examples are the seeds of economically important tropical species, such as cacao, coconut, rubber, avocado and mango. The so-called intermediate seeds (Ellis *et al.*, 1990, 1991), forming a third group of seeds, store better in dry conditions than recalcitrant seeds, but have a much shorter life span than orthodox species. Examples are the seeds of coffee, papaya, neem, *Khaya*, *Lannea* and

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Sclerocarya. These seeds might be sensitive to low temperatures during rehydration.

The majority of studies on desiccation tolerance are focused on changes in organisms during drying and/or dry storage, regrettably without much consideration of the stresses associated with rehydration. It has been argued that successful imbibition leading to the resumption of vital processes should be considered an essential part of the mechanism of desiccation tolerance (Hoekstra *et al*., 2001). During imbibition, desiccation-tolerant organisms (anhydrobiotes) leak solutes into the surrounding medium. This leakage is generally transient and does not lead to severe damage (Senaratna and McKersie, 1983; Hoekstra and Van der Wal, 1988). Lately, this leakage has been attributed to the transiently increased membrane permeability associated with the presence, in plasma membranes, of amphiphilic metabolites that are in the process of partitioning back into the aqueous cytoplasm during rehydration (Golovina *et al*., 1998). Complete loss of cell viability due to irreversible membrane damage may occur when dried anhydrobiotic cells are allowed to imbibe in relatively cold media (Hoekstra *et al*., 1999). This phenomenon is called imbibitional damage, and may lead to reduced germination ability. Elevated initial moisture content and warm imbibition alleviate the stress of rehydration.

Neem is an important tropical tree species, the products of which have multiple uses in Asia and, since its introduction, in the Sahelian regions of Africa (Bellefontaine, 1992). Essentially, the species propagates via seeds. Conflicting results have been published on the physiology and storage of neem seed (reviewed by Hong *et al*., 1996; Poulsen, 1996). Neem seeds have been reported to lose viability within 1 to 4 months during *ex-situ* conservation (Ezumah, 1986; Berjak *et al*., 1995; Gunasena and Marambe, 1995; Msanga, 1996). They have also been reported to display orthodox behaviour (Roederer and Bellefontaine, 1989; Bellefontaine and Audinet, 1993; Tompsett and Kemp, 1996; Sacandé *et al*., 2000b). Other studies concluded that neem seeds belong to the intermediate category of storage behaviour (Gaméné *et al*., 1996; Sacandé *et al*., 1996; Hong and Ellis, 1998; Varghese and Naithani, 2002). Many factors, such as seed provenance, maturity at harvest and conditions of storage and sensitivity to imbibition, have been suggested to explain these apparent controversies. Neem seeds are chilling sensitive at moisture contents (MCs) above 10% (fresh weight basis), but when dried to MCs below 10%, they are sensitive to imbibitional stress, limiting the tolerance to desiccation (Sacandé *et al*., 1998). Although there is evidence of an increase in sensitivity to imbibition with reduction of MC, the MC may not be the main or the only one reason for the sensitivity of neem seeds to imbibition. For example, seed age has been suggested to increase the sensitivity to imbibition (Priestley, 1986; Zeng *et al*., 1998).

The current study is focused on factors involved in the sensitivity of neem seeds to imbibitional stress and aims at characterizing the type of damage inflicted by cold imbibition. The effects of seed MC and ageing on the sensitivity to low imbibition temperatures were investigated. This was performed at an organismal level by germination assay, at a cellular level by a spin-label technique for the quantitative estimation of plasma membrane intactness, and at an ultrastructural level by cryo-planing (Nijsse and Van Aelst, 1999), followed by cryo-scanning electron microscopy (cryo-SEM). Dried seeds of two seed lots that differed in age were allowed to imbibe at a range of temperatures. We attempted to establish whether ageing increases the sensitivity to imbibitional stress in a species that is characterized by low desiccation tolerance and difficult storage behaviour.

Materials and methods

Seed material

Neem (*Azadirachta indica* A. Juss.) seeds were collected at two localities in Burkina Faso – Tougan and Ouagadougou. Mature (yellow) fruits were harvested by hand from at least 20 mother trees in each stand. Previous studies on the developmental stages of neem fruits have indicated that seeds from yellow fruits are physiologically mature (Sacandé *et al*., 1997; Yaméogo, 1997; Neya, 1999). The seeds were prepared locally on the day of collection at the Centre National de Semences Forestières (CNSF) in Ouagadougou, according to the method described by Sacandé *et al*. (1998). After depulping, the seeds were cleaned with water, dried in the shade on a grid for 1–2 d. Seeds, surrounded by an intact endocarp, were then selected and sent by air to Wageningen in cotton bags, arriving 1 week after harvest.

All seeds used in the present experiments were 'naked' seeds, i.e. seeds with the seed coat (endocarp) removed. Endocarp removal provided a more homogeneous germination, enhanced the rate of germination, but had no effect on seedling quality.

Moisture content and germination

Freshly collected seed lots were used directly in the experiments, or first dried to various MCs by exposure, for different lengths of time, to relative humidities (RHs) established above saturated solutions of $MgCl_2$ or LiCl in containers with

circulating air at 20°C. A Rotronic hygroscope (±2% RH, Rotronic AG, Zürich, Switzerland) was used to measure relative humidity. For the ageing experiments, initially high-quality (>90% germination) seeds were equilibrated in circulating air above a saturated solution of $Ca(NO_3)_2$ at 20°C (final MC = 7.0%). After 9 weeks, some of the seeds were dried further above saturated LiCl for a week (final MC = 3.7%). This procedure provided seeds that were 10 weeks old having two different MCs. The remaining 10-week-old, 7.0% MC seeds were then stored for 7.5 months at 15°C in a closed plastic bag (final MC 7.0%). Subsequently, half of this seed lot was after-dried above saturated LiCl for a week (final MC = 4.3%). For simplicity, these MCs are referred to as 7% and 4%. The endocarps were removed just before the imbibition tests.

Seeds (50) were allowed to imbibe by pre-soaking them for 1 h in tap water (100 ml) in beakers kept in incubators set at temperatures between 15 and 40°C. After the soaking treatment, the seeds were sown on moist filter paper in plastic boxes (25 seeds/box) and incubated at a constant temperature of 30°C in a 12h/12h light/dark regime. Germination was scored at least twice a week until all viable seeds had germinated. Seeds were scored as germinated when the radicle had emerged to at least 2 cm.

Three replicates of five seeds were used to determine the MC of seeds during the different experiments. The seeds were weighed before and after drying at 103°C for 17 h. The MC of seeds was expressed on a fresh weight basis.

Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker, Rheinstetten, Germany, model 300E). The water-soluble nitroxide radical, per-deuterated TEMPONE (PDT, where TEMPONE = 4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy; a kind gift of Professor I. Grigoriev, Institute of Organic Chemistry of the Russian Academy of Sciences, Novosibirsk, Russia), was used as the spin probe for testing the integrity of the plasma membrane, according to the method described by Golovina and Tikhonov (1994).

The seeds were first soaked in water at different temperatures for 1 h to let them imbibe, and then incubated in Petri dishes at 30°C for 1 d. After that, axes were isolated and incubated in 2 mM PDT + 120 mM potassium ferricyanide for at least 20 min. Because of spin-label reduction after some time (if incubation periods were longer), the solution was renewed. ESR spectra of PDT in the isolated axes were recorded at 0.5 G modulation amplitude and 18 dB (3.17 mW), to increase the signal/noise ratio without the need for scan accumulation. For each seed batch the number of axes analysed was from 8 to 15. The spectra were recorded for individual axes, and the ratio of amplitudes of water/lipid (W/L) was calculated for each spectrum.

Cryo-planing and cryo-scanning electron microscopy

Non-aged (10-week-old) and aged (10-month-old) seeds, which were dried as indicated above to 4% MC, were used for ultrastructural investigation. The method of cryo-planing for cryo-scanning electron microscopy (cryo-SEM), as described by Nijsse and Van Aelst (1999), was applied to the two neem seed lots. This technique is used to produce flat surfaces (planes) that reveal the internal structures of frozen specimens.

Seeds soaked in water for 1 h at different temperatures were incubated in Petri dishes at 30°C for 22 h. After that, the seeds were cut transversely in half, and the parts with the axes were mounted on to specimen holders, using a drop of Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and immediately frozen in liquid nitrogen slush (–210°C). The frozen samples were cryo-planed longitudinally at –90°C using a cryo-ultramicrotome (Reichert-Jung Ultracut E/FC4D, Vienna, Austria), first with a glass knife for rough planing, and the last sections with an 8-mm-wide diamond knife for fine planing. The samples were stored in liquid nitrogen and later cryo-transferred to a cryo-SEM (JEOL 6300F Field Emission SEM, Japan, Tokyo) equipped with an Oxford 1500 HF cryo-system (Eynsham, UK). The planed samples were freeze-etched for 3 min at –89°C to enhance contrast and remove water vapour contamination, then sputter-coated with platinum, and subsequently analysed at –190°C with an accelerating voltage of 5 kV. At least two seeds per treatment were analysed.

Results

Effects of seed MC and imbibition temperature on germination

Seed batches of different MC, which were obtained during the drying of a freshly collected seed lot, were allowed to imbibe for 1 h at a range of temperatures from 15–40°C, followed by incubation at 30°C. The percentages of germinated seeds are presented in Fig. 1. There were no significant differences in percentage germination for seeds having MCs in the range 27–6%. But at 5% MC, the germination percentage declined significantly when imbibition occurred at 15–20°C, indicating that the seeds had

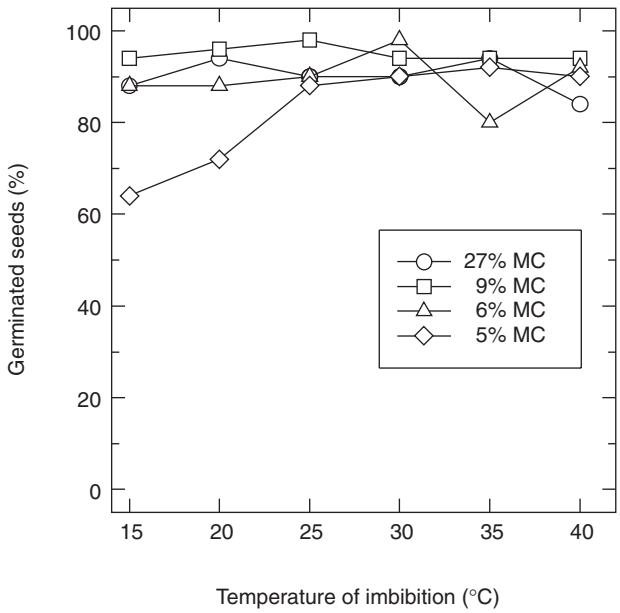

Figure 1. Effect of moisture content (MC) and imbibition temperature on neem seed germination (n = 50). After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C. Fresh seeds (27% MC) were dried at 20°C above saturated salts; the batches of 9% and 5% MC were taken during drying above $MgCl_2$; that of 6% MC during drying above LiCl. Germination data are significantly different [$P < (\chi^2_2 = 5.99)$ 0.05] when they diverge by at least 18% (Fisher's exact probability test).

become sensitive to low imbibition temperatures. These results confirmed that dried neem seeds are sensitive to imbibitional stress, and demonstrate that high viability (>80%) can be retained upon drying to low MCs (5%) as long as the seeds are properly rehydrated, i.e. at 30°C and above. The range of relatively high imbibition temperatures shown in Fig. 1 was chosen because injury can occur even at an imbibition temperature of 20°C in some neem seed lots (Sacandé *et al*., 1998).

Effects of seed age and imbibition temperature on germination

The effect of seed age on the response to low imbibition temperatures was investigated using initially high-quality seed lots. Seeds were examined either after 10 weeks or after 10 months at 7% MC, and after an additional week of after-drying to 4% MC. The 10-month- and 10-week-old seeds are referred to as aged and non-aged seeds, respectively. The seed lots were allowed to imbibe at temperatures ranging from 15 to 40°C (Fig. 2). The non-aged, 7% MC seeds were entirely insensitive to the imbibition temperatures tested, but further drying to 4% MC rendered them sensitive to the low temperature range 15–25°C [Fig. 2a, see also Fig. 1; significance at $P < (\chi^2_2 = 5.99)$ 0.05, Fisher's exact probability test]. Ageing resulted in a decrease in germination percentage of the 7% MC seeds (Fig. 2b), which was significant over the temperature range 15–25°C when compared with the non-aged 7% MC seeds (at $P <$ 0.05, same test). Interestingly, the imbibitional damage of the non-aged, 4% MC seeds was observed over the same temperature range (15–25°C; Fig. 2a). This means that ageing induced a similar sensitivity to imbibition at low temperatures as did drying of the non-aged seeds to 4% MC. When the aged seeds were further dried to 4% MC, germination percentages also decreased further. These percentages were significantly lower than those of the non-aged, 7% and 4% MC seeds at all temperatures tested, except at 40°C ($P < 0.05$, same test). This dramatic increase of the effect of imbibition temperature on germination is caused by both factors – ageing and drying.

To verify whether post-storage drying of the aged seeds from 7% to 4% MC caused a higher sensitivity to low temperature imbibition than similar drying of the non-aged seeds, the extent of reduction of germination was compared. Drying only caused a significantly larger decrease in germination in the aged seeds at 15°C [$P < (\chi^2_2 = 6.50)$ 0.039] and 20°C [$P < (\chi^2_2 = 15.88)$ 0.0004]. In the range 25–40°C, drying caused similar decreases in germination in both the aged and non-aged seeds. This means that ageing amplifies the effect of drying on the sensitivity to imbibitional stress at the low temperatures, whereas at temperatures of 25°C and higher, the effects of ageing and drying are just additive. The maximum effect of warm rehydration was reached at 30°C in the non-aged, 4% MC seeds and at 40°C in the aged, 4% MC seeds.

The aged seeds exhibited a slower start of radicle protrusion, as compared to the non-aged ones (Fig. 3). None of the aged, 4% MC seeds exhibited radicle protrusion after 2 d of incubation, whereas in all the non-aged seed lots, some germinated individuals were noticed. The slow germination could be a sign of reduced vigour associated with ageing. There was no clear influence of the imbibition temperature on the rate of germination for either the aged or non-aged seeds.

Effects of imbibition temperature and seed age on membrane barrier properties

Figure 4 shows two contrasting ESR spectra of PDT. One is from the axis of a viable seed (non-aged; imbibition temperature 30°C) and one from the axis of a non-viable seed (aged, imbibition temperature 0°C). Both spectra are the result of the superposition of two triplets. One triplet originates from PDT in aqueous cytoplasm surrounded by an intact membrane, the

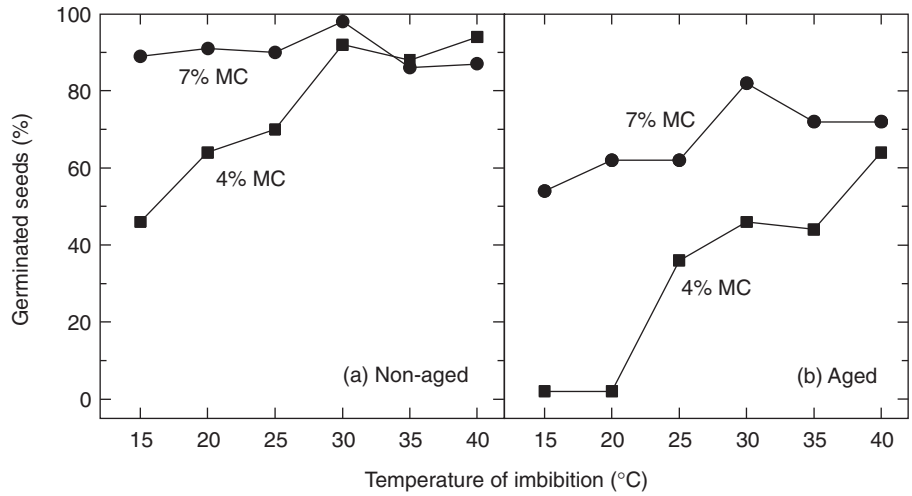

Figure 2. Effect of seed age and imbibition temperature on neem seed germination (n = 50) at two initial moisture contents (MCs) of the seeds (approximately 4% and 7%). After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C. High-quality (initial germination >90%) seed lots were equilibrated for 10 weeks (non-aged) above a saturated $Ca(NO_3)_2$ solution (MC = 7%); some were then stored for another 7.5 months at 15°C in a closed plastic bag (10 months old = aged). The low MC (4%) was obtained by after-drying above saturated LiCl for a week. Germination data are significantly different ($P < 0.05$) when they diverge by at least 18% (Fisher's exact probability test).

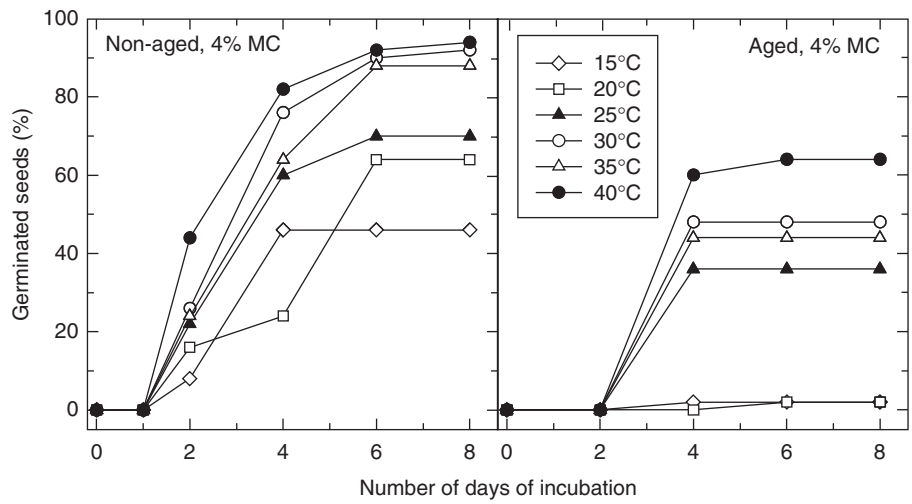

Figure 3. Effect of seed age and imbibition temperature on the rate of germination of the neem seeds [moisture content (MC) = 4%] from Fig. 2. After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C. Germination data are significantly different ($P < 0.05$) when they diverge by at least 18% (Fisher's exact probability test).

other from PDT in oil bodies. The lines of the two triplets are resolved at the right-hand side of the spectra (high-field lines). The amplitudes of the lines designated as W (water) and L (lipid) reflect the amount of spin-probe molecules in the cytoplasm and in lipid bodies, respectively. The ratio W/L, therefore, reports on the cytoplasmic volume of intact cells normalized to the amount of material (lipids) in the sample. On the basis of W/L values, the effects of imbibition temperature and seed age on the average integrity of cellular membranes in an embryo axis were estimated. Figure 5 shows that the W/L values increased with increasing soaking temperature, for both the aged and the non-aged 4% MC seed lots. At each temperature tested, the W/L value for the aged seeds was significantly lower than that for the non-aged seeds. This means that the proportion of intact cells increased with increasing temperature of imbibition, and was higher in the non-aged seeds than in the aged seeds. Fresh neem axes (27% MC)

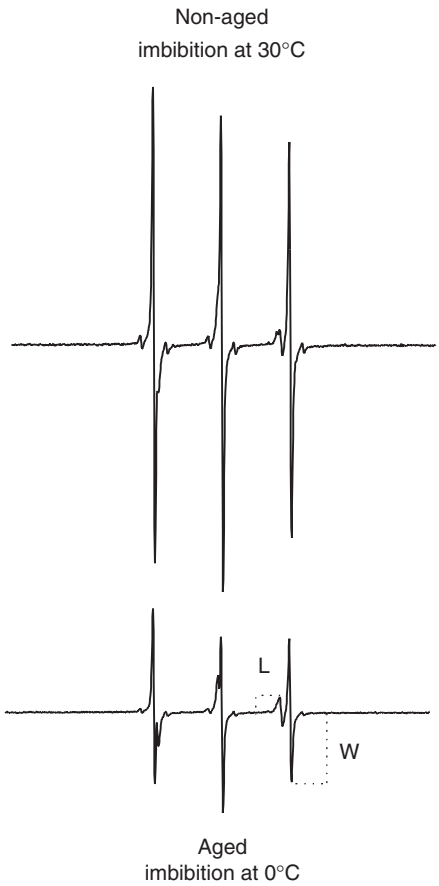

Figure 4. Representative electron spin resonance (ESR) spectra of perdeuterated TEMPONE (PDT) in axes of neem seeds after 1 d of rehydration. Imbibition was for 1 h at 30°C or 0°C, after which the seeds were further incubated at 30°C. Top spectrum: an axis from a non-aged seed (imbibition temperature 30°C, germination > 90%); bottom spectrum: an axis from an aged seed (imbibition temperature 0°C, germination < 2%). The lipid (L) and aqueous (W) cytoplasmic components are indicated in the high-field region (right-hand side) of the spectrum.

had an average W/L value of 16.1, which is similar to the W/L values obtained after imbibition of the non-aged seeds at 30 or 40°C (Fig. 5).

By plotting the W/L values obtained for the axes versus the percentage germinated seeds (4% MC), straight lines could be drawn (Fig. 6), from which the average proportion of live cells at zero percent germination was approximated. With an estimated W/L value of 9 for these dead axes and 16 for highly viable ones, it can be perceived that in non-germinating, imbibitionally injured axes, more than half of all the cells have intact plasma membranes and, thus, may be alive. The two 4% MC seed lots in Fig. 6 differed in that the slope of the plot of the non-aged lot was steeper than that of the aged lot. This

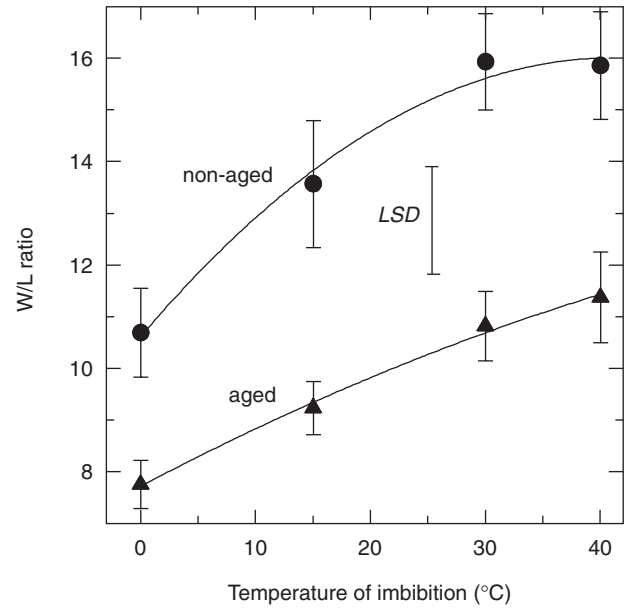

Figure 5. Effect of imbibition temperature on the water/lipid (W/L) ratio, as calculated from electron spin resonance (ESR) spectra of perdeuterated TEMPONE (PDT), in axes of neem seeds [moisture content (MC) = 4%] from the non-aged and the aged seed lots from Fig. 2. After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C; analyses after 1 d. Data (± SE) are means of 8–15 measurements on individual axes. Least significant difference (LSD) at ($P < 0.05$) is indicated.

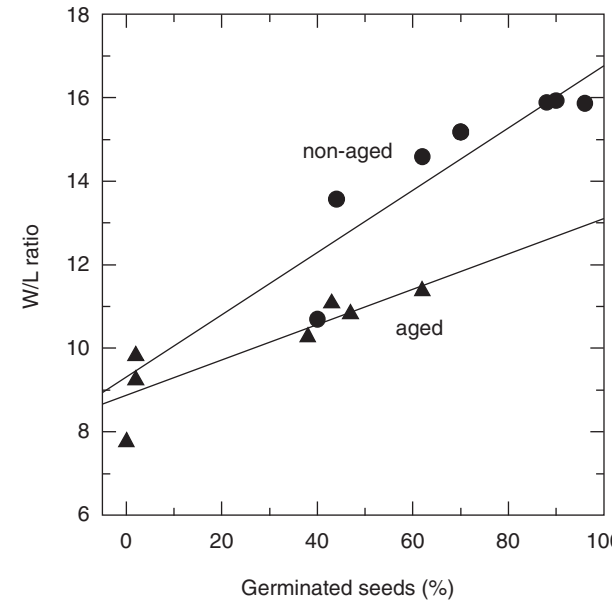

Figure 6. Plot of water/lipid (W/L) ratios in axes of non-aged and aged neem seeds (derived from Fig. 5) versus percentage germination. The different viabilities were obtained as a result of different imbibition temperatures (1 h, followed by incubation at 30°C).

may be explained by the faster germination of the non-aged seeds (cf. Fig. 3). Because axes in these vigorous seeds showed some signs of swelling after 1 d of incubation in water (data not shown), W/L values may be higher than expected on account of the proportion of viable cells, because of cell extension. This could mean that the average proportion of dead cells in imbibitionally injured embryo axes is less than estimated above.

W/L values of single axes from one treatment give insight in the distribution of this parameter, allowing the threshold W/L value to be determined, below which the proportion of live cells is insufficient to support germination and above which there are enough live cells for germination (Golovina *et al*., 1997). Figure 7 shows such distributions for the 4% MC seed lots. For the aged seeds that had imbibed at 0°C, all axes had W/L ratios below 11; whereas for the non-aged seeds that had imbibed at 30°C, all axes had W/L values above 11. Because germination percentages for the aged and non-aged seeds were 0% and 92%, respectively, it follows that a W/L of approximately 11 is the threshold value. Although the relatively low number of axes examined by ESR (10–15) is insufficient to support a firm conclusion about the critical W/L value, it is striking that the distributions of this parameter for the individual axes in the other two treatments of Fig. 7 correspond to the germination percentages obtained. Although ESR data can report on the proportion of dead cells in an axis, ultrastructural analysis is required for the precise localization of these cells.

Effects of imbibition temperature and seed age on ultrastructure

An example of the general morphological structure of a neem seed is presented in Fig. 8a, showing a relatively small embryo axis and two large, oily cotyledons, surrounded by a thin endosperm layer. Figure 8b shows an edited image of Fig. 8a, highlighting the general anatomy (white lines) and areas of dead cells (black areas) after severe imbibitional stress. Cryo-planing was used to analyse the effects of age (10 weeks and 10 months) and imbibition temperature (0°C and 30°C) on the cellular ultrastructure in seeds from the 4% MC lots, 23 h after hydration had commenced.

A perfectly intact ultrastructure of apparently live cells is shown for an endosperm and axis in Fig. 9a

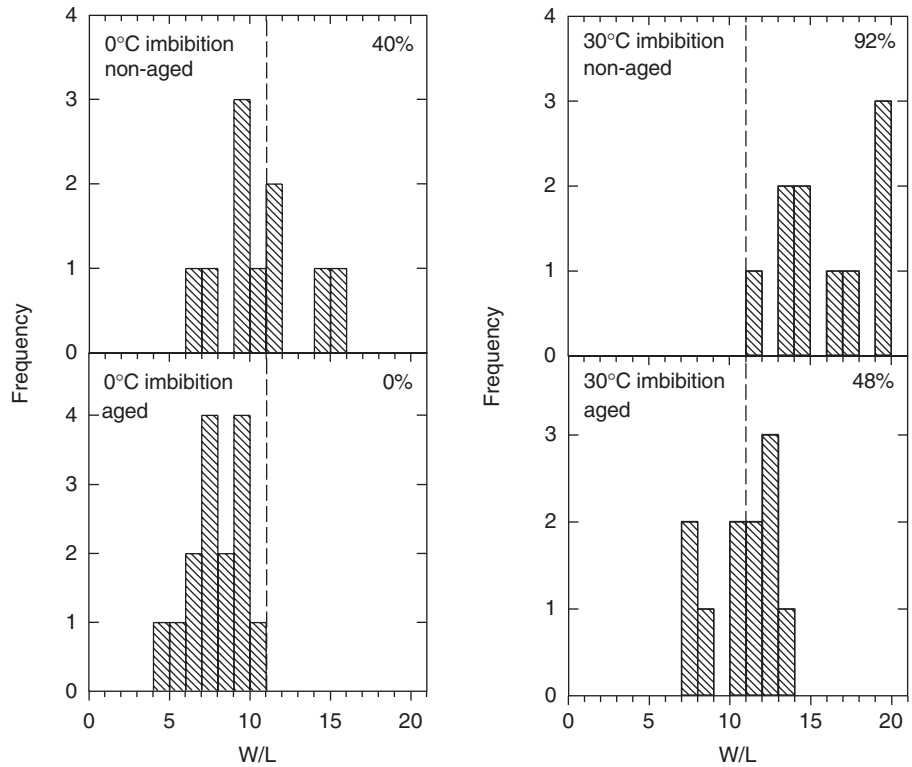

Figure 7. Distribution of the water/lipid (W/L) ratio in individual axes (10–15) of neem seeds from non-aged and aged seed lots [moisture content (MC) = 4%]. Imbibition temperatures were 0°C and 30°C (1 h). Percentage germination is indicated in the figures.

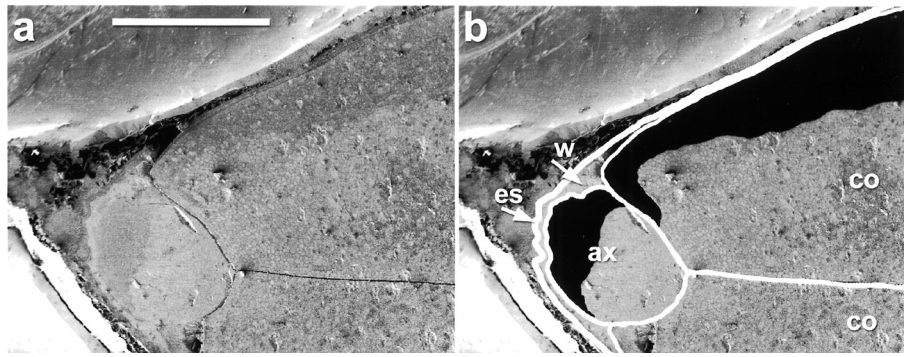

Figure 8. Micropylar end of a cryo-planed, aged (10-month-old) neem seed [moisture content (MC) = 4%], soaked in water at 0°C for 1 h, followed by incubation on moist filter paper at 30°C for 22 h. (a) Non-edited image; (b) edited image, highlighting the general anatomy (white lines) and areas of dead cells (black areas). The embryo consists of two large, oily cotyledons (co) and a small axis (ax). A very thin endosperm (es) surrounds the embryo, leaving spaces of free water (w) in between. Scale bar represents 1 mm; the total length of the seed is approximately 12 mm.

and for a cotyledon and axis in Fig. 9b. These intact cells are characterized by general turgidity and clearly recognizable organellar structures, such as nuclei and small vacuoles. An abundance of small lipid bodies occurred at the periphery of the cotyledon cells, but axis cells had few such lipid bodies (Fig. 9b), which is in accordance with the earlier reported neutral lipid contents of 51% and 14%, respectively (Sacandé *et al.*, 2000a). Almost all cells in the non-aged seeds (MC = 4%) that were allowed to imbibe in water at 30°C had this perfect ultrastructure. The germination percentage of these seeds was generally >90%.

Figure 9c shows an example of mostly intact axis cells covered with a layer of damaged endosperm cells that are characterized by loss of turgor and lack of distinguishable organelles. Figure 9c further shows a few damaged, peripheral axis cells (see also inset). This micrograph originates from a non-aged seed after imbibition at 0°C (approximate seed-lot germination of 40%). Axes of replicate seeds that had received the same treatment showed a larger number of damaged cells at the periphery, particularly at the radicle tip (image not shown). However, this involved only a few percent of all cells in the cross-section. Also, cotyledon cells (up to 10 cell layers) at the periphery of the non-aged seeds suffered injury after the 0°C imbibition (Fig. 9d). The damaged cells are characterized by a dense, amorphous structure and heavily folded cell walls, probably the result of the pressure evoked by swelling cells localized deeper in the cotyledon. Figure 9d shows the boundary between dead and healthy cells, where live, turgid cells with aggregated lipid bodies (up to 15 µm) were noticed. Injury was never found in the interior of the seed tissues.

For the aged seeds imbibed at 30°C (48% germination of the seed lot), injured cells were observed in all three replicates. In two replicates, all endosperm cells were dead, and this coincided with patches of 3–15 peripheral layers of damaged cells in the axis. In the third replicate, patches of dead endosperm cells were noticed (micrograph not shown), coinciding with an ultrastructurally entirely intact axis. The area occupied by damaged cells did not exceed 10% of the total surface area occupied by axis cells. In contrast to the cotyledon cells of the non-aged seeds, those of all aged seeds had lipid body aggregates (up to 20 µm), although cells with non-coalesced lipid bodies were also observed (Fig. 9e). Some irregularities inside these giant aggregates are suggestive of being the remnants of the monolayer membrane from the original lipid bodies. Cells with the giant lipid body aggregates were turgid and had discernible organelles. The fusion of the lipid bodies in the aged seeds was associated with rehydration, because in freeze-fracture images of dried specimens before imbibition, only small non-coalesced lipid bodies were noticed (micrograph not shown). Some cotyledon cells at the periphery were injured, but the area occupied by these cells did not exceed 5–10% of the total cryo-planed surface.

The 0°C-imbibition of the aged seeds (0% germination) caused considerable cellular damage. All endosperm cells appeared dead, while approximately 35% of the axis cells in the cross-section were injured. All injured cells were located at the periphery (Figs. 8b and 9f). Large peripheral areas of the cotyledons had cells with damaged ultrastructure (up to 15 cell layers; Fig. 8b). It was estimated that 15–20% of the total number of cotyledon cells in the cross-section were injured during cold imbibition. As in other treatments, injured cells were never noticed in the interior of the seed tissues, and they were always found in clusters.

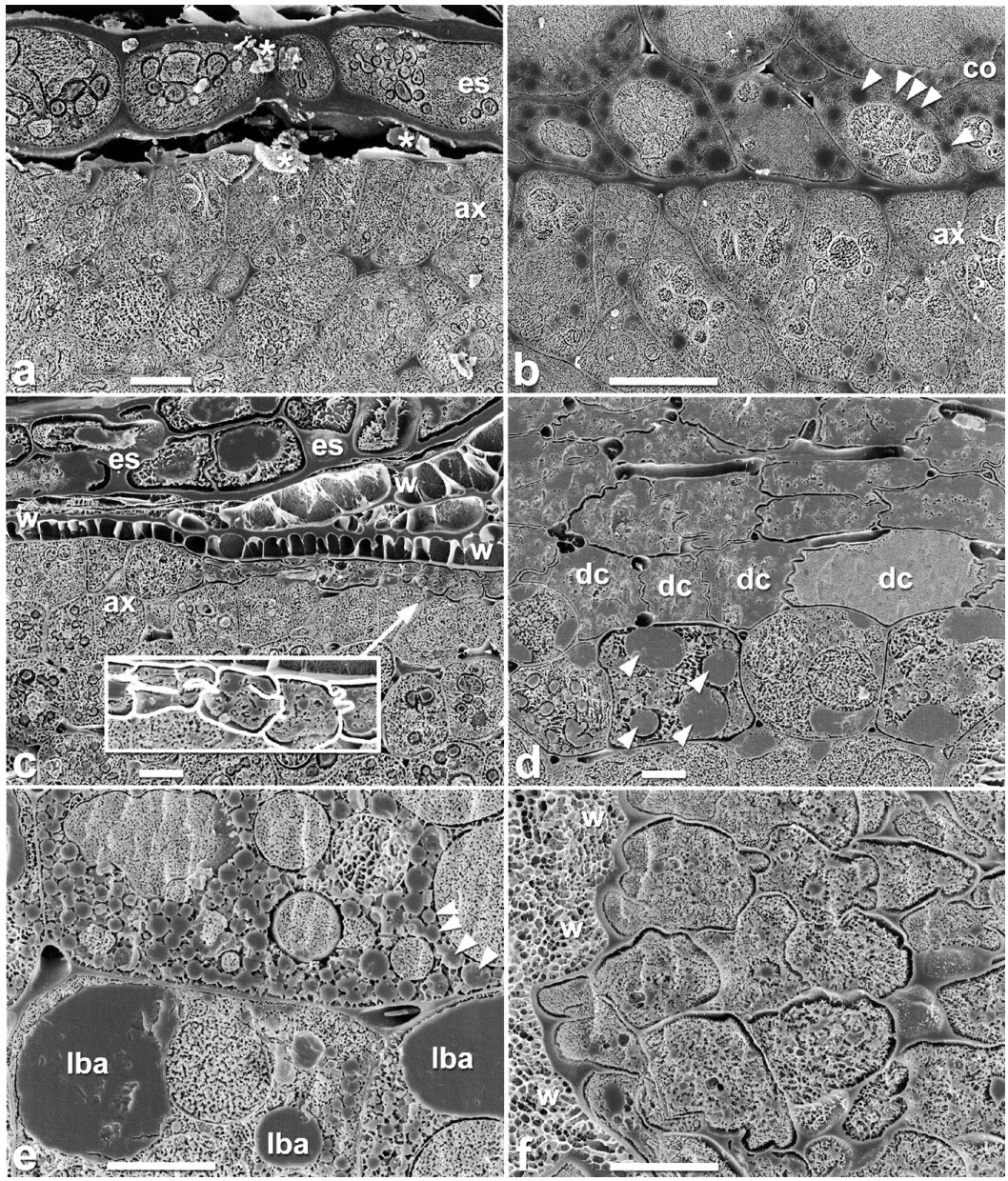

Figure 9. Cryo-SEM micrographs of a cryo-planed neem seed [moisture content (MC) = 4%] soaked in water at either 0°C or 30°C for 1 h followed by incubation on moist filter paper at 30°C for 22 h. (a) Non-aged, 30°C-imbibed seed, showing axis (ax) and endosperm (es) cells. Cells of both tissue types are turgid and compartmentalized, which is a sign of being alive. The debris (e.g. asterisks) originates from the cryo-planing procedure. (b) From the same seed as (a), showing the boundary between axis (ax) and cotyledon (co). All cells are alive. Cotyledon cells contain a high amount of lipid bodies (arrowheads). (c) Non-aged, 0°C-imbibed seed, showing axis (ax) and endosperm (es) with a water-containing space (w) in between. All endosperm cells are dead, which is apparent from the irregular and clumped cell contents. The axis cells are alive, except a part of the epidermis, which is magnified in the inset (cell walls highlighted). The dead epidermal cells lack turgor and have heavily folded walls. (d) Same treatment as in (c), showing cotyledon cells at the boundary of damaged and intact cells, located at 10 layers from the periphery; dead cells (dc) lack turgor, have dense, amorphous contents and have curved walls. Typically, in the neighbouring live cells, lipid body aggregates (arrowheads) can be noticed. (e) Cotyledon cells of aged, 30°C-imbibed seeds. The cell at the top of the micrograph has numerous small lipid bodies (arrowheads), similar to the cells of non-aged seeds. The cells at the bottom have very large lipid body aggregates (lba). (f) Detail of the peripheral dead axis tissue of the aged 0°C-imbibed seed, also shown in Fig. 8. Cell walls and axis surface are folded, and no cellular compartments are discernible; w, water between endosperm (not shown) and axis. All scale bars represent 10 µm.

Discussion

Dried neem seeds have been reported to be sensitive to imbibitional stress (Sacandé *et al*., 1998, 2000a), and the data in Fig. 1 confirm this. When freshly collected seeds are dried to a low water content of about 5%, their germination percentage is still high if imbibition takes place at 30–40°C, but is reduced upon imbibition temperatures ⩽ 20°C. If rehydration is to be considered an essential part of desiccation tolerance (Hoekstra *et al*., 2001), then the problems with neem seeds, as reported in the literature, point to limited desiccation tolerance. Nevertheless, the integrity of cells in seeds that are dried to low MC is maintained as long as these seeds are in the dry state. Aged seeds displayed a considerably reduced germination percentage (Fig. 2). This reduction could partially be reversed by imbibition at the high temperature of 40°C, higher temperatures being ineffective in this respect, even when applied during periods shorter than the 1 h used in this work (unpublished results).

A number of reports link seed ageing with increased sensitivity to imbibitional stress (Priestley, 1986; Zeng *et al*., 1998). Typically, this could also be concluded for neem seeds, on the basis of the data in Fig. 2 (7% MC, non-aged versus aged). While the non-aged 7% MC seeds were not sensitive to differences in imbibition temperature (15–40°C), the aged 7% MC seeds performed worse at 15–25°C. Furthermore, the effect of after-drying to 4% MC was greater in the aged seeds than in the non-aged seeds (particularly the 15–20°C range). To study a possible involvement of ageing in the sensitivity to imbibitional stress in more detail, we resorted to methods that can give an estimate of the proportion and location of dead cells.

Both ageing and imbibitional damage have been linked to the loss of plasma membrane barrier properties (Golovina *et al*., 1997; Hoekstra *et al*., 1999). If not a primary damage, reduced integrity at least will be evident 1 d after the onset of imbibition, when the analyses were performed. The spin-label technique used allows for the calculation of the normalized aqueous volume surrounded by intact plasma membranes (W/L), which is indicative of the viability of the cells in an axis. This method is particularly suitable for small samples and is not sensitive to problems usually encountered with electrolyte leakage measurements (reviewed in Hoekstra *et al*., 1999). Both ageing and low temperatures of imbibition led to a reduction in the proportion of viable cells (Fig. 5). For the calculation of the proportion of dead cells in an axis, the average W/L value of highly viable axes has to be known, which was approximately 16 (Fig. 6). However, the oil content and the extent of cell extension at the sampling time should be the same for the different lots and treatments. Here some problems arose, because the non-aged axes showed signs of swelling, whereas the aged axes did not. The early swelling of the non-aged seeds is corroborated by their earlier radicle protrusion (Fig. 3). Calculations on the basis of a W/L value of 16 for highly viable seeds would probably lead to an overestimate of the proportion of dead cells in the stressed seeds. An extrapolated W/L value of 13 for the hypothetical 100% germinated seeds from the aged lot (Fig. 6) might be more realistic, assuming moderate cell extension at the sampling time. Calculated in this way, the ESR method indicates that germination is not supported when approximately 30% of axis cells have died. This also means that there are still, on average, 70% of cells alive in a non-germinating seed. From the distribution of W/L values in the axes of non-germinating seeds (Fig. 7; Golovina *et al*., 1997), it is clear that there is a large distribution in the number of dead cells, and that cellular ageing further proceeds post-mortem. Also, a value of approximately 35% of dead cells arose from cryo-SEM images of cross-sections of aged axes after 0°C imbibition.

Although electron microscopical analysis cannot quantitatively support the germination and ESR data, it qualitatively contributed to answering the question of whether ageing increases the sensitivity to imbibition. At decreasing imbibition temperatures, the number of affected cell layers at the periphery of axes and cotyledons (and of the endosperm) increased, but cells in the interior of the tissues were never damaged (Figs 8 and 9). Affected cells are characterized by loss of turgor and lack of distinguishable organelles. With ageing, the proportion of affected cells further increased, again always at the periphery and never in the interior of the tissues. The injury type was similar to that in imbibitionally stressed, non-aged cells. Dead layers apparently slow further water influx so that deeper cell layers remain intact upon rehydration. Because damaged cells in aged neem seeds were only encountered at the periphery of the seed tissues and not randomly distributed, it is concluded that ageing increases the sensitivity to imbibitional stress. On a microscopic level, seed ageing is generally observed as patches of dead cells, also in the interior of tissues (ISTA, 1993). This appears to be in contrast to the pattern of ageing in neem seeds. Whether the increased sensitivity of neem seeds to imbibitional stress stems from changed physical properties of the plasma membrane, or from changed properties of the cell walls that allow for an increased water influx, has to be determined.

Ageing also had a specific effect on lipid bodies. Upon rehydration, the small lipid bodies still present in the dried cotyledons fused into giant droplets of up to 20 μm. Cells with these droplets, nevertheless,

showed clearly discernible organelles and were turgid, suggesting that they were still alive. It is suggested that the giant lipid droplets may be involved in the delay of germination of the aged seeds. Lipid-body coalescence has been observed repeatedly in aged seeds (reviewed in Priestley, 1986). In this respect, the occurrence of giant lipid aggregates in the boundary layer between imbibitionally damaged and healthy cotyledon cells (non-aged; Fig. 9d) is indicative of a mild stress that is widespread in cotyledons of aged seeds, even after high temperatures of imbibition.

This work has shown that neem seeds are prone to rapid ageing, apart from their sensitivity to imbibitional stress. Both factors are likely to have led to their reputation as being difficult to store dry. In addition, the seeds are chilling sensitive at moisture contents above 10% (Sacandé *et al*., 1998). For the safe storage of neem seed, we suggest that ageing and the associated increased sensitivity to imbibitional stress should be kept at a minimum, for example by storing seeds at –20°C as soon as the MC during drying has reached values that preclude chilling injury. However, over-drying should be avoided. The maintenance of high neem seed viability after storage at subzero temperatures for more than a decade (Tompsett and Kemp, 1996) and the data from Sacandé *et al*. (1998) on storage at –20°C corroborate this view.

Acknowledgements

The financial contributions of the Dutch Organization for the Advancement of Tropical Research (WOTRO) (O.N.), the Netherlands Science Foundation (NWO) (E.A.G.) and the Netherlands Technology Foundation (STW) (J.N.) are gratefully acknowledged. We thank Sheila Adimargono for discussions and advice on the use of statistical methods.

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Received 29 August 2003
accepted after revision 12 February 2004