Is oxidative stress involved in the loss of neem (*Azadirachta indica*) seed viability?

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Abstract

Neem (*Azadirachta indica*) is a valuable multipurpose tree of tropical arid and semi-arid regions. The use of its seeds is hindered by their short storage longevity. The possible causes of rapid loss of viability were investigated on different seed lots during exposure to 32% and 75% RH at 20°C. Within 6 months the seeds almost lost germinability at 75% RH, whereas at 32% RH viability decreased only slightly. On rehydration, the axis cells from nongerminable seeds had lost turgor, whereas those from viable seeds were turgescent as visualized by low temperature scanning electron microscopy images of fractured axes. Glutathione oxidation status was used to estimate oxidative stress during storage. Oxidative stress was much higher at 75% RH storage than at 32% RH, mainly caused by the rapid loss of reduced glutathione at 75% RH. Oligosaccharides and phospholipids decreased, and free fatty acids increased during storage at the high RH but remained at a constant level at the low RH. However, the degree of fatty acid unsaturation between viable and nonviable seed lots was similar. During the (slow) dehydration of fresh seeds, total glutathione, oligosaccharides and phospholipids accumulated, particularly in the initially more hydrated seeds. We interpret this accumulation as a post-maturation process associated with acquisition of the capability for long-term survival in the dry state. The mass ratio of oligosaccharides to sucrose was 0.19 on average in dehydrated neem seeds. The data suggest that the storage behaviour of neem seeds has features that characterize it as orthodox.

Keywords: *Azadirachta indica*, glutathione, oxidative stress, phospholipids, seed viability, storage, sugars

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Introduction

The seeds of most plant species are shed in a partly dehydrated state and can survive further drying to low moisture contents (MCs). Such dry seeds are usually able to survive for long periods of time. This type of storage behaviour has been designated as orthodox (Roberts, 1973). In contrast, seeds that are readily damaged by drying and, in addition, often sensitive to low temperatures have been denoted as recalcitrant (Roberts, 1973; Roberts and Ellis, 1989). Seeds that have a lethal limit of dehydration falling between these two categories are referred to as intermediate and can generally be stored for intermediate periods (Ellis *et al.*, 1990, 1991, and references therein). Intermediate seeds survive drying to moderately low MCs and are often injured by low temperatures. Because of the sensitivity to desiccation and/or low temperatures, recalcitrant and intermediate seeds have a relatively short life span.

Neem (*Azadirachta indica* A. Juss.) is an important multipurpose tropical tree species, but use of its seeds for propagation purposes is hampered by its difficult storage behaviour. Neem seed storage has variously been described as recalcitrant, intermediate, and orthodox. Germinability of seeds is reduced during

drying, and seed longevity does not exceed a few years under optimal conditions (reviewed by Hong *et al.*, 1996; Poulsen, 1996; Hong and Ellis, 1998).

In desiccation-sensitive seeds, drying increases the production of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide (Leprince *et al.*, 1990, 1994). Production of ROS has been demonstrated for some recalcitrant temperate tree seeds (Finch-Savage *et al.*, 1994) and the intermediate papaya seeds (Magill *et al.*, 1993). ROS formed during drying have been associated with damage to cellular constituents such as proteins, DNA, and membranes, which may ultimately result in cell death (Benson, 1990). Oxidative stress has also been shown to take place during storage of seeds (Hendry *et al.*, 1992) and to be involved in the viability loss of entire plants under environmental stress from chilling, freezing, or exposure to environmental pollution (Bowler *et al.*, 1992). Seeds have several antioxidant systems that may help to prevent oxidative damage. These systems include the enzymes superoxide dismutase, ascorbate peroxidase and glutathione reductase and several non-enzymatic antioxidant compounds like reduced glutathione (GSH), tocopherol and ascorbic acid (Hendry *et al.*, 1992). However, under stress conditions, oxidation may still occur if the cellular antioxidant systems fail to control oxidative injury (Benson, 1990; De Vos *et al.*, 1994). This may be the cause (or effect) of deterioration in seeds (Wilson and McDonald, 1986).

Non-protein compounds with an active sulfhydryl (SH-) group like GSH can protect SH-groups in proteins from harmful oxidation, forming intra-molecular disulfide bridges (S–S) (Navari-Izzo *et al.*, 1997). During dry storage of plant systems, GSH can be oxidized as a result of ROS formation, forming the glutathione disulfide GSSG that can be converted back to GSH after rehydration (Benson, 1990; De Vos *et al.*, 1994; Kranner and Grill, 1996). Thus, the increase in the ratio of GSSG to total glutathione (GSH + GSSG), i.e. the glutathione redox status, reflects oxidative stress in seeds. Other indices of oxidative damage during drying and storage are an increase in ion leakage resulting from membrane breakdown and changes in the content and composition of membrane lipids (Wilson and McDonald, 1986; Benson, 1990). Decreases in phospholipid content and in the level of unsaturation of the esterified acyl chains, and increases in free fatty acid content are associated with oxidative stress (Van Bilsen and Hoekstra, 1993; Van Bilsen *et al.*, 1994).

Desiccation and storage survival may also depend on the ability of seeds to produce sugars that stabilize biomembranes (Crowe *et al.*, 1992) and proteins (Carpenter and Crowe, 1989). The sugars have been hypothesized to replace the lost water by hydrogen bonding to the membrane lipid polar headgroups and the proteins, thus keeping the native structure intact (Crowe *et al.*, 1992; Wolkers *et al.*, 1998). Orthodox seeds generally have an elevated proportion of oligosaccharides to the total amount of soluble sugars, whereas recalcitrant seeds usually lack these oligosaccharides or have low mass ratios of oligosaccharides to total soluble sugars (Steadman *et al.*, 1996). Slow drying of seeds can increase the level of these oligosaccharides and improve desiccation tolerance and storage longevity (Sanhewe and Ellis, 1996a,b; Sinniah *et al.*, 1998; Wolkers *et al.*, 1999). The content and concentration of protective sugars in seeds may change during storage due to metabolic activity depending on seed MC and storage temperature.

In the present paper we investigated whether the ageing pattern and compositional characteristics of neem seeds from Burkina Faso may be associated with a specific type of storage behaviour. For this purpose the changes in viability and ultrastructure were analyzed during drying and storage at two different MCs. The glutathione oxidation status as a measure of oxidative stress was determined, and the compositional changes in phospholipids and sugars were measured. The data are discussed in relation to the reputedly intermediate storage behaviour of neem seeds.

Materials and methods

Plant material

Neem seeds were harvested at Ouagadougou (Ouaga) and Bobo Dioulasso (Bobo)–two different locations in Burkina Faso. Yellow fruits were picked by hand from more than 20 selected adult trees from 30-year-old plantations. Seed preparation was carried out on the day of harvest. Fruits were soaked in water and then rubbed with sand. After depulping, the seeds were cleaned and dried in the shade on a grid for 2 days. Seeds surrounded by an intact endocarp were then collected and sent to Wageningen, the Netherlands, in cotton packets, arriving within 7 days after seed preparation.

Seeds were sampled and exposed to two relative humidities (RH) obtained above saturated salt solutions with circulating air at 20°C ($CaCl_2.6H_2O$ for 32% RH, and NaCl for 75% RH). After storage at these RHs for the appropriate periods, sub-samples from each lot were germinated or stored at –80°C, until use.

Moisture content and germination

Three replicates of five embryos (seeds with pericarp removed) were weighed, dried at 103°C for 17 h and then weighed again for the determination of MCs, expressed as a percentage of the fresh weight (FW) (ISTA, 1993).

The viability of seeds was defined as the germination capacity. Two replicates of 50 seeds were soaked for 4 h at 30°C (50 seeds in 100 ml of water; no supplementary aeration) and then placed on wet filter paper in germination boxes that were placed in the light for 8 h/d at 30°C and in the dark for 16 h/d at 20°C. The seeds were scored twice a week until all viable seeds had germinated. A seed was scored as germinated when a radicle of 30 mm had emerged (ISTA, 1993).

Analysis of non-protein thiols and disulfides

Preparation of seed material and extracts

Seeds that were initially stored at –80°C were lyophilized for 72 h and then kept in a desiccator under vacuum at room temperature until analysis. This lyophilization was necessary to avoid SH-group oxidation during the extraction of non-protein (i.e. acid-soluble) thiols (cf. De Vos *et al*., 1992). All extraction steps were performed at 0–4°C. We used a modification of the method of De Vos *et al*. (1994) for determining non-protein thiols and disulfides. Briefly, about 0.5 g of lyophilized embryos was homogenized in triplicate in 20 ml of an ice-cold solution of 5 mM diethylenetriaminepentaacetic acid (DTPA) in 5% w/v sulfosalicylic acid (SSA), using an UltraTurrax homogenizer operating at maximum speed (3 min). The extracts were placed on ice for at least 10 min to allow proteins to precipitate. The extracts were then centrifuged twice in an Eppendorf centrifuge at maximum speed for 3 min (4°C) to remove the precipitated protein, and the supernatants were put on ice for subsequent analyses.

Assay of acid soluble SH-groups

Total non-protein thiols in the extracts were determined with 6 mM 5,5′-dithiobisnitrobenzoic acid (DTNB, Ellman's reagent). After mixing 300 µl of each supernatant with 630 µl of 0.5 M K_2HPO_4 (giving a pH of approximately 7.0) and 25 µl of the DTNB solution, the absorbance was read after 1 min at 412 nm (absorption coefficient = 13,600) (Ellman, 1959). The absorbance was corrected for DTNB using the extraction buffer (SSA/DTPA) instead of supernatant. Duplicate assays were performed for each extract. The content of total non-protein SH-groups (including GSH) was calculated on the basis of embryo dry weight (DW).

Assay of GSH and GSSG

Both total glutathione (GSH + GSSG) and GSSG were assayed by an enzymatic cycling method with NADPH and GSSG-reductase (EC 1.6.4.2.; Sigma type III from baker's yeast) at 35°C. The reactions involved in this cycling method have been outlined in detail by Anderson (1985). Total glutathione (GSH + GSSG) and GSSG contents were determined separately, and the GSH content was calculated by subtracting the amount of GSSG from the total glutathione level. The contents in the acid supernatants were calculated from standard curves prepared with a standard solution of 0–20 µM GSSG in SSA/DTPA.

For the determination of total glutathione, 100 µl of DTNB, 50 µl of supernatant, 100 µl of a 2.1 mM NADPH solution, and 750 µl of a 0.143 M phosphate buffer, containing 6.3 mM DTPA, pH 7.5, were sequentially pipetted into a 1-ml cuvette. Then, 25 µl of an appropriately diluted solution of GSSG-reductase in the buffer was added and rapidly mixed, and the increase in absorbance was followed at 412 nm. For the GSSG determination, the SH-groups (including GSH) in the embryo extracts were derivatized (for at least 3 h) with 2-vinylpyridine, immediately after the centrifugation steps, to exclude any further oxidation, as follows. Sequentially, 200 µl of supernatant was mixed with 4 µl of 2-vinylpyridine and 20 µl of 50% (w/w) triethanolamine (final pH 6–7) to remove the SH-groups from the extract. This derivatization step was used for both the GSSG standard curve and GSSG analyses of extracts in the same way as indicated for total glutathione.

Analysis of lipids

Five embryos of each lyophilized seed sample were cut into pieces and mixed well. For extraction and determination of lipids the methods described by Hoekstra *et al*. (1989) were utilized. In duplicate, 100 mg of the granulated sample was homogenized with a mortar and pestle and a little sand in 20 ml chloroform:methanol (2:1, v/v) containing internal standards for phospholipids and triglycerides (1.5 mg diheptadecanoylphosphatidylcholine and 30 mg triheptadecanoin, respectively). The homogenate was centrifuged and the supernatant washed with 0.2 volumes of 0.9% NaCl solution. After phase separation the chloroform layer was collected, dried by passage over anhydrous Na_2SO_4 and vacuum evaporated. The residue was resuspended in 1 ml chloroform and separated into a neutral and a polar lipid fraction by passing the material over a SEP-PAK silica cartridge (Waters Associates, Milford, MA, USA, catalog no. 51900). The neutral lipids were eluted with 20 ml chloroform, followed by elution of the polar lipids with 30 ml methanol. Transmethylation of the lipid fractions was carried out with 0.3 M KOH in methanol for 15 min at 70°C with vigorous shaking. After cooling, the methylated fatty acids were phase separated to hexane and dried over anhydrous Na_2SO_4 before GC analysis. The amount of phospholipids and neutral lipids was determined by comparing the total peak surface of methyl esters with that of the heptadecanoic methyl ester (Hoekstra *et al*., 1989).

For the analysis of free fatty acids, the embryos (100 mg) were homogenized in the presence of 0.1 mg heptadecanoic acid as the internal standard. After following the procedures outlined above, the neutral lipid fraction containing the free fatty acids was separated on TLC plates using hexane: diethylether:acetic acid (80:20:1, v/v/v) as the developing solvents. After the plates were sprayed with 0.1% 8-anilino-1-naphthalene sulfonic acid in methanol and inspected with UV light, the free fatty acid band was scraped off and methylated using freshly prepared diazomethane in diethylether.

Fatty acid methylesters were analyzed on a Shimadzu GC8A GC, equipped with a 30 m J&W DB225 megabore column (J&W Scientific, Folsom, CA, USA), coupled to a Spectra Physics SP4100 integrator. Identification was done by comparison with standards and GC-MS analysis as described by Van Bilsen *et al.* (1994).

Carbohydrate analysis

In duplicate, approximately 100 mg of each of the same lyophilized samples was homogenized with a mortar and pestle in 10 ml 80% methanol containing 10 mg melezitose as the internal standard. The samples were kept at 76°C in a water bath for 15 min to extract soluble carbohydrates and to inactivate enzymes. Subsequently, the methanol was evaporated in a Speedvac (Savant Instruments Inc., Farmingdale, NY, USA). The samples were then suspended in 10 ml of milli-Q water. After centrifugation in an Eppendorf centrifuge for 3 min at maximum speed (15,000 rpm), the supernatants were diluted 50-fold with H_2O for HPLC analysis. Carbohydrates were separated with a Dionex HPLC system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector. For the separation of carbohydrates, a Carbopac PA-1 column with guard column was used, operated at 30°C. A 50–200 mM gradient of NaOH in water was used as the eluent, and 1.1 M sodium acetate in 100 mM NaOH was used to clean the column after each run. The data were analyzed using a Spectra Physics integrator model SP 4400 and Spectra Physics software (Labnet, Chromdat, San Jose, CA, USA). Identification of carbohydrate peaks was by comparison with retention times of standard solutions in two different elution programmes (the 50–200 mM NaOH gradient or with 100 mM NaOH).

Low temperature scanning electron microscopy (LTSEM)

After 4 h of soaking in water at 30°C (as for the germination assay), seeds were incubated on wet filter paper for 20 h at 30°C, and axes were subsequently isolated and mounted on stubs with conductive carbon cement (Leit-C, Neubauer Chemikalien, Münster, Germany). The stubs were placed on a specimen holder that was plunged into liquid nitrogen (–196°C). The stubs with the frozen axes were transferred to a cryo-transfer unit (CT 1500 HF, Oxford Instruments, Oxon, UK), consisting of a cryo-preparation chamber at high vacuum (10^{-6} Pa) attached to the LTSEM, and a cryo-stage inside the microscope. The axes were placed inside the cryo-chamber at –85°C, kept there for 2 min to sublimate the contaminating water vapor, and then cross-sectioned with a cold sharp knife. After sputter-coating with 3 nm platinum, the coated specimen was placed inside the LTSEM (JEOL, model 6300 F, Tokyo, Japan) and observed at 1–5 kV. The temperature of the specimens inside the LTSEM was kept at –180°C.

Results

Equilibrium moisture contents and germination capacity of seeds

Seeds from Ouagadougou and Bobo Dioulasso initially germinated at >90%. Under defined conditions of RH at 20°C, fresh seeds reach equilibrium MCs after 3–4 weeks of exposure (Sacandé *et al.*, 1998). For the RHs of 32% and 75% at 20°C, equilibrium MCs were 5.5% and 11.7% (FW basis), respectively. Seed germinability was followed over the 26 weeks of exposure to the two RHs (Fig. 1). Seeds from both sources behaved similarly. High germinability was maintained during the first 6 weeks of exposure to 75% RH, followed by a decrease to <20% at 26 weeks. At 32% RH, germinability dropped to approximately 70–80% after the first 3–6 weeks and then slowly decreased to 60–70% over the next 20 weeks of storage.

Non-protein thiols during dehydration and storage

As thiols are sensitive to oxidation, it is possible that they become oxidized during extraction, resulting in erroneous thiol–disulfide values (De Vos *et al.*, 1994). To determine whether such artefactual oxidation occurred, seeds were homogenized in SSA + DTPA in the presence and absence of an internal GSH standard and subsequently analyzed for both GSH and GSSG. A high recovery of the GSH added (96%) was obtained, which indicated that the extraction and analysis procedures were correct and that the amounts of GSH and GSSG measured corresponded to the amounts actually present in the seeds.

The non-protein thiol contents in the embryos comprise the amounts of GSH and a number of other compounds with SH groups (e.g. cysteine). The changes in content of these thiol compounds were followed during exposure of the seeds to 32% and

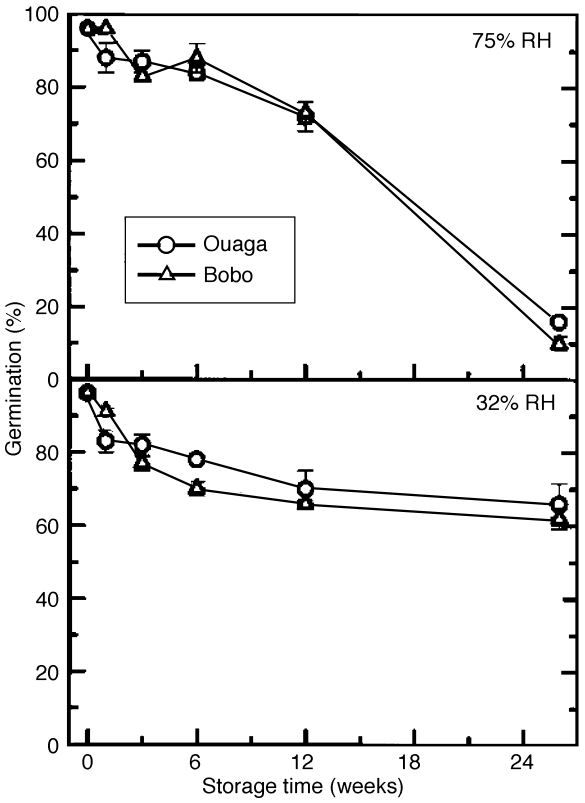

Figure 1. Germinability of neem seeds from Ouagadougou (Ouaga) and Bobo Dioulasso (Bobo), Burkina Faso, after 0–26 weeks of storage at RHs of 75% (11.7% MC, FW basis) and 32% (5.5% MC, FW basis), at 20°C. Each data point is the mean percentage of germinated seeds (2 ¥ 50 seeds). SD is represented when larger than the symbols. Data are significantly (*P*£0.05) different when they diverge by 15% or more (χ^2 test).

75% RH at 20°C (Fig. 2). Analyses showed significant initial differences in the content of total non-protein thiols between the seed sources. The embryos from the seed lot with the highest MC (from Bobo; 25.0% MC) had an initially lower amount of SH-containing compounds than those that were drier at the onset of the exposure to the RHs (from Ouaga; 14.8% MC). Other, more immature samples that were more hydrated at the onset of drying (41.3% MC) had even lower initial amounts of SH-compounds (data not shown). During drying to equilibrium MCs (first 3 weeks of storage), the initially low contents in the Bobo seeds rose to levels that were of the same order as those in the initially drier (Ouaga) samples (Fig. 2). Upon further storage, the thiol contents changed depending on the equilibrium MC that the seeds had attained. The high moisture condition (75% RH, giving 11.7% MC) led to a rapid decrease in content during storage for both seed sources, whereas in the low moisture condition (32% RH, giving 5.5% MC),

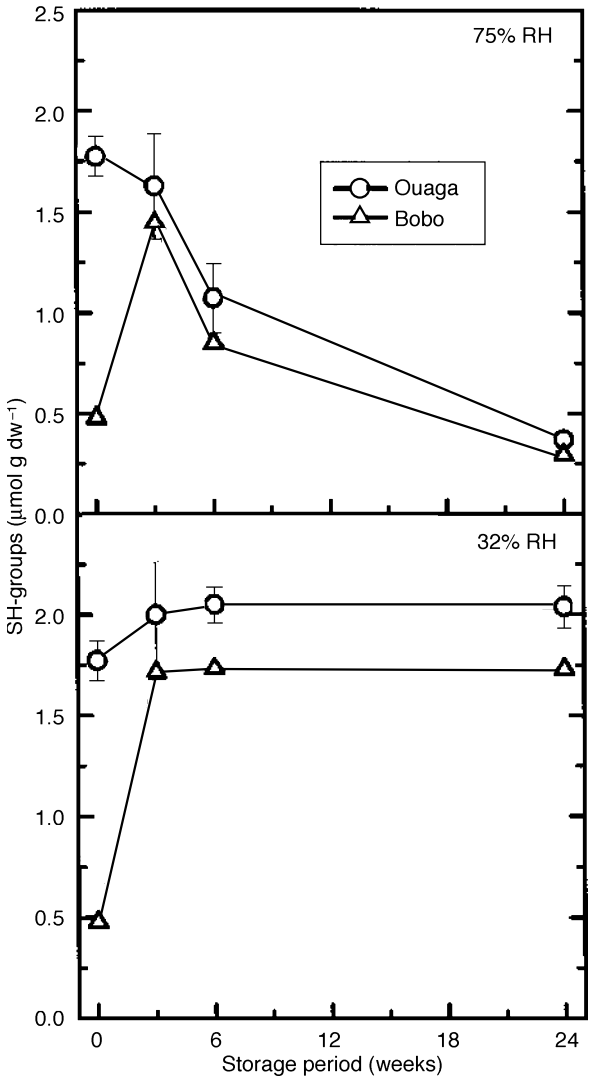

Figure 2. Effect of storage on total non-protein SH-content of neem embryos. Seeds from Ouaga and Bobo were stored for 24 weeks at 20°C at 32% or 75% RH. Each data point is the mean of three replicates from extracts of five embryos. SD is represented when larger than the symbols.

the contents stabilized after 3 weeks when the seeds had reached equilibrium MC. Other seed sources displayed essentially the same pattern (data not shown) as those shown in Fig. 2.

Glutathione redox status

A very similar pattern of changes in GSH content with drying and further dry storage was observed (Fig. 3) as found for the total non-protein thiols in Fig. 2. After an initial increase in the content of the originally more hydrated sample, most likely associated with afterripening processes, a decrease in the amount of GSH occurred in the seed lots stored at

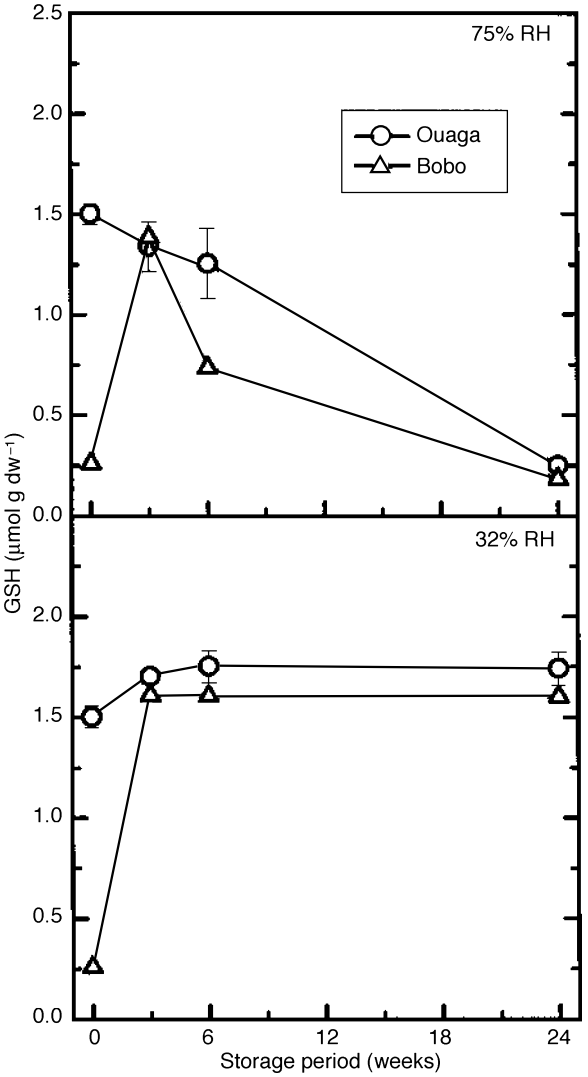

Figure 3. Change in GSH content in embryos during storage of neem seeds from Ouaga and Bobo at 20°C at 32% and 75% RH. Each data point is the mean of three replicates from extracts of five embryos. SD is represented when larger than the symbols.

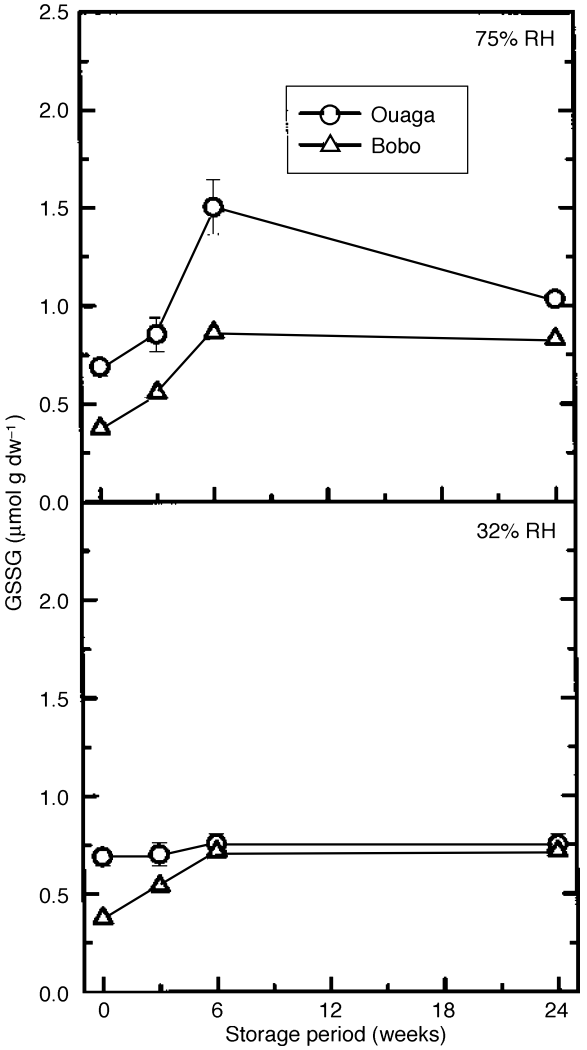

Figure 4. Change in the GSSG content in embryos during storage of neem seeds from Ouaga and Bobo at 20°C at 32% and 75% RH. Each data point is the mean of three replicates from extracts of five embryos. SD is represented when larger than the symbols.

75% RH, whereas the seed lots stored at 32% RH had stable amounts during the entire storage period. The amount of GSH during storage under both RH conditions was slightly less than that of the total of non-protein SH-compounds (Fig. 2) in all extracts, which means that GSH comprised most of the non-protein thiols present in the seeds. A sample of seeds from green fruits containing the highest water content, had the lowest GSH content at the onset of drying, which increased to values comparable with those initially found in the drier samples from the yellow fruits (data not shown).

The amounts of GSSG increased with the water loss of the samples during the first 3–6 weeks of exposure (Fig. 4). After 6 weeks this increase levelled off, or the GSSG contents slightly decreased (in the case of storage of the Ouaga seeds at 75% RH). For the levels of total glutathione (not shown), this has the following consequences. During the first 3–6 weeks, the increase in GSH in most of the samples and the simultaneous increase in GSSG led to a net increase in the total glutathione levels. However, during further storage at 75% RH, the decrease in GSH and the more or less stable level of GSSG led to a net loss of total glutathione during storage. During further storage at 32% RH, the amount of total glutathione did not change much.

The ratio of GSSG to total glutathione can be considered as a measure of the cellular redox status

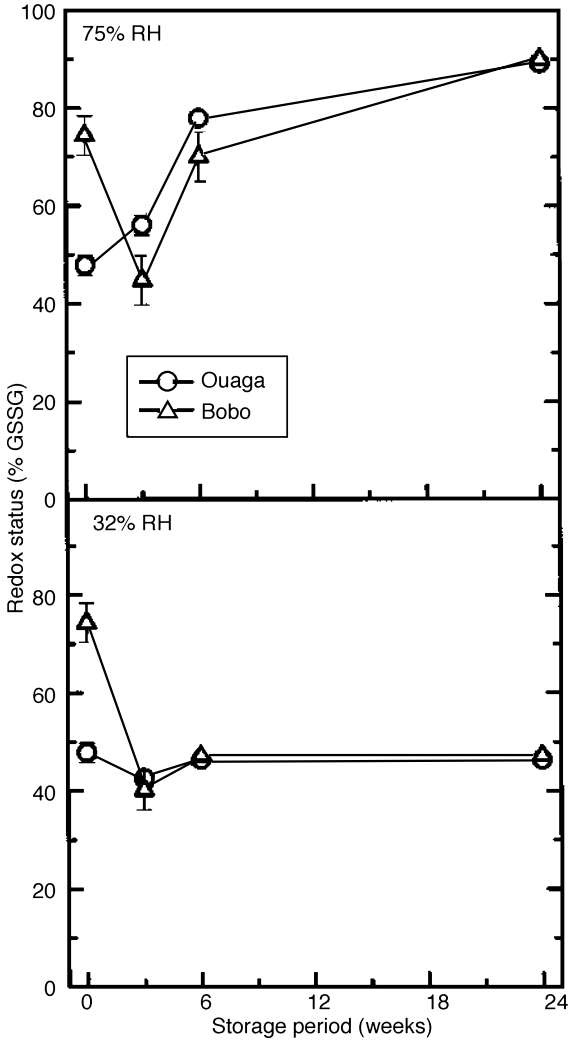

Figure 5. Change in the glutathione redox status [(S-) groups in GSSG expressed as a percentage of (S-) + (SH-) groups in total glutathione] in embryos of neem seeds stored under conditions of 32% and 75% RH at 20°C. Data give an estimate of oxidative stress and are the means of three replicates from extracts of five embryos. SD is represented when larger than the symbols.

(De Vos *et al*., 1994; Kranner and Grill, 1996). In the seeds stored at 75% RH the proportion of GSSG increased up to 90% (Fig. 5), which suggests oxidative stress during storage. The initial reduction in this ratio during drying for the wettest seeds can be explained by the fact that the production of GSH, associated with afterripening, occurred at a higher rate than that of GSSG. Under conditions of 32% RH, the redox status remained stable and low over the storage period. We conclude that the seeds kept at 75% RH experienced oxidative stress, whereas those at 32% RH did not.

Lipids during dehydration and storage

In an attempt to measure possible increases in lipid peroxidation products over storage as an indication of oxidative damage, thiobarbituric acid reactive substances were measured at 532 nm according to the method of De Vos *et al.* (1994). The method did not show significant differences between seeds kept under the different storage conditions (data not shown). This could indicate that there was no peroxidation, or that the lipids did not contain sufficient poly-unsaturated fatty acids to allow detection of changes in absorption at 532 nm.

Table 1 shows the amounts of phospholipids and free fatty acids during dehydration and subsequent storage of the Bobo seed lot (25.0% initial MC) at 32% and 75% RH and 20°C. During drying (first 6 weeks of storage), the phospholipid (PL) content increased, particularly when the drying occurred at 32% RH. This content remained unchanged during the next 18 weeks of storage at 32% RH. However, at 75% RH there was also a breakdown of the phospholipids, associated with an increase in the cellular level of free fatty acids. Compositional analysis of the acyl chains of the phospholipids showed that oleic acid (18:1) was the dominant fatty acid and that there was very little linolenic acid (18:3) present. Slight changes in composition could be observed during drying and storage. At 75% RH there was a slight but significant

Table 1. Free fatty acid (FFA) and phospholipid (PL) contents and fatty acid composition of neem seeds from Bobo Dioulasso. The initially fresh seeds had 25.0% MC (FW basis). Data are means of two extractions. The double bond (DB) index (number of double bonds per acyl chain) is also indicated. Palmitic acid, 16:0; stearic acid, 18:0; oleic acid, 18:1; linoleic acid, 18:2; linolenic acid, 18:3.

Treatment	FFA-content (mg/g DW)	PL-content (mg/g DW)	Mol % fatty acids in PL					DB index
			16:0	18:0	18:1	18:2	18:3	
Fresh, no drying	0.4	5.1	21.2	8.1	50.7	17.0	2.9	0.94
32% RH								
6 weeks	0.3	10.0	20.5	8.1	51.4	18.4	1.6	0.93
24 weeks	0.3	10.4	21.4	6.8	54.3	16.1	1.3	0.91
75% RH								
6 weeks	2.1	7.7	18.3	8.8	56.0	15.4	1.5	0.91
24 weeks	7.2	6.4	18.7	8.2	54.5	16.5	2.1	0.94
LSD (P = 0.05)	2.1	1.2	1.9	0.8	2.0	2.0	0.5	–

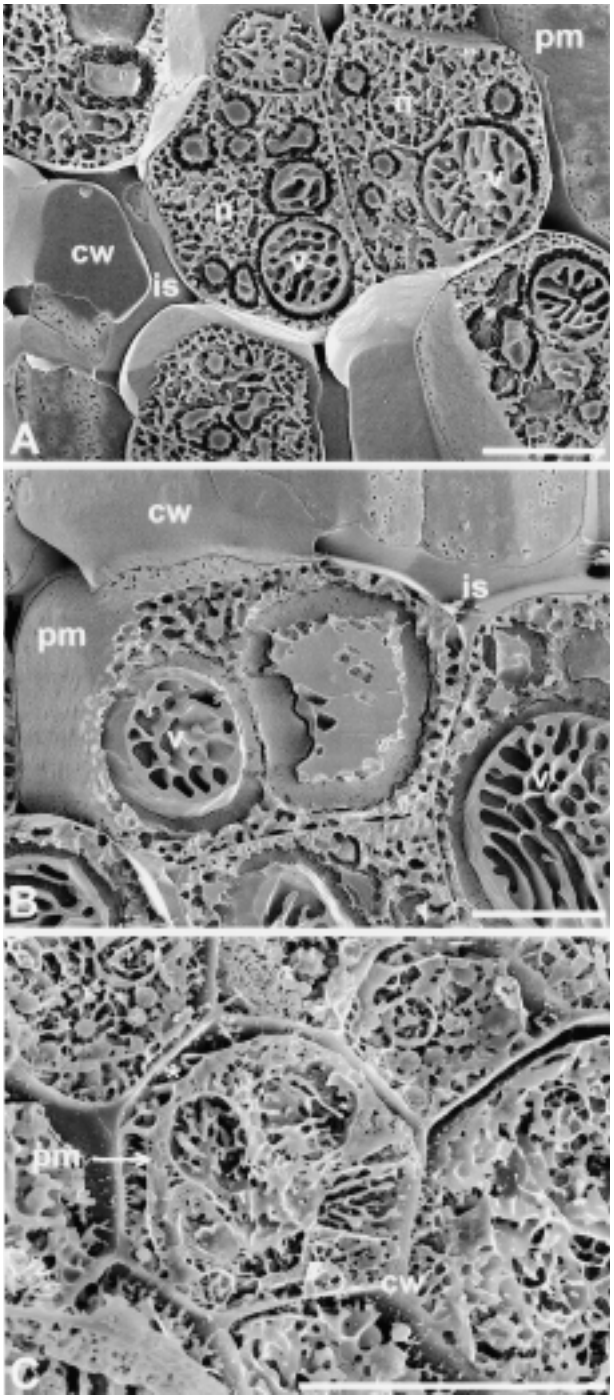

Figure 6. LTSEM micrographs of cryo-fractured axes from neem seeds that were soaked for 4 h in water at 30°C, followed by incubation on moist filter paper for 20 h at 30°C: (A) fresh seed; (B) a seed stored for 6 months at 32% RH and 20°C; (C) a seed stored for 6 months at 75% RH and 20°C (bars = 10 µm). cw = cell wall, is = intracellular space, n = nucleus, pm = plasma membrane, v = vacuole. Asterisk: space between plasma membrane and cell wall due to turgor loss.

decrease in palmitic (16:0) and linolenic acids, and a slight increase in oleic acid compared with the composition of fresh seeds, whereas at 32% RH linolenic acid decreased and the amount of oleic acid was higher after 24 weeks. The number of double bonds per acyl chain did not change consistently during storage at any RH (Table 1). Neutral lipids comprised almost 50% of the DW, with a considerable similarity in acyl chain composition to that of phospholipids (data not shown). They had low levels (1–2%) of eicosenoic acid (20:1) and erucic acid (22:1) that were not found in phospholipids. The free fatty acid content did not increase at 32% RH storage, but increased considerably at 75% RH.

Ultrastructural changes of fresh and stored axes

After 24 h of water uptake in Petri dishes, fractured cells of isolated axes were inspected by LTSEM. Figure 6A shows that the axis cells from fresh seeds had a turgescent appearance with large vacuoles visible, typical of viable cells. Axis cells from seeds that had been stored for 6 months at 32% RH and 20°C had an appearance similar to those of fresh seeds (Fig. 6B). However, axis cells from seeds stored for 6 months at 75% RH had the typical appearance of debilitated cells that had lost turgor (Fig. 6C).

Sugars during dehydration and storage

Sugar analysis revealed the presence of the oligosaccharides raffinose and stachyose, the disaccharide sucrose, and the monosaccharides glucose and fructose. Figure 7 shows the changes in raffinose, stachyose, and glucose contents with dehydration and storage of the seeds from Bobo. During drying to 5.5% MC under conditions of 32% RH (first 6 weeks), the contents of the oligosaccharides and glucose increased and then stabilized. The fructose levels were very low throughout (data not shown). However, when the seeds were dried and stored at 11.7% MC at 75% RH, the increases in oligosaccharides and glucose contents were confounded by a decrease that continued over the entire storage period. The amount of sucrose under both conditions of RH was in the range of 6.4 to 9% of DW during the storage period, with that at 75% RH being slightly below that at 32% RH (data not shown). The seeds from Bobo had oligosaccharide contents that were low (Fig. 7) compared with those from the three other seed lots that were tested. The average sugar contents in the four seed lots after drying at 32% RH (6 weeks storage) were: raffinose, 5.6 ± 2.6 (SD) mg/g DW; stachyose, 7.2 ± 4.0 mg/g DW; sucrose, 69.0 ± 19.4 mg/g DW; glucose, 3.4 ± 0.7 mg/g DW, with negligible amounts of less than 0.3 mg/g DW for fructose. These sugar contents from the

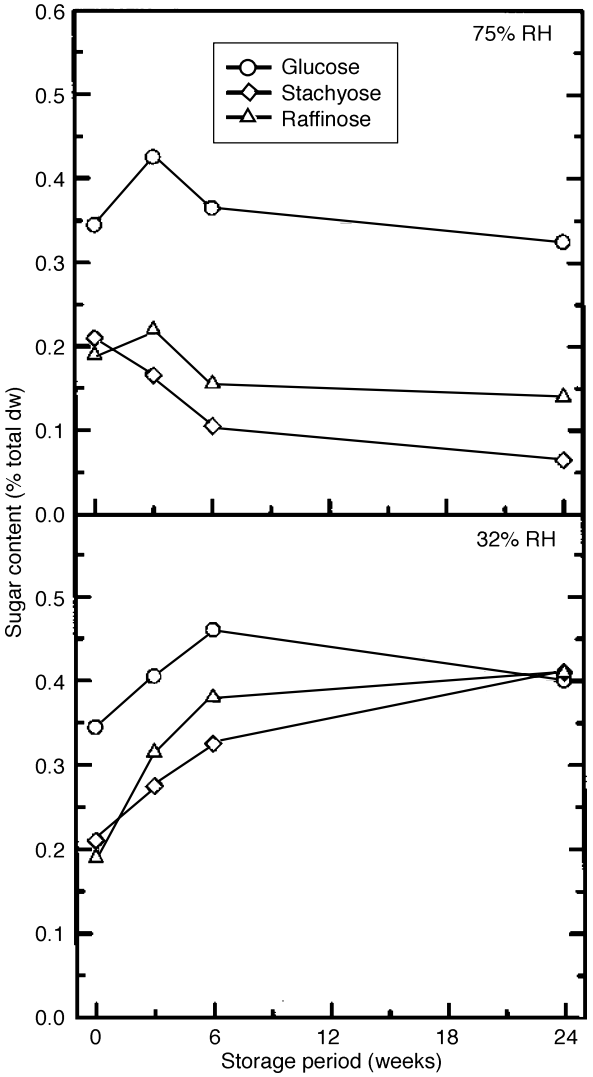

Figure 7. Composition and changes in sugars (glucose, stachyose and raffinose) in embryos of neem seeds from Bobo stored at 32% and 75% RH at 20°C. Each data point is the mean of two determinations from samples of five embryos.

four lots gave an average oligosaccharide/sucrose mass ratio of 0.19.

Discussion

Oxidative stress and compositional changes in a number of neem seed lots from two sources were studied at two humidity regimes, in an attempt to understand the rapid viability loss of neem seeds during drying and storage. Whereas the germination capacity after 6 months at 20°C decreased from 96% to 60–70% under storage conditions of 32% RH, germination was less than 20% after 6 months at 75% RH (Fig. 1). These data are consistent with other survival data of neem seeds during dehydration and storage (reviewed by Hong *et al.*, 1996; Poulsen, 1996). Seed storage behaviour of neem is controversial. It has been characterized as recalcitrant (Ponnuswamy *et al.*, 1990; Maithani *et al.*, 1989) on the basis of its rapid loss of viability when seeds are stored at 12% MC (FW basis), which agrees with the rapid deterioration that we observed in seeds stored at 75% RH (11.7% final MC). Neem seeds have also been categorized as displaying intermediate storage behaviour, because 50% of seeds have been reported to survive several months of storage after dehydration to water contents as low as 4–6% (Gaméné *et al.*, 1996; Hong and Ellis, 1998; Sacandé *et al.*, 1996, 1998). This agrees generally with the levels of survival observed in our storage experiments at 32% RH (5.5% equilibrium MC). However, orthodox behaviour has also been claimed on account of more than a decade of successful dry storage at –20°C (Tompsett and Kemp, 1996).

The short longevity of seeds at 11.7% MC is not surprising, because deteriorative processes at such water contents may be prominent, particularly in oily seeds. Because 50% of the DW of neem seeds is in the form of oil (Table 1; Sacandé *et al.*, 2000), the actual water content in the cytoplasm outside the oil will be twice as high as might be expected, i.e. 20.9% on a FW basis. Under these conditions the cytoplasm is not in the glassy state (Sacandé *et al.*, 2000). It has been suggested that, in general, the rate of deterioration of seeds and pollen is considerably lower when the cytoplasm is in the glassy state than when it is in the liquid state (Sun, 1997; Buitink *et al.*, 1998). At 32% RH the cytoplasm in neem seeds has been shown to be in the glassy state (Sacandé *et al.*, 2000), supporting this concept.

Our data on the extent of oxidative stress during drying and subsequent storage at 32% and 75% RH (Fig. 5) concur with the existence and absence of cytoplasmic glasses, respectively. Seeds exposed to high RH suffered oxidative stress, whereas dry seeds (32% RH) did not. The glutathione oxidation status was used to estimate oxidative stress in general (De Vos *et al.*, 1994). In metabolizing tissues GSSG is considered to be reduced immediately back to GSH by the action of glutathione reductase. However, because glutathione reductase presumably cannot be active in seeds in the dry state, desiccated tissues accumulate GSSG depending on the extent of oxidative stress (De Vos *et al.*, 1994; Kranner and Grill, 1996).

Under storage conditions of 75% RH, a loss of total glutathione was observed – to a lesser extent in GSSG than in GSH – leading to a noticeable shift in the glutathione redox status towards a more oxidized form. In contrast, there was no loss of total glutathione at 32% RH storage. Storage at 75% RH

also led to decreases in oligosaccharide and phospholipid contents, which did not occur at 32% RH.

An interesting observation was made when comparing seed samples of initially different MCs with respect to their glutathione, oligosaccharide and phospholipid contents. The more hydrated samples accumulated these compounds during equilibrium drying (first 3 weeks) to a constant water content (brought about by either 32% or 75% RH), whereas the initially drier samples already contained elevated amounts of these compounds. We interpret this to result from afterripening (or post-maturation) processes induced by slow drying, during which the seeds become better able to withstand complete water loss. In our opinion, this is a clear indication that neem seeds display typically orthodox rather than recalcitrant behaviour. Although recalcitrant seeds do possess antioxidant mechanisms (reviewed by Pammenter and Berjak, 1999), these mechanisms tend to become insufficiently effective during dehydration, because these seeds cannot down-regulate metabolism in the way that orthodox seeds do.

Loss of seed viability has been linked to increased lipid peroxidation, particularly of the poly-unsaturated fatty acids (Wilson and McDonald, 1986), which can lead to disrupted membranes and the associated leakage of endogenous solutes. We were unable to detect signs of such lipid peroxidation when measuring thiobarbituric acid reactive substances. The low levels of poly-unsaturated (18:3) acyl chains in the neutral lipids and phospholipids (Table 1) can explain this because the assay is particularly sensitive to the peroxidation products of the poly-unsaturated lipids. Also, the analysis of the fatty acid composition in the phospholipids does not indicate that lipid peroxidation occurs to any considerable extent during storage at 75% RH. This observation is in agreement with the results of others (Priestley and Leopold, 1979; Senaratna *et al*., 1988; Van Bilsen and Hoekstra, 1993; De Vos *et al*., 1994). However, during storage at 75% RH free fatty acids in the neem seeds accumulated and the phospholipid content decreased – a phenomenon that has been observed in many other systems during (accelerated) ageing (Priestley and Leopold, 1979; Senaratna *et al*., 1988; Van Bilsen and Hoekstra, 1993; Van Bilsen *et al*., 1994). It has been proposed that a mechanism other than peroxidation is responsible for the acyl chain de-esterification (Senaratna and McKersie, 1986). The larger free fatty acid accumulation in neem seeds compared with the loss in phospholipid content may be attributed to a contribution of free fatty acids originating from the neutral lipids. The supposition that the lipid changes in the seeds stored at 75% RH reduce the barrier properties of the plasma membrane is supported by the LTSEM micrograph (Fig. 6C), showing the loss of turgescence of the axis cells after 6 months under this RH condition.

The occurrence of di- and oligo-saccharides has been linked with the acquisition of tolerance to desiccation (Horbowicz and Obendorf, 1994) and improvement of storage longevity. Particularly, the mass ratio of oligosaccharides to sucrose has been implicated in these capabilities (Steadman *et al*., 1996). Neem seeds accumulated oligosaccharides (Fig. 7) in amounts comparable to those found in orthodox seeds. The average mass ratio of oligosaccharide to sucrose of 0.19 for two batches of the seeds from both locations is in the typical range for orthodox seeds. Recalcitrant seeds are generally characterized by mass ratios of oligosaccharides to sucrose of <0.083 (Steadman *et al*., 1996).

Taken together, the dehydration-induced increase in substances that are considered to promote desiccation tolerance, the relatively high oligo-saccharide content, and the ability to survive long-term dry storage imply that neem seeds have orthodox rather than intermediate or recalcitrant storage behaviour. Their extreme sensitivity to imbibitional stress (Sacandé *et al*., 1998) may have contributed to their reputation as being difficult to store. In addition, dehydration histories of the original seed samples may lead to differences in the amounts of protective substances in the dehydrated seeds and might also help to explain variations in the extent to which neem seeds can be successfully stored.

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