

# **Stress, Storage and Survival of Neem Seed**

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# **Stress, Storage and Survival of Neem Seed**

**Moctar Sacandé**

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## Propositions (Stellingen)

1. It is difficult to say whether dry neem seeds are dead or alive. Their viability can be tested by vital staining or standard germination. However, if none germinate, the viable seed may have been killed by handling rather than they being previously dead.

*This thesis*

2. Although being the only obvious means of testing for viability, standard germination tests do not reveal how, when and where damage occurs in seeds.

*This thesis*

3. The high intrinsic gel-to-liquid transition temperature ( $T_m$ ) of membranes in tropical seeds is responsible for their extreme sensitivity to imbibitional and chilling stress below these temperatures.

*This thesis*

4. The insecticidal and insect-repellent properties of compounds in neem kernels have attracted worldwide attention. However, the availability of good quality seeds limits their exploitation.

*This thesis*

5. A plant is just a seed's way of making another seed.

*Bradford KJ and Cohn MA (1998) in Seed Science Research 8, 153-160; after Richard Dawkins' Selfish Gene (Dawkins, 1976).*

6. The challenge today is no longer to decide whether the conservation of genetic resources is a good idea, but rather how it can be implemented in the interest of nationals and within the means of each country.

*Brundtland GH, 1987. Our common future. Oxford University Press, UK.*

7. All reactions are slowed down to a vanishing point, that is reactions at  $-200^{\circ}\text{C}$ , if at all possible in the solid state of the matter, would be about eight million times slower than at  $20^{\circ}\text{C}$ .

*Becquerel, 1951 quoted by Keilin, 1959. Proc R Soc London B Biol Sci 150: 149-191.*

8. The concept of life as applied to an organism in the state of anhydrobiosis becomes synonymous with that of the structure, which supports all the components. Only when the structure is damaged or destroyed does the organism pass from the state of anabiosis, or latent life, to that of death.

*Keilin, 1959. Proc R Soc London B Biol Sci 150: 149-191.*

9. Science is like a hungry furnace that must be fed logs from the forests of ignorance that surround us. In the process, the clearing we call knowledge expands, but the more it expands, the longer its perimeter and the more ignorance comes into view.

*Matt Ridley (1999) Genome. London 4<sup>th</sup> Estate.*

10. Development aid should always help us to put an end to aid.

*Thomas Sankara (1949-1987). Late President of Burkina Faso (1983-1987).*

11. It is circumstances which show what men are.

12. What we call the beginning is often the end, and to make an end is to make a beginning. The end is where we start from.

*Eliot TS (1942). Little Gidding. Collected Poems 1909-1962. (1963 edn). London, Faber & Faber.*

These propositions belong to the PhD thesis entitled: "Stress, Storage & Survival of Neem Seed" by Moctar Sacandé.

Wageningen, 26 April 2000

*For my parents Hjs. Asseta Sakandé/Nikiema and Late Saidou Sakandé*  
*For my friends and brothers*  
*For Abidine, Naïma and Valerie*

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## Preface

The first time that I met the Neem tree was when I was a child, growing up in a large family in Bobo Dioulasso – the second city of Burkina Faso. My father used to bring back sticks collected from the neem trees that lined the wide earth avenues of the town. When we misbehaved my father threatened to use the sticks on us, though he did not often have to carry out the threat. One such stick was carefully planted in a corner of the compound to remind us to behave and to produce lots more sticks. Today it is a big tree. I firmly believe that without my late father's loving discipline I would never have achieved all that I have today. This thesis on neem is dedicated to him.

Although discipline may be a little out of fashion these days, I think that it gave me a good basis for my career. What I have learned through the planning, execution and finally the writing of this thesis has been another major step forward for me. I have learnt how to face and to overcome many difficulties, both technical and social. What has helped me most has been the ready collaboration and tolerance of colleagues, the support of my friends and the faith my family had in me.

Scientific curiosity was one of the reasons that led me to work on the biology of neem seed. The other reason was to do something of immediate practical significance. It has therefore been particularly satisfying to be able to use the insights gained from explorations of cell physiology, biochemistry and biophysics to understand and predict how tropical seeds will perform in storage. I am very encouraged to see that our results are beginning to have an impact on the way that recalcitrant and intermediate tropical tree seed species are handled and stored in international projects and at my home institute, the Centre National de Semences Forestières (CNSF) in Ouagadougou, Burkina Faso.

This thesis could not have been completed without the contributions and the collaboration of the CNSF, the Laboratory of Plant Physiology of Wageningen University and the Plant Research International/Centre of Plant Breeding and Reproduction Research (PRI/CPRO), in the Netherlands. It benefited from the inputs of many partners who are co-authors of the published papers that form chapters of this thesis. I would therefore like to take this opportunity to express my sincere gratitude to all who have helped me in one way or another.

Firstly, Prof Cees M. Karssen who accepted me under his supervision despite his full agenda as rector magnificus of the WU. Prof Linus van der Plas, head of the Laboratory of Plant Physiology and acting promotor is acknowledged for his wise advice.

My cordial and special thanks go to Dr Folkert A Hoekstra, my main supervisor, for his personal commitment and interest in my research which dates back to his assistance with my MSc research. His close supervision and generous help in technical discussions took the research to a different level and greatly improved the standard of this thesis. First of all I got used to, and now I miss the smell of Havana cigars during long hours behind the computer, working with him on the scientific papers that came from the research project. Apart from his immense contribution to my scientific development I also thank him for ensuring the financial support for my stay

in his laboratory. I sincerely thank you Folkert and your family for making my problems yours.

The Department of Reproduction Technology at CPRO kindly hosted me for my practical studies. Dr Raoul Bino's interest in my research and his encouragement resulted in the setting up of this project in 1995. The co-operation between the CNSF and the CPRO, which began a few years earlier with visits from Dr Lieke H. Kraak, has been strengthened through these studies. Later, in 1996, no effort was spared to invite the Director of CNSF to the CPRO to discuss possibilities for further technical collaboration. Dr Kraak helped broaden my views about seed research when I joined the Seed Physiology Section at CPRO. Her successor, Dr Ric De Vos contributed to my work from the formulation of the research proposal through to the laboratory work. I very much appreciated his collaboration and supervision. To Dr Steven PC Groot, who recently took over the section I also express my sincere thanks for his efforts. I appreciated working with Jaap van Pijlen who assisted with many of the germination experiments.

The technical and scientific discussions of the Seed Group at the Plant Physiology Department of Wageningen University, were the medium that nurtured my scientific growth. Working with Julia Buitink was a real pleasure for me and I greatly appreciated the initiation to DSC. To Dr Olivier Leprince, *'toute ma reconnaissance pour ton aide et tes commentaires qui ont souvent amélioré mes analyses'*. I thank Mark Alberda for his trouble-shooting on computers and for the FTIR experiments, Dr Elena Golovina for generating and interpreting EPR data, Adriaan van Aelst for helping with electron microscopy. My special thanks go to Dr Valerie Curtis, for her support and help with my English.

The collaborative nature of the project and the interest it has generated brought me into contact with international institutes and universities. I wish especially to mark the contribution of the late Dr Abdou Salam Ouédraogo of IPGRI (may he rest in peace), who died recently in a plane crash in Ivory Coast. I benefited from his advice as a 'koro' and as a model of the success that determination and faith in oneself can bring. I wish to thank Drs. Jan Engels and Florent Engelmann (IPGRI, Rome) for their interest and financial support for some of the studies; Profs. Daniel Côme, Francoise Corbineau (Université Pierre et Marie Curie, Paris), Patricia Berjak (University of Natal, Durban) and HPM Gunasena (University of Agriculture, Peradenia, Sri Lanka) for the opportunity to work in their laboratories.

The work was conceived as collaborative research project, which was part of the programmes of the CNSF and managed by the CNSF administration. I wish to thank the team from CNSF and especially Soumaila Bancé, Martinus de Kam, Albert Nikiema, Sibidou Sina, Lambert Ouédraogo, Raymond Balima, Joséphine Yaméogo Mathurin Sanou, Ms. Haoua Ouédraogo, Sylvie Yaméogo, Asséto Ouédraogo, Juliennne Botoni, Cecile Kaboré, Lucie Foro, and Mamadou Tou, Mamadou Sidibé, Moumouni Zida, Hamado Derra.

I could not have completed this work without the assistance of my family and friends at large. My mother Hj. Asseta Sakandé/Nikiema has been a great support. To my brothers Hj. Adama Sakandé and Dr Souleymane Sakandé many thanks for your moral support and permanent encouragement. Your help with my many duties in

Burkina during my absence was greatly appreciated. The Curtises, my beloved family, are gratefully acknowledged for their help and contributions. To the Damibas, Bayalas, Sedogos and Toés, many thanks for all the arrangements you have helped with for my family and your real friendship and support.

I gratefully acknowledge the financial support of the Government of Burkina Faso, the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), the International Plant Genetic Resources Institute (IPGRI) and DANIDA, and for the use of facilities at CPRO, at the Wageningen University and at the CNSF, which made my studies possible.

## List of abbreviations

$\Delta H$	enthalpy
ANOVA	analysis of variance
C value	constant DNA value of a haploid genome
CNSF	Centre National de Semences Forestières
CPRO	Centre for Plant Breeding and Reproduction Research
Da	Dalton
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
DTNB	5,5'-dithiobisnitrobenzoic acid
DTPA	diethylene-triamine-pentaacetic acid
DTT	dithiothreitol
DW	dry weight
EF	exoplasmic face
EPR	electron paramagnetic resonance
FFA	free fatty acid
FTIR	Fourier transform infrared spectroscopy
FW	fresh weight
G <sub>0</sub> phase	quiescent phase of cell-cycle
G <sub>1</sub> phase	pre-synthesis (first gap) phase of cell-cycle
G <sub>2</sub> phase	post-synthesis (second gap) phase of cell-cycle
GC-MS	gas chromatography – mass spectroscopy
GSH	(reduced) glutathione
GSSG	oxidised (disulfide) glutathione
H <sub>II</sub> phase	inverted hexagonal phase
HPLC	High Pressure Liquid Chromatography
IMP	intra-membrane particle
IPGRI	International Plant Genetic Resources Institute
ISTA	International Seed Testing Association
LN <sub>2</sub>	liquid nitrogen
LSD	least significant difference
LTSEM	low temperature scanning electron microscopy
MC	moisture content
Mes	morpholino ethanesulfonic acid
PAGE	PolyAcrylamide Gel Electrophoresis
PF	protoplasmic face
PL	phospholipid
RH	relative humidity
ROS	reactive oxygen species
S phase	Synthesizing phase of DNA
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
SH-group	sulphydryl group
SSA	sulfo-salicylic acid

TBS	Tris-buffered saline
TEM	transmission electron microscopy
TEMPONE	4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy
T <sub>g</sub>	glass transition temperature
TLC	thin layer chromatography
T <sub>m</sub>	gel-to-liquid transition temperature
WAP	weeks after pollination
WC	water content
WOTRO	Netherlands Organization for the Advancement of Tropical Research

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## General introduction

Moctar Sacandé

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### 1. The challenge of conserving tropical tree seeds

All cultivated plants arose from wild plant species. The problem of how best to store their seeds has been an important issue since man first began to domesticate plants, somewhere between 10,000 and 5,000 years ago (Diamond, 1997; Tudge, 1998). Selecting seeds from the best plants – from the tiniest of herbs to the tallest tree – and storing them till the next growing season was the process that gave us the crop plants we use today. Because seeds are often produced in abundance, particularly when they serve as a food source (crops, cereals), it was an easy step for mankind to exploit plants. However, as early farmers soon discovered, seed storage was not always straightforward. Some seeds were easy to store for long periods of time, whereas others remained viable only for short periods. The latter species were probably not perpetuated through their seeds.

Today many plant species, both wild and cultivated, are under threat. The conjunction of environmental degradation due to drought and human pressures, for example, overexploitation, pollution, and intensive animal husbandry in several regions of the world is greatly contributing to the reduction of plant gene-pools and subsequently of the number of species (Brundtland, 1987).

Genetic resources and threatened species in particular can be safeguarded by conservation *in-situ* in the natural environment and *ex-situ* in gene banks. *Ex-situ* conservation requires that large numbers of seeds of many species be stored over long periods of time. Research into seed physiology and storage behaviour is an essential component of *ex situ* conservation activities for national and regional genebanks. Until recently, priority was given to food and crop species of economic importance. However, tropical tree seeds are an increasing focus of attention in the face of threats to sensitive tropical environments, and awareness of the need to conserve biodiversity (Brundtland, 1987; UNCED, 1992). Knowledge of the storage behaviour of tropical seeds is a prerequisite for their preservation (Hong *et al.*, 1996).

Many conservation programmes are hampered both by a lack of understanding of the biology of tropical tree seeds and by practical problems with their storage. In seed science and technology, seeds are usually characterised with reference to their storage behaviour. Seeds that are shed in a partially dehydrated condition can generally be further dried to low water contents (5%) and stored for many years at low, or even subzero temperatures. These seeds are from plants that include many crops, both perennials and annuals. They are easy to handle and store in a predictable manner, and have been designated as having orthodox storage behaviour (Ellis and Roberts, 1980). In contrast, seeds with recalcitrant storage behaviour are those that get damaged early during dehydration and cannot be stored for long periods of time (Roberts, 1973). A large number of tree seeds from the humid tropics display such recalcitrant storage behaviour, presumably because they are adapted to warm humid conditions. Examples are the seeds of economically important tropical species such as cacao, coconut, hevea, avocado and mango. Seeds that have a lethal limit of dehydration falling between these two categories are referred to as intermediate, and can generally be stored for periods of intermediate length (Ellis *et al.*, 1990, and references therein). They are often chilling sensitive, as are recalcitrant seeds. In general, all seeds can be described as exhibiting storage behaviour that falls on a continuum somewhere between orthodox and recalcitrant (see review by Berjak and Pammenter, 1997). Comprehensive data classifying seeds into orthodox, recalcitrant or intermediate categories are available for only about 7000 plant species (see review by Hong *et al.*, 1996), which comprises 2-3% of all the plant species of the world.

The practical significance of these behaviours pertains to seed longevity. Due to their sensitivity to desiccation and/or low temperatures, recalcitrant or intermediate seeds often do not survive storage for a whole year, or even from harvest till the next sowing season. For recalcitrant seeds, the prospect of manipulating storage conditions without damage is very limited (King and Roberts, 1979). For intermediate seeds, however, relatively long storage periods can be achieved in carefully defined and controlled environments (Hong and Ellis, 1998, and references therein).

It is believed that the seeds of most tropical forest trees display recalcitrant or intermediate storage behaviour. If such seeds are to be conserved for the sake of biodiversity and for use in planting programmes, then more needs to be known about their physiology. A first step may be to screen priority tropical tree seed species for survival when they are dried (IPGRI/DFSC project, 1996). Further research is also needed into the mechanisms underlying the sensitivity to desiccation of tropical seeds.

We have chosen to investigate the longevity of neem (*Azadirachta indica*) seeds as a model of complex storage behaviour for several reasons. These include (i) the importance of neem as a priority species in planting programmes in Africa and its uses in agroforestry systems in many developing countries; (ii) the fact that the neem tree is propagated mainly via seeds, despite there being many difficulties with their storage and very little data on their physiology: the seeds are reputedly classified as recalcitrant or intermediate; (iii) the possibility of collecting seeds from several distinct accessions, and (iv) the fact that the seeds lack dormancy and germinate without delay or specific pretreatment. The objective of the work reported in this thesis is to provide insights into the mechanisms associated with the short life span of tropical tree seeds and thus help improve their storability.

## 2. *Azadirachta indica* A.Juss., the Neem tree

*Azadirachta indica* is a member of the Meliaceae family, the mahagonies, and is native to the dry forest zones of the Asian sub-continent including India, Sri Lanka, Pakistan, Bangladesh, Malaysia, Myanmar and Thailand. The tree is found both cultivated and growing in its natural habitat in these zones. The botanical nomenclature of neem, *Azadirachta indica* A.Juss., was established by the French botanist Antoine Laurent de Jussieu (Tewari, 1992). The genus *Azadirachta* A.Juss. described in 1830 comprises at least three species of Indo-Malaysian origin including *Azadirachta excelsa* (Jack) Jacobs, mostly thriving in Malaysia, and *Azadirachta siamensis* Valetton, mainly growing in Thailand (Suthi and Pirom, 1989; Schmutterer and Doll, 1993). The one that concerns us here, *Azadirachta indica* A.Juss., has three synonyms including *Antelaea azadirachta* (L.) Adelbert, *Melia azadirachta* L. (often confused with *Melia azedarach* L.) and *Melia indica* (A.Juss.) Brandis (von Maydell, 1986). There are many common names for the neem tree in countries where it has been introduced in Africa, South America, and Australasia.

Neem has become widely distributed by introduction into the dry, arid and semi-arid tropical and subtropical zones. The expansion was (and still is) driven by the tree's intrinsic value, its easy adaptation, and its many uses. In Africa, different routes of spread have been documented. Between 1919 and 1927, Brigadier-general Sir Frederick G. Guggisberg, governor of Gold Coast (now Ghana), introduced seedlings (or seeds) from India, and the first seedlings were planted in the northern territories (National Research Council USA, 1992). Today, neem is found throughout Ghana and the Sahelian region including Burkina Faso. Countries such as Sudan, Nigeria and Senegal had neem plantations before the 1950s (Schmutterer, 1995). In the last decades, the tree has also been introduced into Latin America. The biggest plantation of neem is in the Arafat plain near Mecca in Saudi Arabia where it provides shade for pilgrims (Ahmed *et al.*, 1989). The neem tree thrives in adverse environments because of its low demand for water and nutrients. Under favourable conditions it grows rapidly and may even become an "invasive" plant under conditions of high precipitation, as birds and bats spread the seeds.

Neem is an evergreen, small to medium-sized (5 to 20 m high), fast growing tree. Two thirds of the height may be reached after 3 to 5 years. The first fruits can be harvested after 5 years and the first timber crop after 5 to 7 years. Neem has no particular site requirements and is very resistant to drought since it is able to grow with only 150 mm of annual rainfall, and heat, but it is sensitive to cold. The species is highly suitable for the improvement of degraded and nutrient-poor soils (Von Maydell 1986) and is therefore considered important in reforestation programmes to combat desertification. The tree provides heavy wood which is resistant to termites and other wood-destroying insects, and is used for timber and for fuel. Neem is hence a priority species for reforestation and for multiple forestry products.



The use of neem has beneficial ecological consequences, including the improvement of the soil micro-climate and the saving of indigenous woody species, which would, in its absence, have been felled for fuel. The cultivation and utilisation of neem have a long tradition in India where nearly every part of the tree is used. These numerous uses have been reviewed in the report on neem of the National Research Council (1992) of the USA. One key practical use of neem is in the control of farm and household pests (Schmutterer, 1995). Extracts from seeds and leaves make highly effective insecticides: they attack many pestiferous species, are biodegradable, appear unlikely to quickly lose their potency due to a buildup of genetic resistance in the pests, seem to leave people, vertebrates and beneficial insects unharmed, and their effectiveness equals that of DDT, Dieldrin and other synthetic pesticides. According to the National Research Council (1992), neem is likely to provide non-toxic (for human) and long-lived replacements for some of today's most suspect synthetic pesticides (Immaraju, 1998). In addition, neem extracts have fungicidal and nematocidal effects, and kill snails. Neem is renowned for its healing properties. Neem extracts used in human and veterinary medicine are mostly based on its merits as a general antiseptic: neem preparations are effective as fungicides, antibacterial, antimalarial and antiviral agents. Moreover, the seeds contain up to 50% oil.

The tree is generally propagated via seeds in nurseries. However, the seeds are reputed to have a short viability, which is the main problem in using neem in agroforestry and reforestation programmes. Although much has been written about neem, only a few publications concern the physiology of the seeds.

### **3. Anhydrobiosis (life without water)**

Survival in the dry state to bridge periods of unfavourable conditions is widespread in both the animal and plant kingdoms. Nematodes, rotifers, tardigrades, *Artemia* cysts, bacteria, fungal spores, algae, yeast cells, mosses, ferns, pollens and seeds of higher plants and even whole Angiosperm plants can exhibit phases of anhydrobiosis (reviewed by Crowe *et al.*, 1997a, and references therein). This term was introduced by A. Giard in 1895 (quoted by Keilin, 1959) and is generally used for organisms and organs that are desiccation tolerant. Although such organ(ism)s seem to be dead, life and metabolism are only suspended. Periods of suspended life may last for several years without much reduction of viability. Metabolism and vital functions are resumed when the organ(ism)s experience favourable environmental conditions. Many scientists have worked on anhydrobiosis and its underlying mechanisms since it was first noticed by Antonie van Leeuwenhoek in 1702 (cited by Keilin, 1959). When Van Leeuwenhoek put some dry sediment from the gutter of his roof into water, he saw hundreds of microscopic "animalcules" swimming around. The observation led to the idea that life could come forth from dead organic material.

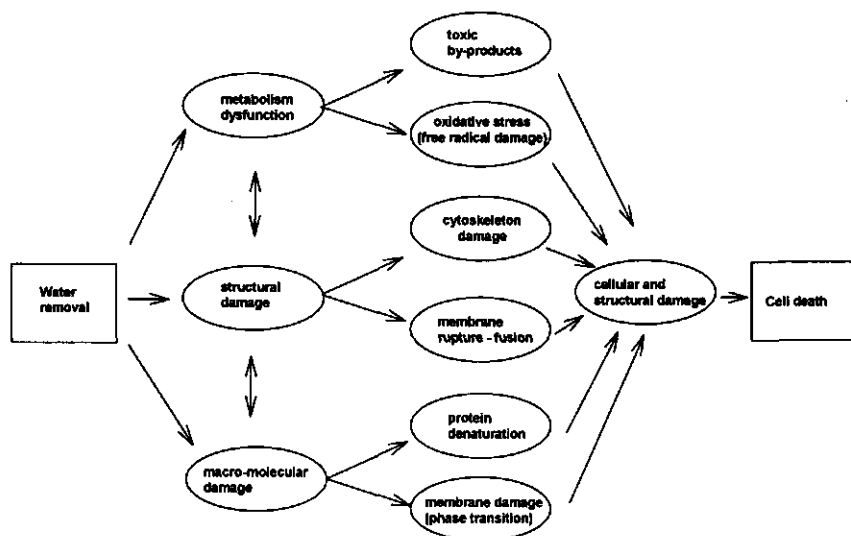
### Dehydration-induced damage

While desiccation-tolerant organ(ism)s are able to survive almost complete dehydration, desiccation-sensitive specimens lose considerable amounts of cellular solutes during dehydration and rehydration. Their cellular organisation is severely damaged, leading to the death of the organ(ism). This indicates that low water contents may affect the ability of the plasma membrane to function as a semipermeable barrier. Because membrane stability has been associated with desiccation tolerance, a considerable research effort has been devoted to membrane behaviour, which is discussed in the next section.

When organ(ism)s experience water loss, the chance of molecular interactions in the cytoplasm increases as a result of increased solute concentration and a closer proximity of cellular structures. This may lead to membrane fusion and denaturation of proteins, which is incompatible with life (Figure 1.1). It has also been demonstrated that drying interferes with normal cellular processes resulting in an increased production of reactive oxygen species, such as hydrogen peroxide and superoxide (Leprince *et al.*, 1990, 1994). If the production of reactive oxygen species cannot be appropriately controlled by the cellular antioxidant systems, these reactive species are able to damage cellular constituents such as proteins, DNA and membranes (oxidative damage). Loss of cellular vigour and, finally, cell death may occur.

It has been suggested that the stage of cell cycle activity at seed desiccation is one of the key factors related to desiccation tolerance and storage longevity (Bino *et al.*, 1992; Saracco *et al.*, 1995; Liu *et al.*, 1996; Van Pijlen *et al.*, 1996). Arrest of the cell cycle activity at the stage where the DNA content per nucleus is at its lowest is thought to predispose embryos to better resistance to stress conditions (Deltour, 1985). At this stage, the DNA is probably the least vulnerable to mutation-inducing factors.

Understanding how seeds tolerate water loss is therefore important for developing strategies that can improve the survival of seeds under conditions of decreasing water availability.



**Figure 1.1.** Diagram summarising causes and mechanisms of seed death. In desiccation intolerant seeds a combination of factors may explain the loss of viability below certain critical water content during dehydration.

### Mechanisms of desiccation tolerance

Orthodox seeds and, to a certain extent, intermediate seeds have the competence to prepare themselves to withstand desiccation through biochemical, biophysical and physiological adaptations during drying. The hormone abscisic acid (ABA) appears to regulate the induction of desiccation tolerance in orthodox seeds (Koomneef *et al.*, 1989). Absence of and/or insensitivity to ABA renders seeds recalcitrant (Ooms *et al.*, 1993a, 1993b). The lack of ABA action in developing mutant seeds of *Arabidopsis* can be compensated for by slow drying at the appropriate stage of seed development, which renders the seeds desiccation tolerant (Ooms *et al.*, 1994). Slow drying also appears to confer desiccation tolerance on ABA-treated somatic embryos of carrot (Tetteroo *et al.*, 1994, 1995). By contrast, recalcitrant seeds from, e.g., *Landolphia kirkii* appear to survive some level of dehydration only when they are fast-dried (Pammenter *et al.*, 1991). It is thought that in systems which are not responsive to slow drying, rapid drying at least limits the extent of desiccation injury. The rate of dehydration obviously is an important factor determining the level of desiccation tolerance of seeds.

*Dehydration to low water contents: hydrogen-bonding interactions*

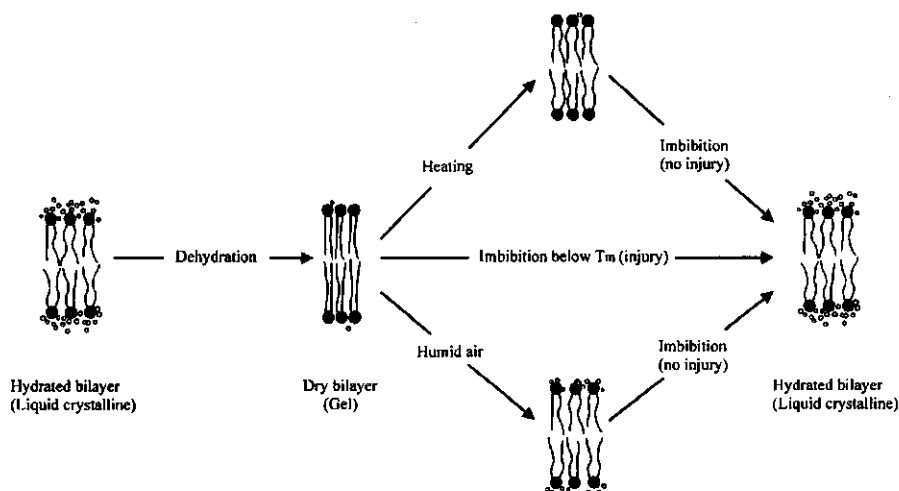
Biological membranes are particularly sensitive to dehydration. They generally consist of about 50% phospholipids, 40% proteins, and 10% steroids (e.g., in Robertson, 1983). Their biophysical behaviour is determined principally by phospholipids. Because of their amphipathic character, phospholipids in water tend to aggregate in ordered lamellar bilayer structures. This bilayer structure, illustrated in Figure 1.2 (modified from Crowe *et al.*, 1989b), is hydrophobic (non-polar) in the acyl chain region and hydrophilic at the (polar) phosphate headgroup region, and forms an integral part of biological membranes. The polar headgroup lines the aqueous medium on both sides of a membrane. Phospholipid bilayers mainly exist in two forms: a rigid gel phase and a fluid liquid-crystalline phase. At the transition temperature ( $T_m$ ) the bilayer changes from the liquid crystalline phase to the gel phase, or vice versa, depending on whether cooling or heating is applied (see Figure 1.2).

Much information concerning the effect of dehydration on membranes has come from studies on model membranes composed of pure phospholipids. With the loss during drying, of the water molecules which are hydrogen-bonded to each polar headgroup (generally below 0.25-0.30 g H<sub>2</sub>O/g dry weight) the lateral spacing between the phospholipids is reduced (Chapman *et al.*, 1967). This leads to increased opportunities for van der Waals interactions between the acyl chains of the phospholipid molecules and, consequently, to the formation of the gel phase (reviewed in Crowe and Crowe, 1988). Thus, the  $T_m$  may increase by as much as 70°C during dehydration. Liposomes that pass through a phase transition leach all entrapped solutes, at least when there is water available for the transport of these solutes (Crowe *et al.*, 1989a). Apparently it is the phase transition, i.e. the occurrence of the two phases at the same time, which renders the liposomes transiently leaky. Defects at the boundary between gel phase domains and the liquid crystalline phase are held responsible for the increased permeability (Hammoudah *et al.*, 1981).

Adaptation in the phospholipid composition of the membranes by insertion of acyl chains with a higher degree of unsaturation would make membranes in the organ(ism) more liquid and reduce  $T_m$ . An increased ordering during dehydration would thus be avoided. However, phospholipid compositions have not been observed to change much with imminent desiccation (Hoekstra and van Roekel, 1988). Research efforts have mainly focused on the effects of extramembrane compounds, such as sugars, on the phase behaviour of the dry membrane.

Di- and oligo-saccharides, which are abundant in desiccation-tolerant organisms, are particularly effective at suppressing the dehydration-induced increase in  $T_m$  by hydrogen bonding to the polar headgroups (Crowe *et al.*, 1986). Thus, the individual phospholipids remain spaced, with reduced opportunities for interactions between the acyl chains. This hydrogen bonding of sugars with the phosphate of the headgroups is known as the "water replacement theory" (Crowe *et al.*, 1984; Clegg, 1986). Moreover, the sugars prevent membrane fusion by the timely formation of a glassy matrix (Crowe *et al.*, 1997b). Both properties are crucial in the protection of liposomes from dehydration

stress. These findings have resulted in important pharmaceutical applications (drug application via stabilised, dry liposomes).



**Figure 1.2.** Diagram depicting the phospholipid bilayer structure and possible changes with dehydration. As a hydrated bilayer (water molecules are represented by small open circles) in the liquid crystalline phase is dehydrated, it may enter the gel phase, depending on the temperature. If this dry bilayer is heated above  $T_m$  (upper pathway) or partially hydrated by exposure to water vapour (lower pathway), it can pass through the phase transition in the absence of bulk water. Under such conditions it will not undergo a phase transition when it is placed in water and it will not leak. In contrast, if the dry bilayer is not heated to above its  $T_m$  before it is returned to water (middle pathway), it will undergo a phase transition during rehydration and can be expected to leak (redrawn from Crowe *et al.*, 1989b).

Membranes in dry desiccation-tolerant organ(ism)s usually have a slightly increased melting temperature, but above 20°C they usually occur in the liquid crystalline phase (Hoekstra *et al.*, 1997). It is not certain whether endogenous sugars are the sole compounds involved in the suppression of  $T_m$  in these organ(ism)s. Partitioning of cytoplasmic amphiphilic compounds into membranes during dehydration may also contribute to this suppression (Golovina *et al.*, 1998). Besides the fluidisation of membranes, these amphiphiles often have effective antioxidant properties. However, the current opinion has it that sugars play a pivotal role in desiccation-tolerant systems.

Sugars may not only be involved in the protection of membranes as earlier mentioned, but also in hydrogen bonding to proteins (Carpenter and Crowe, 1988; Prestrelski *et al.*, 1993; Wolkers *et al.*, 1998b) and in the formation of glasses (reviewed in Slade and Levine, 1994). In protein model systems, protein secondary structure is retained in the native aqueous conformation during drying by interaction with sugars, whereas without sugars the proteins denature. In dry seeds, the native aqueous secondary

structure is maintained during dehydration and storage (Golovina *et al.*, 1997; Wolkers *et al.*, 1998a). A glassy cytoplasm is considered important in relation to the long-term

stability of desiccation-tolerant organisms (Leopold *et al.*, 1994; Buitink *et al.*, 1998). A glass is a liquid with very high viscosity, having solid-like properties, the occurrence of which has been established in seeds and pollen below water contents of 0.1 g H<sub>2</sub>O/g dry weight at 20°C (Williams and Leopold, 1989; Buitink *et al.*, 1996).

Besides the prevention of protein denaturation by hydrogen bonding with sugars, proteins may be protected by some specific dehydration-induced, heat-soluble, hydrophilic proteins [= Late embryogenesis abundant (Lea) and small heat shock proteins] (Dure III *et al.*, 1989; Close, 1996). These specific proteins have been suggested to exert chaperone-like activities and may act during the early stages of dehydration. These hydrophilic proteins may also be involved in the protection of cells against dehydration stress through other mechanisms, among them ion sequestration and replacement of the hydrogen bonding function of water. Some of these hydrophilic proteins have been localised in close proximity to membranes (Helm *et al.*, 1993). A Lea (dehydrin)-like HSP-12 protein could reduce dehydration-induced leakage of solutes from liposomes, but success depended on the charge of both the liposome and the protein (Sales, 1999).

Although the above-described mechanisms prevent or postpone formation of the gel phase during drying, it has nevertheless been established that membranes in dry desiccation-tolerant systems can exist in a rigid gel phase-like condition without leading to loss of life in the organ(ism) (Hoekstra *et al.*, 1991, 1992; reviewed by Crowe *et al.*, 1992). Deep-freeze storage (-18°C) certainly provides the conditions under which such highly ordered membranes can be expected to occur. Dry seeds and pollen survive deep freezing, but careful rehydration after thawing may be necessary (see section on rehydration). However, the formation of the gel phase domains in membranes entails the risk of lateral phase separation of membrane proteins. Because these membrane proteins cannot properly accommodate in a membrane in gel phase, they tend to aggregate (Platt-Aloia and Thomson, 1987). Lateral phase separation is considered fatal for the survival of cells, because phase-separated components cannot easily resume their original position in the membrane. In desiccation-tolerant dry cells this phenomenon has never been observed, even when membranes exist in gel phase (Hoekstra *et al.*, 1999; Leprince, personal communication). It is possible that the mobility of membrane proteins under these conditions is so slow that phase separation becomes extremely unlikely, at least on a realistic time scale.

#### *Dehydration to intermediate water contents*

As mentioned above, reduced membrane integrity is among the first noticeable signs of injury for desiccation-sensitive organ(ism)s. However, it occurs in recalcitrant seeds at already elevated moisture contents, usually above 0.4 g H<sub>2</sub>O/g dry weight, which is much higher than the moisture contents at which membranes pass into the gel phase and also much higher than the moisture contents at which glasses are formed during dehydration

(approximately 0.1 g H<sub>2</sub>O/g dry weight). It is therefore likely that hydrogen bonding interactions with sugars and immobilisation in the glassy state, as discussed above, are

more important for long term storage stability in the dry state than for desiccation tolerance *per se*.

Cells are more than just membranes filled with a sugar and protein solution. They are metabolically active entities that carry out reactions involving electron transfer. Hence, there are problems when water is removed because free radicals are generated by a partially repressed respiratory system (Leprince *et al.*, 1994). Insufficient control of metabolism (Leprince *et al.*, 1999, 2000) may lead indirectly to peroxidized lipids, free fatty acid accumulation, and loss of phospholipids, with increased  $T_m$ , reduced membrane integrity, and, probably, lateral phase separation as the results (Senaratna and McKersie, 1986; Hoekstra and Golovina, 1999). It might therefore be expected that desiccation tolerance be associated with an effective defence mechanism against free radicals. A controlled partitioning of amphiphilic antioxidants into membranes during dehydration may be part of just such a defence mechanism. For this to occur, the level of antioxidants and free radical scavenging or processing systems has to be sufficient. In general, seeds contain several systems that help to prevent oxidative damage. These mechanisms include enzymes such as superoxide dismutase, ascorbate peroxidase and glutathione reductase, and several antioxidant compounds including glutathione, tocopherols and ascorbic acid (Leprince *et al.*, 1993).

A further source of dehydration damage is the possible fusion between membranes, when insufficient carbohydrates are available. In liposomes, fusion commences in the absence of carbohydrates when the moisture content falls below 1.5 g H<sub>2</sub>O/g dry weight (Hoekstra *et al.*, 1997). The effect of such fusion on cells is dramatic, because compartmentalisation is lost at relatively high water contents, leading to self-digestion of the tissue. Lateral phase separation and further loss of membrane integrity may result. Fusion can be prevented by compatible solutes, such as soluble sugars, glycine betaine, and proline (Rudolph and Crowe, 1985; Anchordoguy *et al.*, 1987), which are often encountered in drought-tolerant plants and desiccation-tolerant organisms (Zhang *et al.*, 1982; Navari-Izzo *et al.*, 1992; Kets *et al.*, 1996). These compounds seem to act by their preferential exclusion from the immediate vicinity of macromolecular surfaces (membranes and proteins), which amplifies the macromolecule-water interaction (Arakawa and Timasheff, 1985). When the hydration shell of these macro-molecules is gradually lost below 0.2 - 0.3 g H<sub>2</sub>O/g dry weight, proline is not an effective protectant, but sucrose, for example, is (Crowe *et al.*, 1990).

### **Rehydration of the dry organ(ism) (imbibition)**

Successful imbibition without loss of viability can be considered as a part of the mechanism of desiccation tolerance. During imbibition, most initially dry desiccation-tolerant seeds leak intracellular solutes into the surrounding medium. This leakage generally declines soon after imbibition is complete and does not lead to severe damage (Senaratna and McKersie, 1983; Hoekstra and Van Roekel, 1988). This

leakage has recently been attributed to transiently increased membrane permeability associated with amphiphiles that are still residing in the membranes (Golovina *et al.*, 1998). Permeability is reduced to the low predrying levels when amphiphiles have

repartitioned from the membranes back into the cytoplasm. However, irreversible membrane damage and complete loss of viability can occur when dry anhydrobiotic organ(ism)s are plunged into cold medium. Prehydration from the vapour phase and preheating prevent this type of permanent damage. It has been shown that the damage occurs when the membranes are in gel phase before imbibition (Crowe *et al.*, 1989b; Hoekstra *et al.*, 1992). Humid air pretreatment and preheating also lead to melting of the membranes, from which it has been deduced that a membrane phase transition during imbibition is involved. It was recently suggested that it is the rigidity of membranes in gel phase which causes the inability of cells to withstand contact with water (Hoekstra *et al.*, 1999). The permanent leakage and death that occur when seeds are imbibed at low temperatures and low initial water contents are therefore believed to have a mechanical cause. A number of seeds, mostly of tropical origin are sensitive to imbibitional stress. Examples of such seeds are those of maize, sorghum, cotton, soybean and mungbean. In other anhydrobiotic systems, such as pollen, yeast and nematodes similar problems have been encountered (Van Steveninck and Ledebor, 1974; Crowe and Madin, 1975; Hoekstra and Golovina, 1999).

#### Ageing of dehydrated anhydrobiotic organ(ism)s

If organs such as recalcitrant seeds and intermediate seeds to a lesser extent, cannot be dried to the air-dry state without losing viability, they can be stored in a (partially) hydrated state at low, above zero °C temperatures to reduce their metabolic activity as much as possible. Alternatively these organ(ism)s may be cryopreserved. We do not discuss this approach further because cryopreservation is not in the scope of this thesis. For tropical recalcitrant seeds, low above-zero temperature storage is often not a good option. This is because of their chilling sensitivity, which may be caused by the elevated  $T_m$  of the membranes (Crowe *et al.*, 1989c). However, the longevity of organ(ism)s stored at elevated water contents is generally short. In a partially repressed metabolic system, oxidative damage is common, particularly if the cellular antioxidant systems fail to control oxidative injury (Benson, 1990; De Vos *et al.*, 1994).

If organisms survive dehydration to the air-dry state, their longevity is considerably extended, particularly at subzero temperatures. The formation of ice is excluded under those conditions because only nonfrozen water is present. Orthodox seeds can thus survive for periods up to 20 years in ambient conditions, with the average somewhere around 5 years. However, dry organ(ism)s nevertheless age under dry conditions. Metabolic reactions proceed in dry seeds, albeit at a very low rate, and nonenzymatic reactions can occur, both adding to deterioration with time (Hendry, 1993). Oxidative damage may also occur during storage (Benson, 1990, Hendry *et al.*, 1992; De Vos *et al.*, 1994) and rehydration (Puntarulo *et al.*, 1991, Simontacchi *et al.*, 1993). Seeds are endowed with several antioxidant systems that may help to prevent oxidative



damage, as previously discussed. The tripeptide glutathione (GSH), for example, can be oxidised during oxidative stress, forming GSSG that, in turn, is recycled using the NADPH-dependent enzyme GSH reductase (Benson, 1990). The extent of the decrease of GSH

and the increase of GSSG reflects the level of oxidative stress in dry seeds. In the case of deteriorating seeds, the GSSG is not properly reduced upon rehydration.

A reduced ability to retain solutes upon rehydration in aged organ(ism)s is one of the first manifestations of reduced integrity of the plasma membranes. In contrast, protein secondary structure is very stable, even long after death (Golovina *et al.*, 1997). During ageing there is a slow accumulation of free fatty acids and a loss of phospholipids, which is thought to be caused by the activity of free radicals (Van Bilsen and Hoekstra, 1993). Enzymes such as phospholipases may also be active in the dry state (Oliver *et al.*, 1997). In the course of ageing, the organ(ism)s gradually become more sensitive to imbibitional damage, which possibly reflects the increase in  $T_m$  of the membranes (Van Bilsen *et al.*, 1994). Ageing rates vary between different species and even within one species originating from different locations. The developmental stage of seeds at harvest and the length of the afterripening period (slow or fast drying) influence post-harvest storage longevity (Hong and Ellis, 1992; Sanhewé and Ellis, 1996). Extended periods of slow drying are thought to promote the production of antioxidant systems and protectants of macromolecular structures and thus to contribute to the extension of life span.

It has been shown that molecular mobility in the cytoplasmic glass also determines the rate of ageing (Buitink *et al.*, 1999). If molecular mobility is increased by heating or the addition of water as a plasticizer of the glass, the ageing rate is considerably increased. Ageing of organ(ism)s proceeds faster when their cytoplasm is out of the glassy state than when it is in it (Buitink *et al.*, 1998).

#### 4. Aim and outline of the research

Neem is an important multipurpose tree species in many developing countries, including Burkina Faso. Because of its many uses, the tree is in high demand from farmers. The tree is usually propagated via seeds in local nurseries. However, the seeds are thought to have a short storage life: seed viability is generally lost within 3 to 4 months after harvest. This rapid loss of viability reduces the availability of neem seeds for tree planting programmes. The National Research Council (1992) of the USA, stated that "*research is particularly needed to develop methods to extend the period of viability of neem seeds*". This project was therefore aimed at increasing the life span of neem tree seeds in storage, in order to facilitate and increase the use of neem in agroforestry and reforestation programmes in Burkina Faso and other Sahelian countries.

The storage behaviour of neem seed is controversial. It has variously been described as recalcitrant, intermediate, and orthodox (see overview by Poulsen, 1996; Hong *et al.*, 1996; and references therein). Some studies (Ouédraogo *et al.*, 1985, Ezumah, 1986) suggest that neem seeds have recalcitrant storage behaviour: viability was lost within a few months of storage and, according to Ezumah (1986), storage at ambient temperatures (about 28°C) was better than at 6°C. However, studies of the

Kenya Forestry Seed Centre (1992) in cooperation with the Royal Botanic Garden at

Kew, England, and of CIRAD-Forêt, France (Bellefontaine and Audinet, 1993) indicate that neem should be regarded as orthodox, because seeds from some lots were still able to germinate after storage for up to 10 years at low moisture contents (5%) and temperatures (+4 and -20°C). However, recent studies carried out independently at

the University of Reading, England (Dr. R.H. Ellis, personal communication) and at CPRO-DLO, Wageningen, The Netherlands (Gaméné *et al.*, 1994) suggest that storage behaviour is intermediate. These conflicting results may indicate that there is considerable variation in the extent of desiccation tolerance (and low temperature tolerance) between lots of neem seeds from Africa. The acquisition and extent of desiccation tolerance of neem seeds are probably determined by several factors including the maturation stage of fruit/seed at harvesting, and the temperature and rate of drying. Which of these factors it is that determines the level of desiccation tolerance is as yet unknown. In practice the life span of neem seeds appears variable and difficult to control. At CNSF (1993) for example, seed viability is frequently lost within a few months after harvest. However, the fact that seeds from some lots have been successfully stored for more than one year indicates that seed behaviour may potentially be orthodox and, consequently, that long-term storage may be possible. More detailed research into the intrinsic and environmental factors that determine the level of desiccation tolerance and life span of neem seed is essential to be able to develop the methods for extending the longevity of neem seeds.

In this project, the major mechanisms involved in desiccation tolerance and storage longevity were explored in an attempt to understand the loss of viability during handling and storage of neem seeds. Developing seeds and seeds from different sources were used as experimental materials for this purpose. Different combinations of water content and storage temperature were employed to identify optimum storage conditions. Further physiological, biochemical and biophysical experiments were carried out to study the causes of loss of viability upon dehydration, rehydration, and during storage.

During the entire period of seed development, the morphological and physiological characteristics of seeds were examined in relation to the germination capacity and storage capability (Chapter 2). The hypothesis that cell cycle events at harvest and storability are related was also tested on seeds of different maturity.

Storage experiments were undertaken using conditions that were expected to influence storage longevity (Chapter 3). This study used seeds from different sources in Burkina Faso (African Sahel) and from Sri Lanka (Asia) in a multifactorial approach. Seed survival was evaluated over periods of time. Because dried neem seeds became extremely sensitive to imbibition stress, particularly at low temperatures, experiments were carried out to characterise the damage with respect to conditions of rehydration preceding seed germination. The involvement of oxidative injury and changes in lipids and sugars in the loss of viability was investigated during dehydration and subsequent storage of the seeds (Chapter 4).

A study of seed-water relations was undertaken to understand the role and properties of water in neem seeds using differential scanning calorimetry and by constructing water sorption isotherms of different seed tissues (Chapter 5). The amounts of non-frozen water in neem embryonic axes and cotyledons were specified. This information is essential with respect to the potential for cryo-preservation of seeds. Thermal transitions were studied in the same tissues. State-phase diagrams were

constructed for neem seeds based on thermodynamic principles, which allow us to speculate about seed storage longevity. This study, together with the storage data from Chapter 3, offered new insights in the storability of neem seeds. These diagrams highlighted options for short-term or long-term storability.

The often-encountered chilling sensitivity led us to study the properties of neem seed membranes. In Chapter 6, particular emphasis was placed on the integrity of (plasma) membranes during dehydration, storage at different (also subzero) temperatures, and rehydration. Attempts were made to measure *in-situ* phase transitions and lateral phase separation of membranes. Electron microscopy techniques helped to visualise the nature and sites of the damage to membranes and provided evidence of lateral phase separation.

Finally we attempted to build a model for the storage longevity of neem seeds. This may be used to develop protocols to extend the longevity of tropical seeds that behave similarly. The general discussion (Chapter 7) describes recommendations for the handling and storage of neem seeds to improve their storage longevity.

This thesis describes a systematic attempt to understand the peculiar behaviour of neem seeds in storage and to gain insight into the causes of their rapid loss of viability.

## Cell cycle events in developing neem seeds: Are they related to intermediate storage behaviour?\*

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### Summary

Seeds of neem (*Azadirachta indica* A. Juss.), a multipurpose tropical tree, have limited desiccation tolerance. Whether their intermediate storage behaviour might be caused by an elevated level of 4C nuclei in the embryo at maturity was investigated. Morphological development of neem seeds was monitored on selected trees in Ouagadougou, Burkina Faso. Developing seeds were tested for germinability, and cell cycle events were determined by using flow cytometry and analysing the level of  $\beta$ -tubulin. Germination could occur after 8 weeks of seed development, but normal seedlings resulted only after 10 weeks or more. The change in fruit colour from green to yellow began after approximately 12 weeks of seed development. Immature, 4-week old embryos about 2 mm in length had 15% of their cells in G<sub>2</sub> phase and 60% in the G<sub>1</sub> phase of the cell cycle, respectively, as indicated by 4C and 2C nuclear DNA levels. During maturation, the proportion of G<sub>2</sub> cells declined to 3% at full maturity and slightly further after drying, and the proportion of G<sub>1</sub> cells increased to approximately 90%. A strong  $\beta$ -tubulin signal was observed in tips of young embryonic radicles and cotyledons, but a weak or non-detectable signal was found in 9-week-old ones and in those from green-mature and yellow fruits. Because DNA replication and  $\beta$ -tubulin levels were almost negligible at seed maturity, as in orthodox tomato seeds, it is suggested that these factors are not involved in the intermediate storage behaviour of neem seeds.

## Introduction

The developmental stage of seeds at harvest plays an important role in postharvest storage longevity (Hong and Ellis, 1992; Sanhewe and Ellis, 1996). Furthermore, this longevity depends on plant species and external factors such as moisture content, temperature and the composition of the gaseous atmosphere during storage. Seeds eventually deteriorate, even under optimal storage conditions (Chin and Roberts, 1980). Three main categories of seed storage behaviour are currently recognised. Roberts (1973) defined orthodox and recalcitrant seeds, as those that survive long term dry storage and those that cannot withstand dehydration, respectively. Recently, a third, intermediate category was identified comprising seeds that can withstand dehydration to a certain extent but have reduced longevity (Ellis *et al.*, 1990).

One factor that may be related to desiccation tolerance and storage longevity is the stage of cell cycle activity at seed desiccation (Bino *et al.*, 1992; Saracco *et al.*, 1995; Liu *et al.*, 1996; Van Pijlen *et al.*, 1996). Arrest of the cell cycle activity at the stage where the DNA content per nucleus is lowest has been suggested to render embryos more resistant to stress conditions (Deltour, 1985). During the cell cycle four different phases can be distinguished. In the S (synthesis) phase, DNA replication occurs. The S phase succeeds the G<sub>1</sub> (first gap) phase and precedes the G<sub>2</sub> (second gap) phase during which the amount of DNA per cell completely doubles. G<sub>2</sub> is normally followed by a cell division or mitosis. In diploid somatic cells at the G<sub>1</sub> phase, the nuclei contain 2C DNA, whereas the nuclei in the G<sub>2</sub> phase have 4C DNA. The 'C' stands for the 'constant' representing the DNA content of haploid tissue. After mitosis, the divided cells re-enter the G<sub>1</sub> phase.

It was observed that in desiccation-tolerant (orthodox) seeds from several plant species, in the mature stage (Bino *et al.*, 1992), or earlier during seed maturation (Bino *et al.*, 1993), most nuclei contain 2C DNA. This indicates that the cell cycle activity of the seed is arrested predominantly in the G<sub>1</sub> phase and marks the end of active growth and acquisition of desiccation tolerance (Liu *et al.*, 1996). As most metabolic activity in seeds is regulated by the availability of water (Roberts and Ellis, 1989), it can be expected that in orthodox seeds, cell cycle activity will subside along with the decline in water content during maturation. Under natural conditions, the moisture content in desiccation-intolerant (recalcitrant) seeds declines relatively little compared to that in orthodox seeds. If the cell cycle is regulated through water content, as suggested by Brunori (1967), the amounts of nuclear DNA in mature orthodox and recalcitrant seeds are expected to differ. Indeed, for recalcitrant *Avicennia marina* seeds, cell cycle activity is not or is hardly arrested upon shedding (D.J. Osborne, P. Berjak, pers. comm.) and this also holds for the recalcitrant sycamore (*Acer pseudoplatanus*) seeds (Finch-Savage *et al.*, 1998). Moreover, desiccation-tolerant *Daucus carota* somatic embryos that are produced by slow drying, have a reduced proportion of 4C DNA nuclei when compared with non-dried or rapidly dried somatic embryos (Tetteroo *et al.*, 1995). Rapid drying leads to loss of viability in these somatic embryos.

Another way to estimate cell cycle activity is by analysing the accumulation of  $\beta$ -tubulin (De Castro *et al.*, 1995). During seed imbibition, the structural protein,  $\beta$ -

tubulin, which is a major component of microtubules, accumulates prior to DNA replication.

Neem, (*Azadirachta indica* A. Juss.) seeds have been described as desiccation-tolerant (Roederer and Bellefontaine, 1989; Tompsett, 1994), moderately desiccation-tolerant but chilling-sensitive (Sacandé *et al.*, 1997), and desiccation-sensitive (Ezumah, 1986; Maithani *et al.*, 1989). These discrepant findings may be due to differences in developmental stage between the seed lots analysed, because it is difficult to estimate the developmental stage accurately on the basis of shape, size and colour of the fruits. More detailed information is thus required on the physiological maturity of the embryos. Furthermore, it is of interest to study whether the poor storability of neem seeds is related to an arrest in the G<sub>2</sub> phase of the cell cycle.

In the present paper we investigated the morphological characteristics, the germinability and the cell cycle activity of neem seeds during development and at maturity. The data on DNA levels and the kinetics of  $\beta$ -tubulin accumulation at seed maturity are discussed in relation to the intermediate storage behaviour of neem seeds.

## Materials and methods

### *Plant material*

Neem seed development was monitored on selected trees during the production period from February to June 1996 at Ouagadougou Protestant College in Burkina Faso. Hand pollination and labelling of individual flowers are difficult in neem trees due to their panicle structure and the small flower size. Hence, a different method was used to estimate pollination dates. As pollination results in corolla disintegration (Gilissen and Hoekstra, 1984), this recognisable event was chosen as a marker of zero time of fruit and embryo development. Because the period of seed development is relatively long (13 to 15 weeks), small errors in the determination of the exact number of days after pollination are not overly important. Small branches bearing fruits at different stages of development were harvested and investigated at Wageningen (Netherlands) within two days of picking.

### *Germination tests and moisture content determination*

The germinability of isolated embryos at various stages of development was tested using the fresh fruit samples. Directly after excision, embryos were placed on wet filter paper and incubated under photoperiodic conditions of 6 h in the light at 30°C and 18 h in the dark at 20°C. Fruit and embryo sizes were measured with a Vernier calliper.

Germination tests (replicate of 50 seeds each) and moisture content determinations were carried out according to the conditions recommended by the International Seed Testing Association (ISTA, 1993). Seedling quality was scored as normal when primary leaves had developed and secondary roots were visible. Data were analysed statistically using ANOVA.

*Determination of the amount of nuclear DNA*

Quantities of nuclear DNA were analysed in the course of seed development using radicle and cotyledon tips. The tips (1 mm) from 5-6 embryos were chopped with razor blades into 1 ml of nuclear isolation buffer (0.2 M mannitol, 10 mM Mes, 10 mM NaCl, 10 mM KCl, 10 mM spermine tetrahydrochloride, 2.5 mM Na<sub>2</sub>-EDTA, 2.5 mM dithiothreitol (DTT), 0.05% v/v Triton X-100, pH 5.8). The mixture was sieved through 88 µm nylon mesh, and the filtrate was collected in test tubes. Then 0.04 ml of 1 mg ml<sup>-1</sup> propidium iodide (Molecular Probes, Eugene, OR, USA) solution was added. Propidium iodide binds to DNA to give fluorescence, thus enabling measurement of the nuclear DNA quantities. The DNA content of isolated nuclei was measured 10 min after sample staining using a flow cytometer (Coulter Corp, Miami FL, USA, model Epics XL-MCL). Excitation of DNA-bound propidium iodide was performed by a 488-nm Argon Ion Laser and fluorescence was detected over the range 605-635 nm. MultiCycle for Windows Cell Cycle Analysis software version 3.0 (Phoenix, Flow Systems, Inc., San Diego, CA, USA) was used for curve fitting of the fluorescence frequency distributions.

*Detection of β-tubulin*

Western blots were used to detect the presence of β-tubulin in extracts of 1 mm tips of radicles and cotyledons from seeds at different stages of development. After excision, 30 fresh radicle or cotyledon tips were transferred into Eppendorf tubes, frozen in liquid nitrogen and subsequently ground to a powder using an Ultraturrax (Janke & Kunkel, Staufen, Germany). Protein extraction, analysis and electrophoresis (PAGE) were performed according to the method of De Castro *et al.* (1995). Briefly, 100 µl of modified Laemmli buffer (SDS-PAGE), consisting of 1 mM Tris-HCL, 12.5% (v/v) glycerol, 1.5 mg ml<sup>-1</sup> DTT and 2% (w/v) SDS, pH 6.8, were added directly to the frozen powder. After mixing, the samples were boiled for 10 min and centrifuged for 7 min at 17,000 g.

The protein concentration of the supernatant was measured following microprotein assay procedures (Bio-Rad, München, Germany, cat. no. 500-0006) modified according to De Castro *et al.* (1995), using bovine serum albumin as the standard.

Samples containing 30 µg of protein were loaded onto a precast 7.5% SDS homogenous ExcelGel (Pharmacia, Uppsala, Sweden, cat. no. 18-1016-86) for PAGE and separated (80 minutes at 600 V, 50 mA, as recommended by the manufacturer). Three different concentrations (1, 10 and 30 ng) of pure bovine brain tubulin (Molecular Probes, Eugene, OR, USA) were used as reference samples. After PAGE, proteins were electrotransferred overnight from the gel to a Hybond-polyvinylidene difluoride membrane (0.45 µm, Amersham International, Amersham, UK) using a Novablot electrophoretic transfer unit (Pharmacia, Uppsala, Sweden) operating at 0.8 mA/cm<sup>2</sup> and 30 V at 4°C. The transfer buffer consisted of 25 mM Tris, 192 mM glycine and 10% (v/v) methanol (pH 8.7).

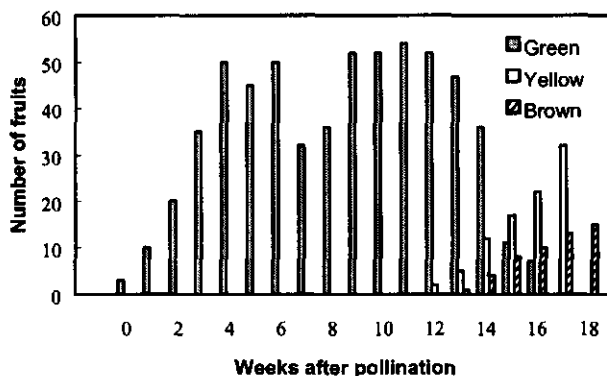
All steps of the immunodetection procedure were performed at room temperature with gentle agitation on a roller incubator. After blotting and subsequent washing in

Tris-buffered saline (TBS, pH = 7.5), the membranes were blocked in 1% (w/v) blocking solution (Boehringer, Mannheim, Germany, cat. no. 10-96-176) for 1 h and probed with 1  $\mu\text{g ml}^{-1}$  mouse monoclonal anti- $\beta$ -tubulin antibody (Boehringer, Mannheim, Germany, clone KMX-1 cat. no. 1111876). The membranes were washed three times in large volumes of TBS and subsequently with blocking solution. Thereafter, the membranes were incubated with 50 mU  $\text{ml}^{-1}$  peroxidase-conjugate secondary antibody, diluted in 0.5% (w/v) blocking solution for 1 h. For detection of peroxidase activity, the immunoblot was incubated with a premixed detection solution consisting of a 100:1 mixture of prewarmed substrate solution A and starting solution B (Boehringer Mannheim, Germany, cat. no. 1-500-694), for 1 min, and fitted into a film cassette between two pieces of overhead sheets. Then several sheets of photographic films (Hyperfilm-ECL, Amersham International, Amersham, UK) were exposed for periods of 20 s to 30 min under safelight in a dark room, and developed according to the manufacturer's protocol.

## Results

### *Characterisation of seed development*

Neem seed development was monitored weekly on selected branches of three different trees during the flowering period. The flowers withered in the second week after opening, after which fruits started to develop (Fig. 2.1). All fruits present during the observation period were counted. The transient decrease in average number of fruits per branch is due to the abscission of some of the young fruits and the development of new fruits thereafter. Figure 2.1 shows that 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 by then.



**Figure 2.1.** Phenology of neem seed growth. Fruit formation starts with flower wilting in the second week after bud opening, which is referred to



as week zero after pollination. The data are means of the number of fruits formed on branches of three different trees.

In a separate trial we attempted to link the fruit and embryo length to the number of weeks of development. For that purpose, several hundreds of fruits were collected at each sampling date during the flowering season. The largest 20% of the fruits were selected, and the lengths of the fruit and its embryo (after excision) were measured. Widths are considerably less than lengths and more difficult to measure with precision. They were not taken into further consideration. Assuming that the largest fruits were from pollinations at the onset of the flowering season, the relationship between seed developmental period and length of the fruit and embryo was established (Fig. 2.2A). Embryos could be isolated from the 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. Thus, it can be seen that the developmental stage of the seed is not easily derived from the fruit length. However, other morphological characteristics of the fruit (endocarp) gave additional information on the developmental stage of the seed (Table 2.1). Before the fourth week, the fruits had a transparent gelatinous endocarp and distinctive embryos were not visible to the naked eye. From 4 weeks onwards, the embryo underwent a rapid extension up to ten weeks (Fig. 2.2A). Initially, we thought that the ratio of embryo length to fruit length would compensate for random variation in individual fruit/embryo size and would provide the best calibration curve for estimation of developmental stage. However, the coefficient of variation of this ratio was generally larger than that of embryo length, indicating that an above average size fruit does not automatically contain an above average size embryo. The curve of Figure 2.2A representing the embryo length during seed development was therefore used to estimate unknown times of development for individual seeds.

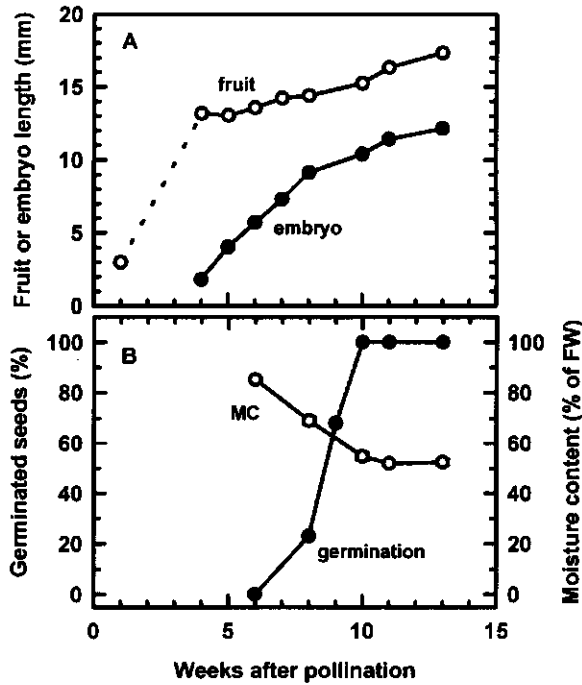
#### *Germination and water content*

Embryos acquired the capacity to germinate after 6 weeks, when embryos were half the length of the fruits (Fig. 2.2B). Full germinative capacity with 100% normal seedlings was reached at 10 weeks, coincident with the cessation of embryo elongation. Abnormal seedlings with defective hypocotyl extension were obtained at intermediate times of development. The moisture content (fresh weight [FW] basis) decreased from 85% at six weeks, to a stable level of approximately 51% after 10 weeks. This level was reached simultaneously with the acquisition of the maximum germination capacity.

#### *Nuclear DNA levels*

Flow cytometric analysis of DNA quantities in individual nuclei provided frequency distributions of nuclei having 2C and 4C DNA, which resembled those shown elsewhere (Bino *et al.*, 1993; Tetteroo *et al.*, 1995). The percentage of 4C DNA nuclei was calculated from the frequency distributions produced at regular time intervals during development and is presented in Figure 2.3A. It decreased from 15% of the total in young 4-week-old embryos to a stable low level of approximately 3% after 10 weeks of development. This stable level of 3% was reached coincident with the attainment of maximum embryo length (cf. Fig. 2.2A) and germination capacity (cf. Fig. 2.2B). It occurred at the still elevated, constant moisture content of 51% and also included nuclei of embryos from brown fruits. The curves of the 4C DNA

percentages with time of development were similar for both the nuclei from radicle tips and those



**Figure 2.2.** Relationship between seed developmental period and length of the fruit and its embryo (A). Data are averages of approximately 48 individual measurements. LSD ( $P=0.05$ ) was 0.53 and 0.25 mm for fruit length and embryo length, respectively. The decrease in moisture content (on a FW basis) and the acquisition of germination capacity during development are represented in (B). SE values never exceed symbol size.

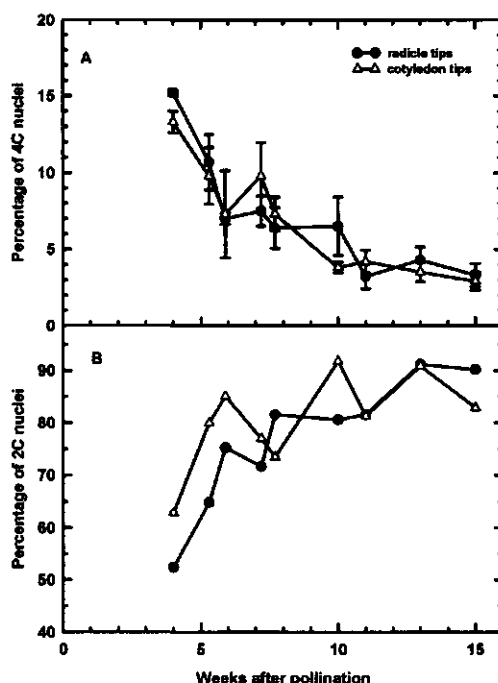
**Table 2.1.** Phenotype and characteristics of developing fruits and embryos of neem. Flower wilting associated with pollination marked the onset of the fruit developmental period (WAP = weeks after pollination).

WAP	Fruit	Endocarp	Embryo
4	green; firm	transparent	light green
5	green; firm	transparent	light green
6	green; firm	soft	light green
7	green; firm	± firm	dark green
8	green; firm	± firm	dark green
10	green; hard	± hard	dark green

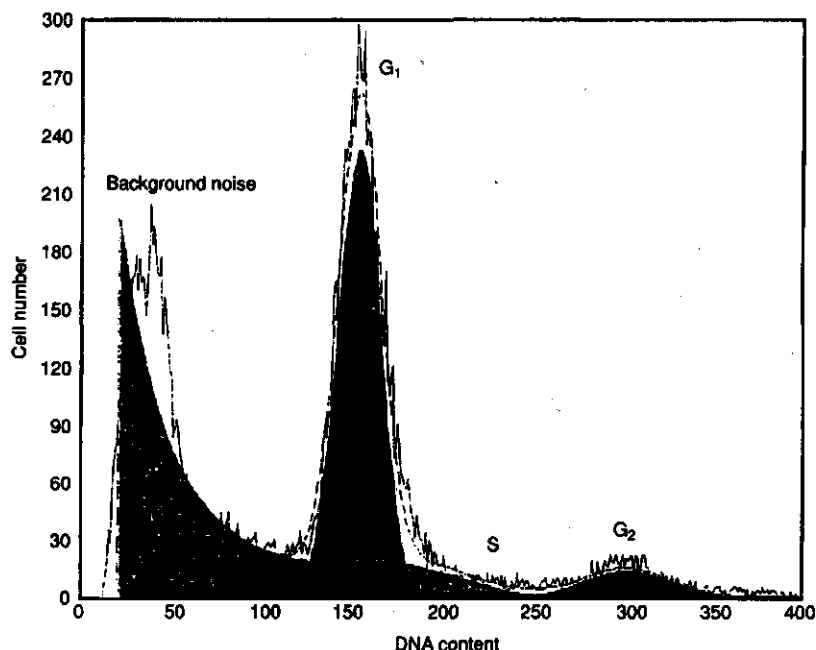
11	green; hard	hard	dark green
13	yellow; soft	hard	dark green

from cotyledon tips. A slight further decrease in 4C DNA quantity to 1.4% was observed upon drying the seeds (from yellow fruits) to 8% moisture content (on a FW basis). For comparison, the 4C DNA frequency was calculated for nuclei of the young first leaf of a seedling, giving a value of 14.8%, which is similar to that found for the 4-week-old embryos.

The proportion of the different fractions contributing to the frequency distributions was estimated in more detail using MultiCycle software. An example of such a curve fitting is given in Figure 2.4, representing the proportions of nuclei having 2C, S and 4C DNA levels. Using this software, 2C DNA percentages were calculated for the same set of samples as in Figure 2.3A with time of development (Fig. 2.3B). The percentage of nuclei with 2C DNA increased from approximately 60% at 4 weeks of development to 90% in mature embryos.



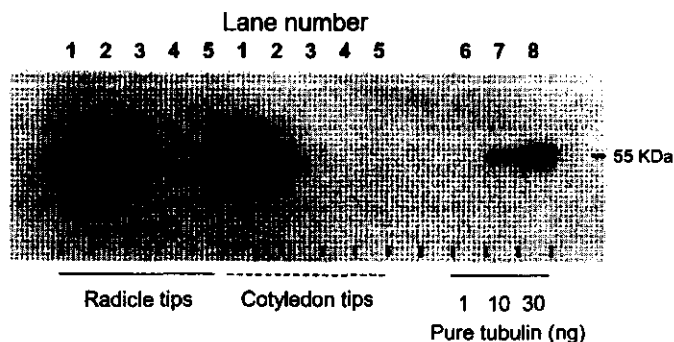
**Figure 2.3.** Nuclear 4C (A) and 2C (B) DNA amounts obtained by flow cytometric analyses of nuclei isolated from radicle and cotyledon tips of developing neem seeds. The number of weeks after pollination (WAP) was estimated from the embryo length curve in Figure 2.2A. Data in (A) are averages of 3 replicates  $\pm$ SE. Data in (B) were calculated with the software outlined in Figure 2.4.



**Figure 2.4.** Example of curve fitting of a flow cytometric curve (4-week-old radicle tip) using MultiCycle software for Cell Cycle Analysis, showing proportions of nuclei in G<sub>1</sub> (2C DNA), S and G<sub>2</sub> (4C DNA) phases of the cell cycle.

#### *$\beta$ -Tubulin accumulation*

A  $\beta$ -tubulin signal of about 55 kDa was immunodetected in extracts from both radicle and cotyledon tips of developing neem embryos (Fig. 2.5). Pure bovine brain tubulin was loaded onto the gels as the reference. The intensity of the  $\beta$ -tubulin signals in radicle tips decreased with time of development, being highest in 5- to 7-week-old embryos, the youngest tested. The signal intensity was much less in radicle tips of the 9- to 10-week-old embryos and lowest in those of mature embryos from yellow fruits. High intensity signals of  $\beta$ -tubulin were found in cotyledon tips from the 5- to 7-week-old embryos, whereas signals were lacking in the older specimens. The most intense  $\beta$ -tubulin signals coincided with the period with a high proportion of nuclear 4C DNA.



**Figure 2.5.** Western blot analysis of  $\beta$ -tubulin following SDS-PAGE of 30  $\mu$ g amounts of proteins extracted from embryo root tips and cotyledon tips of developing neem seeds. Lanes 1 to 5 (radicle tips and cotyledon tips) from 5.5-week-old, 6.8-week-old, 9.4-week-old, mature-green fruits and yellow fruits, respectively. The number of weeks after pollination was estimated from the embryo length curve in Figure 2.2A. Pure bovine  $\beta$ -tubulin (1, 10 and 30 ng) was used as the standard (55 kDa). The film was exposed for 1.5 min.

## Discussion

Most orthodox seeds (e.g. legumes and cereals) are shed in a relatively dry condition and can be further dried to low moisture contents (<5%) without losing viability. They can be stored successfully for many years at ambient temperatures. In contrast, recalcitrant seeds are characterised by the absence of maturation drying and are shed at moisture contents >50% (on a FW basis). They are readily damaged upon drying and are often sensitive to low temperatures (Roberts, 1973; Roberts *et al.*, 1984). Recalcitrant seeds are particularly common in the moist humid tropics. The so-called intermediate seeds survive drying to moderately low moisture contents (8-10% on a FW basis) but are often injured by low temperatures (Ellis *et al.*, 1990; Ellis, 1991). Due to their sensitivity to desiccation and/or low temperatures recalcitrant and intermediate seeds are reputed to have a relatively short life span.

Neem seed has been designated as being recalcitrant (Ezumah, 1986), but also as being orthodox (Tompsett, 1994). In our hands, neem seeds had intermediate storage behaviour and were cold sensitive (Gamené *et al.*, 1996; Sacandé *et al.*, 1997). It could be stored best, for at least one year, at approximately 8% moisture content and 15°C (M. Sacandé, unpublished results; Chapter 3). However, storage survival also

depended on the maturity of the seeds. Yellow fruits (about 13 weeks old) provided better quality seeds than did mature-green or brown fruits. One reason for the

controversy in the literature concerning the storage behaviour of neem seeds may be the fact that seeds had been harvested at different developmental stages.

Considering the relatively small increase in fruit length during development from 4 weeks after pollination onwards, it is difficult to estimate with precision the developmental state of an individual seed. Embryo length increased considerably between 4 and 10 weeks after pollination and is, therefore, a much better criterion for estimating the state of development (Fig. 2.2A). We could distinguish three main phases of neem seed development: (1) the early stage with active DNA replication up to 6 weeks after pollination, corresponding to histo-differentiation; (2) the median stage between 7 and 10 weeks of fruit formation, when cell cycle activity slows down and cell expansion takes place, mainly in the cotyledon, and (3) the maturation phase from about 10 weeks onwards, when cell cycle events come to a standstill. The maturation stage is also characterised by the attainment of maximum germination and a stable, relatively high moisture content of approximately 51% (on a FW basis) (Fig. 2.2B). Although seed desiccation is generally important in the transition from the developmental mode to the germination mode (Kermode and Bewley, 1985; Xu and Bewley, 1994), fresh excised neem embryos from 10-week-old fruits germinated and developed into normal seedlings (100%). During development, neem seeds did not go through maturation drying, as most orthodox seeds do.

Cell cycle activity subsides along with the decline in water content during maturation. Differential cessation of DNA synthesis and mitotic activity during dehydration may lead to an increased proportion of nuclei having 2C DNA (Brunori, 1967). This may be the reason for the low 4C and the high 2C DNA amounts in nuclei of orthodox seeds (Bino *et al.*, 1992; 1993; for a review see Bewley and Black, 1994) and in slowly dried somatic embryos (Tetteroo *et al.*, 1995). We found the highest proportion 4C DNA (ca. 15%) in embryos from immature green fruits containing transparent gelatinous endocarp (4 weeks after pollination; Fig. 2.3A). The proportion of 4C DNA decreased over the course of seed development and attained a stable low level (3%) at the maturation stage. This is surprising, considering the fact that this decrease in 4C DNA in the embryo cells occurred in the presence of ample water, that is most probably maintained by the fleshy fruit. The low 4C DNA frequency and the high 2C DNA frequency, indicating the arrest of the cell cycle at the presynthetic G<sub>1</sub> phase, may be a reflection of restrained metabolism in the embryos before they were dry.

Deltour (1985) postulated that nuclei having 4C DNA are more sensitive to stress. This was further elaborated by Saracco *et al.* (1995), who found that primed seeds with a high frequency of 4C nuclei are less resistant during storage. Under natural conditions, the moisture content in desiccation-intolerant (recalcitrant) seeds does not fall as much as in orthodox seeds, which may allow a higher proportion of nuclear 4C DNA in the former. When water contents are then lowered rapidly, the situation may be fixed (cf. Tetteroo *et al.*, 1995).

Overviewing the occurrence of 2C and 4C DNA quantities in nuclei of the different seed categories, a confusing picture emerges. Mature recalcitrant *Aesculus hippocastanum* seeds have cells mostly in the G<sub>0</sub>/G<sub>1</sub>-phase (SPC Groot, unpublished), whereas a number of orthodox seeds are arrested for a considerable extent in the G<sub>2</sub>-phase (Bino *et al.*, 1993). In the case of intermediate neem seeds, embryos from mature-green, yellow and brown fruits showed no significant differences in their generally very low 4C DNA frequencies (Fig. 2.3), but storage longevity differs considerably between them (Sacandé *et al.*, 1997; 1998; Chapter 3). From this we conclude that the differences in longevity between seeds from mature-green, yellow and brown fruits are not linked with possible differences in DNA amounts in the nuclei. Furthermore, we conclude that the intermediate storage behaviour of neem seeds is not due to elevated frequencies of 4C DNA in the mature hydrated and dry embryos. In this respect, results are similar to those obtained for desiccation-tolerant mature and dry tomato seeds, where the embryo radicle tips contain mainly nuclei with amounts of 2C DNA (5-11% of the nuclei in this tissue have 4C DNA) (Lanteri *et al.*, 1993; Bino *et al.*, 1992, 1993; Liu *et al.*, 1994).

The expression of  $\beta$ -tubulin, a protein required for passage through the cell cycle is associated with the onset of nuclear division following imbibition of tomato seeds and precedes visible germination (De Castro *et al.*, 1995). In dry tomato seeds  $\beta$ -tubulin is below detection levels. During neem seed development, the level of  $\beta$ -tubulin was correlated with the frequency of 4C nuclei and was very low in mature seeds. The decrease in  $\beta$ -tubulin signal was inversely proportional to the stage of maturity. Because the frequency of 4C nuclei and  $\beta$ -tubulin levels were almost negligible at seed maturity and followed the same pattern as in orthodox tomato seeds, we suggest that these factors are not involved in the intermediate storage behaviour of neem seed.

### **Acknowledgements**

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## A multifactorial study of conditions influencing the longevity of neem seeds\*

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### Summary

The longevity of neem, *Azadirachta indica* A. Juss., seeds from African Sahelian (Burkina Faso) and Asian (Sri Lanka) provenances, was studied over two years of storage under different conditions of moisture and temperature. After drying to equilibrium moisture content (MC) at different relative humidities at 20°C, seeds were placed in open storage at 20°C or hermetically sealed in packets at temperatures ranging from -20 to +20°C. There was hardly any difference in storage behaviour between seed batches/lots, whatever their provenance. Seeds originating from mature yellow fruits lived longer than seeds from green or brown fruits. In all storage experiments with seeds having MCs  $\geq 10\%$ , viability was preserved best at 10-15°C, indicating that neem seed is chilling (and freezing) sensitive. There was no survival longer than 2 years under these conditions. At MCs of 4-8%, seeds were considerably more tolerant to low temperature storage and had 40-60% viability after 2 years at all temperatures tested (-20 to +20°C). However, the seeds were sensitive to imbibitional stress, which could be alleviated by imbibition at temperatures of 25-30°C or above. The difficult storage behaviour of neem seed seems to stem from: 1) the sensitivity to low temperatures at MCs  $\geq 10\%$ ; 2) the extreme sensitivity to imbibitional stress after storage  $\leq 8\%$  MC; 3) underestimation of the water activity due to the high oil content of the neem seeds, causing unexpected metabolic stress in the higher MC and temperature range.



## Introduction

In arid and semi-arid tropical zones, high quality tree seeds are required both for reforestation and for the *in-* and *ex-situ* preservation of forest genetic resources. The seeds of many plant species are easy to handle and store for long periods of time, whilst others are very difficult and need special treatment to maintain viability. Easy maintenance of viability over extended periods of time depends on the seed's ability to withstand desiccation (Roberts, 1973; Roberts and Ellis, 1989; Kraak, 1993). The mechanisms of acquisition of desiccation tolerance, and the internal and external factors involved, are only partially understood (Kraak, 1993; Leprince *et al.*, 1993; Tetteroo *et al.*, 1996). The capacity of seeds to tolerate desiccation and survive for extended periods in the dry state depends on a number of intrinsic factors including the protection of macromolecular structures against dehydration stress (Crowe *et al.*, 1992; Ooms *et al.*, 1993; Crowe *et al.*, 1997a; Hoekstra *et al.*, 1997). Longevity further depends on a number of external factors such as temperature, moisture content (MC), and composition of the gaseous atmosphere.

Roberts (1973) defined seeds that can be dried to low moisture contents ( $\leq 5\%$ ) without damage as orthodox and those that are killed during dehydration as recalcitrant. Ellis *et al.* (1990, 1991) designated a third category of seeds that they called intermediate, e.g., coffee and papaya. Due to their sensitivity to desiccation and/or low temperatures, recalcitrant and intermediate seeds have a relatively short life span during storage. At present, often it is not possible to store them for one year, or even from harvest till the next suitable sowing season. Viability preservation during storage, e.g. in seed gene banks thus presents a major problem. In-depth knowledge of how to improve handling and storage would help meet the growing demand for such seeds for reforestation programmes and seed banks.

Neem, *Azadirachta indica* A. Juss., is an important multipurpose tree species used almost all over the tropics, particularly in reforestation in the Sahel region of Africa, as well as in rural areas of Asia and South America. Moreover, seeds and leaves are used to produce medicines, insecticides (azadirachtin), oil and cosmetics (manufactured margosa soap and toothpaste). The neem tree is a polygamous species, protandric bisexual flowers and male flowers occurring on the same individual (Schmutterer, 1995). The fruits start ripening approximately 10 weeks after flowering and are shed in the 15<sup>th</sup> week (Maithany *et al.*, 1989; Sacandé *et al.*, 1997). One kilogram of seeds contains approximately 3300 - 6500 individuals (Von Maydell, 1986; Tewari, 1992; CNSF, 1993). The seeds are non-dormant, but soaking them in water for a few hours before sowing helps to improve their germination rate and capacity. The first seeds start germinating after five days at temperatures of between 20 and 30°C (Sacandé *et al.*, 1996). Under natural conditions, the seeds, which mature during the rainy season, germinate on the ground within a few days of shedding.

The few papers published on the storage and physiology of neem seed report conflicting and contradictory results (see Hong *et al.*, 1996 for an overview; Poulsen, 1996). Neem seed has been described as recalcitrant (Ezumah, 1986; CNSF, 1993; Gunasena and Marambe, 1995; Msanga, 1996), losing viability within 1 to 4 months.

Other studies indicated that neem seed displays orthodox behaviour (Roederer and Bellefontaine, 1989; Dickie and Smith, 1992). Recent investigations with seed lots from Burkina Faso suggest that neem seed exhibits intermediate behaviour (Gamené *et al.*, 1996; Sacandé *et al.*, 1996). These apparent divergences in the ability to preserve viability may be due to differences in seed provenance, maturity at harvest, or conditions of storage. Alternatively, there may have been differences in the way in which seeds had been handled before storage or germinated.

In the present paper, the survival of neem seeds from African and Asian provenances over a two year period is described. In a multifactorial study, the effects of provenance, seed maturity, storage temperature and MC after drying were analysed. Special attention was paid to the mode of rehydration of dry samples. We aimed to determine the combinations of seed MCs and temperatures for optimum storage survival.

## Materials and methods

### *Plant material*

Neem seeds were harvested in Burkina Faso and in Sri Lanka in 1994 and 1995 (see Table 3.1 for details). All the seeds used in these experiments came from yellow fruits, unless stated otherwise. The fruits were picked by hand from a minimum of 25 mother trees, using the same methods in Burkina Faso and in Sri Lanka (FAO Neem Network, 1995). In Burkina Faso, fruits were collected from areas having widely different rainfall: Dori, Koupela, Ouagadougou and Bobo Dioulasso. In Sri Lanka, fruits were collected from one provenance in the northern dry zone in the Maha Illuppallama region. For experiments concerning the influence of stage of maturity, green, yellow and brown fruits were collected separately. Green fruits (10-12 weeks after pollination) contain physiologically mature seeds with a completely developed embryo and hard endocarp (Sacandé *et al.*, 1997); yellow fruits were harvested at approximately 12-13 weeks after pollination, and brown fruits at >13 weeks, just before shedding.

The preparation of seeds from fruits was carried out locally on the day of harvesting. The fruits were soaked in water and then rubbed with sand. After depulping, the seeds were cleaned with water and dried in the shade on a grid for two days. Mean temperatures in Ouagadougou (Burkina Faso) ranged between 25-40°C and between 35-60% relative humidity (RH); in Peradeniya (Sri Lanka) between 25-30°C and 50-80% RH. Seeds surrounded by an intact endocarp were then selected and sent by air cargo to Wageningen, The Netherlands, in cotton bags, arriving one week after harvest.

### *Determination of MC and viability*

The MC of the whole seed (including endocarp) was determined by monitoring weight loss during drying, and by the low-constant-temperature oven method for tree seeds, following the International Seed Testing Association rules (ISTA, 1993). Five seeds in duplicate were cut and weighed before and after drying at  $103 \pm 2^\circ\text{C}$  for 17 hours, and the MC was calculated as the percentage of water on a fresh weight (FW) basis.

**Table 3.1:** Characteristics of neem seed sources from locations in Burkina Faso and Sri Lanka

	Burkina Faso				Sri Lanka
	Dori	Koupela	Ouagadougou	Bobo Dioulasso	Maha Illuppallama
Latitude	0°	0.5° W	1.5° W	4.5° W	80-81° E
Longitude	14° N	12.2° N	12.5° N	11° N	7-8° N
Rain (mm/year)	350-400	600-700	650-750	900-1000	> 750
No of trees	35	25	25-35	25-33	30
Dates of harvest	-	-	28/4/94	27/4/94	-
	-	-	29/7/94	29/7/94	-
	25/7/95	12/11/95	25/7/95	25/7/95	30/8/95

The viability of the seeds was determined on the basis of the percentage of germinated seeds, with sample sizes from 45-100, depending on availability. Before the germination test, seeds were held for 24 hours at room temperature. The seeds were pre-soaked in tap-water for 4 hours at approximately 22°C before sowing in transparent germination boxes containing wet filter paper. The boxes with seeds were placed in an incubator which was set at 30°C during the day (8 hours) and 20°C during the night (16 hours). The filter paper was renewed every 8 days. Germination of the seeds was scored twice a week for at least four weeks, until all viable seeds had germinated. The seed was scored as germinated when the endocarp had opened and the radicle had emerged (ISTA, 1993). Seedlings were scored as being normal when the cotyledons were fully expanded for more than 75% of their area and the radicle was at least 2 cm long without infection.

Statistical analysis of the germination data was carried out using the  $\chi^2$ -test. The level of significance employed was 5%.

#### *Seed desiccation*

Upon arrival, the seed lots were divided into sub-samples that were placed at four different RHs at a constant temperature of  $20 \pm 2^\circ\text{C}$  until they reached equilibrium MCs (Fig. 3.1). The different RHs were obtained in a cabinet ( $10 \pm 2\%$  RH) and in closed drums with air circulating above saturated salt solutions (Rockland, 1960;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  for 32% RH,  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  for 53% RH and NaCl for 75% RH). In the course of the drying procedure, the MC of the seeds was regularly monitored, either by recording the weight loss, or by using the oven-drying method. Seeds having MCs that differed from equilibrium MCs were thus obtained. The level of desiccation tolerance was indicated by the percentage of seeds germinated.

#### *Storage experiments*

The effect of storage temperature on seed viability was investigated using seeds at different stages of maturity and from different locations that had been dried to between 4 and 38%, on a FW basis. A factorial combination of MCs and storage temperatures was applied as storage conditions. The seeds from both provenances, i.e. Burkina Faso (Koupela) and Sri Lanka, had similar histories with regard to all handling procedures.

They were dried and subsequently packed into laminated aluminium foil packets (8  $\mu$ m aluminium - 12  $\mu$ m Pol/25 PE) and hermetically sealed with an electric sealer that produces both pressure and heat. The seeds (on average 100 in each packet) were then stored at different constant temperatures. The high moisture seeds with 33 to 38% MC, were stored at 5, +10, +15 and +20°C. Seeds with approximately 4, 6, 8, 10 and 12% MC were stored at -20, -10, 0, +5, +10, +15 and +20°C.

Each treatment combination of MC, temperature and storage duration was represented by a sample in one packet. The entire content was used to determine the percentage of germinated seeds at storage intervals of three months, over the entire storage period.

## Results

### *Germination of seeds*

Maximum seed germination was obtained within 4 weeks of incubation in the germination cabinet. Nearly all (98%) of the seeds that germinated produced normal seedlings, for both fresh and stored lots (data not shown). All the non-germinated seeds were rotten. We did not attempt to control fungal growth in this study, although a few types of fungi (*Aspergillus*, *Fusarium* and *Penicillium*, C.K. Langerak, pers. com.) were observed on seeds during germination.

### *MC of seeds in different RHs*

After three weeks under the different conditions of RH at 20°C, the seeds were close to equilibrium MC (Fig. 3.1). These MCs varied between 3.8 and 13.3%, depending on the RH applied. The coefficient of variation of the MC under one RH condition was usually less than 8%. The rate of moisture loss for the samples from Burkina Faso and Sri Lanka was similar in each RH, and the final moisture levels between the provenances in each RH were not significantly different.

### *Desiccation tolerance*

Fresh seeds of approximately 35% MC from both provenances, having initial germination percentages of approximately 98%, were exposed to a range of RH conditions at 20°C. Moisture contents were determined at intervals over four weeks, and the seed germination capacity was tested. Figure 3.2 shows the effect of dehydration on the percentage of germinated seeds obtained in the germination test. Below 10% MC, germination decreased steeply, to reach a low value of approximately 35% at 4% MC. Slower drying rates by first drying for 6 weeks at 75% RH followed by drying at 32% RH, resulted in percentages of germinated seeds similar to those obtained after faster drying at constant 32% RH. The final MC appears therefore to determine the percentage of germinated seeds in the germination test. There were no major differences in sensitivity to drying between seeds from the two provenances. No seed lots died completely during drying to 4% MC.

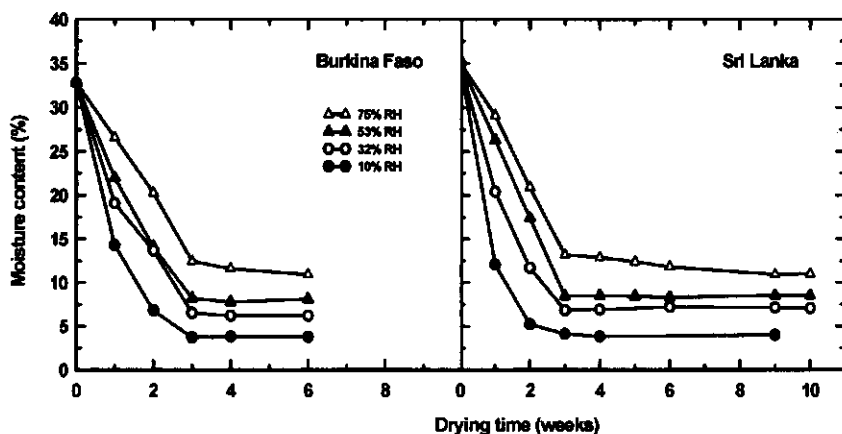


Figure 3.1. Progression towards equilibrium MCs under different RHs at 20°C of neem seeds from Burkina Faso and from Sri Lanka. The data points are means of five replicates of five seeds. LSD values at  $P=0.05$  for the equilibrium MCs was 0.56 for the Burkina Faso material, and 0.71 for the Sri Lankan material.

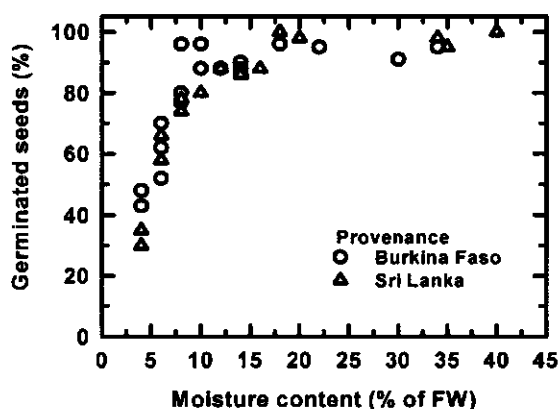


Figure 3.2. The effect of dehydration on the percentage of germinated neem seeds. Seeds were exposed to a range of RH conditions at 20°C and sampled over a four week period. For each data point 100 seeds were tested. Data points are significantly ( $P \leq 0.05$ ) different when they diverge by 15% or more ( $\chi^2$ -test).

#### *Effect of MC (in open storage) on storage longevity*

Figure 3.2 implies that drying to approximately 8% MC does not significantly affect viability. In order to investigate the effect of MC on storage longevity, a seed lot from Burkina Faso was stored at 20°C in RH conditions of 75%, 53% and 32% (Fig. 3.3). These RH conditions resulted in MCs of 12.4%, 8.0% and 6.4%, respectively. The germination capacity of all the samples decreased during storage. The seeds stored at 75% RH all died within 12 months. While the 75% RH condition led to a pattern of viability loss typical of fast ageing seeds, i.e. a cumulative normal distribution of negative slope (Ellis and Roberts, 1980), the seeds stored under the drier conditions exhibited biphasic patterns of viability loss. After an initial rapid fall in germination with drying during the first 3 months of storage, the remainder of the seeds retained viability for at least 12 months. This suggests that there may exist two populations of seeds which differ in their ability to survive dry storage.

#### *Effect of geographical origin of seeds on storage longevity*

To analyse whether the biphasic pattern of viability loss observed during storage also occurs with seeds from other locations with different climatic conditions, seed lots from three locations in Burkina Faso were exposed to 53% RH for three weeks at 20°C (giving 8.3% MC), then hermetically sealed in laminated aluminium foil packets and stored at 20°C. Figure 3.4 shows the similar biphasic pattern for the seeds regardless of their provenance, suggesting that the biphasic pattern is not associated with specific location or climate. Figure 3.4 also shows how hermetic storage after equilibration to 53% RH for 3 weeks gave almost identical survival curves as open storage (cf. Fig. 3.3).

#### *Effect of stage of maturity of seeds on storage longevity*

Because the maturity of the seeds at harvesting might effect the rate of ageing, we investigated the storage behaviour of seeds from green, yellow and brown fruits, harvested at 10-12, 12-13 and >13 weeks after pollination, respectively. The seeds were stored at 20°C, after exposure to 53% RH at 20°C for 3 weeks (giving 8.2% MC) and enclosure in the laminated aluminium foil packets. Figure 3.5 shows that the retention of viability during storage at 20°C was better with seeds from yellow fruits than from green or brown fruits. Also in seed lots from other locations and crop years, the seeds from yellow fruits survived longer during storage than those from green or brown fruits (data not shown).

#### *Effects of temperature on storage longevity*

Freshly isolated seeds from both Burkina Faso and Sri Lanka had MCs of 33 and 38%, respectively. When these seeds were sealed in packets to reduce further water loss and exposed to temperatures ranging from 5-20°C, they completely lost viability over storage periods of one and three months. After the first month, viability was lower at 5°C and 20°C than at 10 and 15°C (Fig. 3.6). We interpret this apparent temperature optimum to mean that neem seed is cold-sensitive, whilst seeds at 20°C metabolise at a high rate and may have produced toxic by-products because they were sealed in a

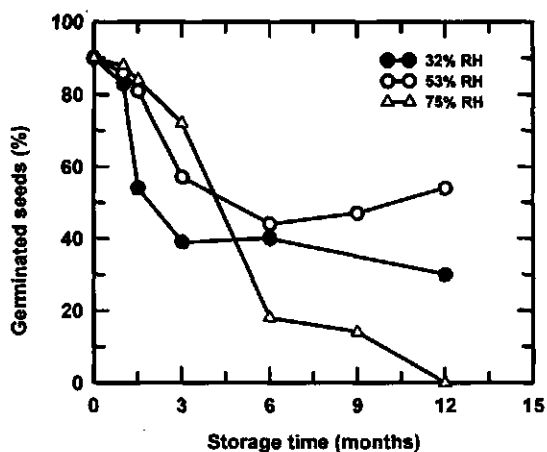


Figure 3.3. The effect of the RH during open storage at 20°C on the percentage of germinated seeds (Ouagadougou provenance). For each data point 100 seeds were tested. Data are significantly ( $P \leq 0.05$ ) different when they diverge by 15% or more ( $\chi^2$ -test).

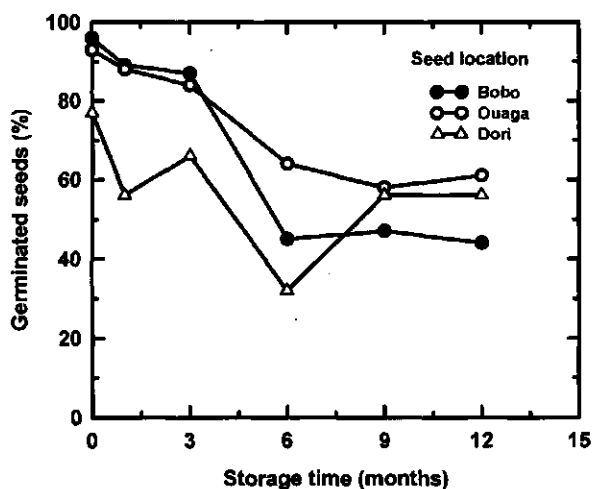


Figure 3.4. Effect of hermetic storage at 20°C (after previous exposure to 53% RH for 3 weeks, giving 8.3% MC on average) on percentage of germinated seeds from different geographical locations in Burkina Faso. The seeds were collected at Dori, Ouagadougou and Bobo Dioulasso. For each data point 100 seeds were tested. Data are significantly ( $P \leq 0.05$ ) different when they diverge by 15% or more ( $\chi^2$ -test).

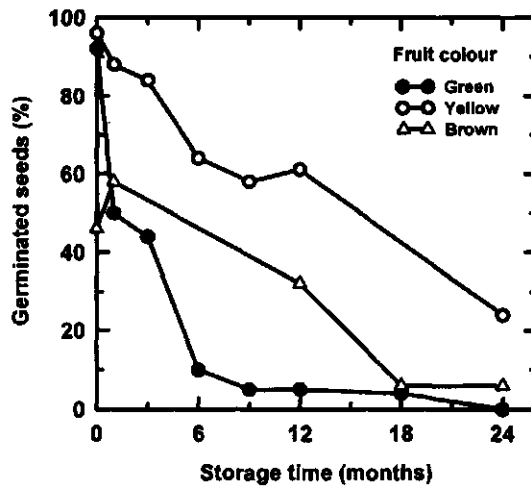


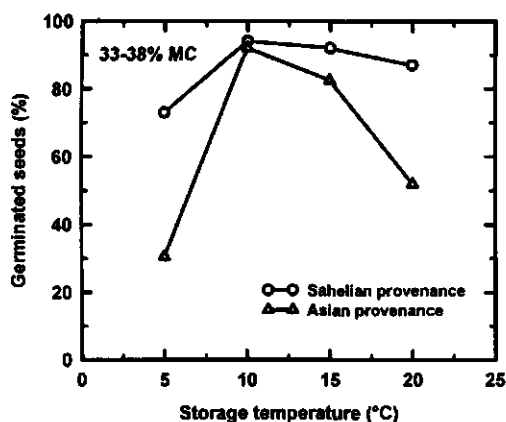
Figure 3.5. Influence of the stage of maturity of neem seeds on the percentage of germination during hermetic storage at 20°C over a two year period (after previous exposure to 53% RH for 3 weeks). The seeds were from green (young), yellow (mature) and brown (overripe) fruits harvested in Ouagadougou. For each data point 100 seeds were tested. Data points are significantly ( $P \leq 0.05$ ) different when they diverge by 15% or more ( $\chi^2$ -test).

packet. Storage at lower MCs would therefore be expected to increase storage longevity.

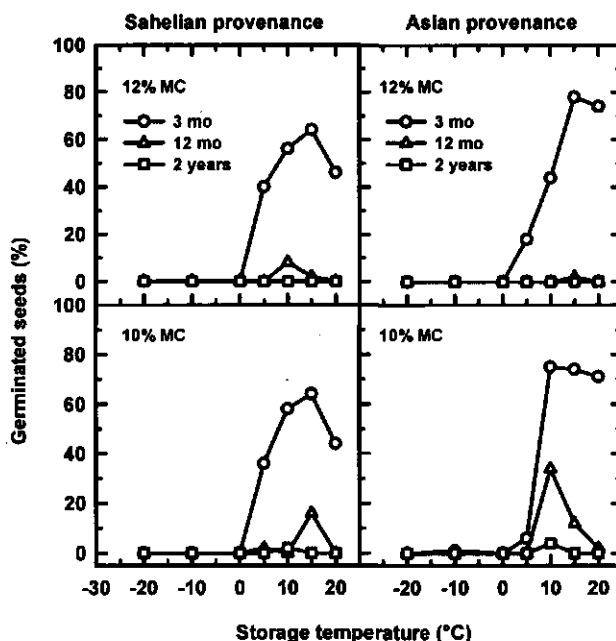
Figure 3.7 shows that storage longevity improved when the seeds were dried to 10 or 12% MC before hermetic storage. However, these partially dried seeds were still cold-sensitive. Whilst viability was between 60 - 80% after 3 months of storage at 15°C, it was 0% at or below 0°C. At 12 months of storage, most 12% MC seeds had died, whereas better survival was found in the 10% MC seed lot (the 10 and 15°C treatments). Increasing the soaking temperature to 30-35°C before incubation, which might reduce imbibitional damage (see next paragraph), did not improve the survival results (data not shown).

The data presented in Figures 3.4 and 3.5 suggest that longevity for up to 12 months is achievable at 20°C at MCs of approximately 8%. To analyze whether seeds of 8% MC or less are still sensitive to low temperatures, samples were dried to approximately 4, 6 or 8% MC, hermetically sealed, and stored for 2 years at temperatures ranging from -20 to +20°C. Survival ranged from 40 to 60% for all storage temperatures and MCs tested for the Burkina Faso samples (Fig. 3.8A). The sensitivity to low-temperature storage was apparently lost in these dry samples. The Sri Lankan provenances still exhibited some degree of low-temperature sensitivity during storage at low MCs (Fig. 3.8B). It should be noted that with these dry samples the soaking was performed at 35°C, because soaking at 22°C with these low MC samples reduced the percentage of seeds that germinated.

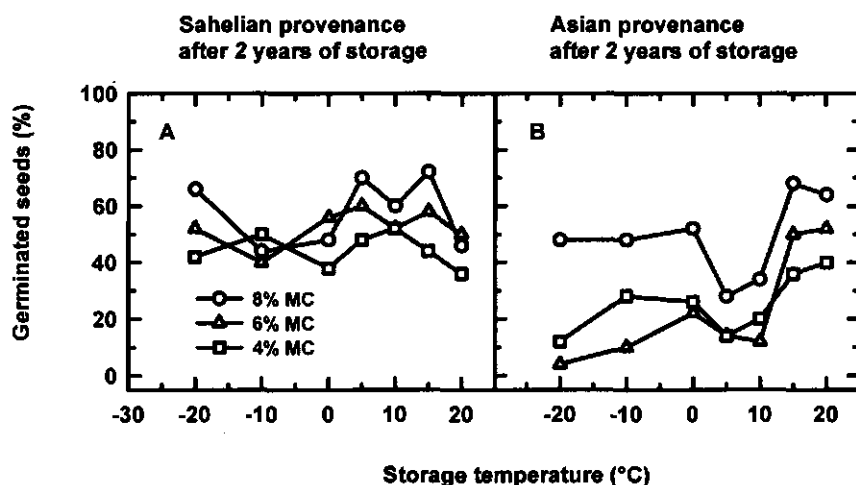




**Figure 3.6.** Effect of storage temperature on viability of neem seeds after one month of hermetic storage. The seeds were from Sahelian (33% MC, Burkina Faso) or Asian (38% MC, Sri Lanka) provenances, had initial viabilities of 95 and 98%, respectively, and had not been exposed to a drying treatment. For each data point 100 seeds were tested. Data points are significantly different ( $P \leq 0.05$ ) when they diverge by 15% or more ( $\chi^2$ -test).



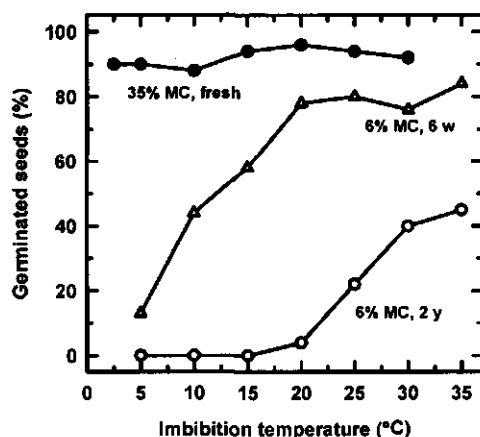
**Figure 3.7.** Effect of temperature on longevity in hermetic storage of neem seeds at 12 and 10% MC (established by previously exposing them to 75% and 53% RHs at 20°C for 3 weeks, respectively) before hermetic storage. The seeds were from Sahelian and Asian provenances, with initial viabilities as in Fig. 3.6. For each data point 100 seeds were tested. Data are significantly different ( $P \leq 0.05$ ) when they diverge by 15% or more ( $\chi^2$ -test).



**Figure 3.8.** Effect of temperature on percentage germinated neem seeds dried to 4, 6 and 8% MC (after previous exposure to 10%, 32% and 53% RHs at 20°C for 3 weeks, respectively) before hermetic storage. The seeds were from Sahelian (A) and Asian (B) provenances, with initial viabilities as in Fig. 3.6. The temperature of imbibition was 35°C. For each data point 50 seeds were tested. Data are significantly different ( $P \leq 0.05$ ) when they diverge by 22% or more ( $\chi^2$ -test).

#### *Imbibitional damage*

Since it appeared during the experimentation that the imbibition temperature of 22°C might be suboptimal, particularly for aged dried seeds, a more detailed study was conducted to reveal possible imbibitional damage. Figure 3.9 shows the percentage of germinated seeds in a fresh sample and two dried samples (6% MC), after soaking for 4 h at a range of temperatures. Germination percentage improved for the 6% MC samples with increasing temperature of soaking to reach an optimum between 20–35°C for non-aged and 30–35°C for aged seeds. The fresh sample (35% MC) was not sensitive to 4 h of cold soaking, the germination percentage remaining at around 90% for all tested temperatures.



**Figure 3.9.** Effect of imbibition temperature (over 4 hours) on germination behaviour of neem seeds from Ouagadougou. Following this imbibition period, the seeds were incubated at 20–35°C for germination. The history of the seeds tested was freshly harvested (35% MC), or stored dry (approx. 6% MC) for 6 weeks or 2 years. The initial viability of all the seed lots was >90%. For each data point 45 seeds were tested. Data are significantly different ( $P \leq 0.05$ ) when they differ by 25% or more ( $\chi^2$ -test).

## Discussion

Conflicting reports on how to store neem seeds and on their sensitivity to dehydration have appeared in the literature. Survival of seeds with high MCs (30–40%) is in the range of a few to 12 weeks (Chaisurisri *et al.*, 1986). Partly dried seeds with 10–12% MC can be stored for a only few months (Ponnuswamy *et al.*, 1990), which led to the idea that neem seed is recalcitrant. However, the storage of seeds with low MCs of 5 to 8% and at 4°C resulted in 40% viability after a number of years (Roederer and Bellefontaine, 1989; Dickie and Smith, 1992; Bellefontaine and Audinet, 1993). Nevertheless, neem seed does not behave like truly orthodox seed that can tolerate drying to very low MCs (1–2%) and storage at low temperatures. A factor related to maturity or provenance could be involved in this unusual storage behaviour.

To identify the optimum conditions for harvest, handling and storage of neem seeds, we subjected a number of seed lots of different origins and stages of maturity to various treatments. Seeds that were obtained from green, yellow, and brown fruits differed in their response to storage, those from yellow fruits being superior (Fig. 3.5). We found that decreases in germination capacity occurred during both drying and subsequent storage (up to 2 years). Since a decrease rather than a complete loss of viability was observed, neem seed cannot be considered to be recalcitrant. Truly recalcitrant seeds would have been completely unable to survive drying to 4% MC.

However, the significant loss of viability indicates that neem seed poses more problems with drying and storage than an orthodox seed type would do.

To be able to store neem seeds safely for 12 months or longer, their MCs should be reduced to approximately 8% or below (Figs. 3.3, 3.4 and 3.5). In our study the optimum storage conditions were 8% MC at 10–15°C (Fig. 3.8). These conditions are in accordance with those mentioned by Hong and Ellis (1998), who found 40% germination after 8 months of storage. Other studies using neem seeds exclusively from Burkina Faso support our results (Gamené *et al.*, 1996; Sacandé *et al.*, 1996).

In many of the storage experiments with the driest seeds (4% and 6% MC), damage seems to have occurred during drying to equilibrium MCs, rather than during subsequent storage. It appears that two populations of seeds exist in one sample: one that deteriorates fast during equilibration to low MC and another that performs better. This biphasic viability loss during storage was observed with all the seed lots, irrespective of provenance. All the seed lots dehydrated at about the same rate, and no particular differences between samples, seed types or provenance were noticed during the drying process. We suggest that this biphasic pattern of viability loss during storage might be associated with problems during rehydration rather than during dehydration, as we discuss in the next sections.

Chilling sensitivity was particularly prominent in our material (Figs. 3.6 and 3.7), and is also known in other intermediate and recalcitrant seed types of tropical origin (Corbineau and Côme, 1988; Chin *et al.*, 1989; Tompsett, 1994). The chilling sensitivity of the fruits and leaves of tropical plants has been attributed to a conformational transition in cell membranes from the liquid crystalline to the gel phase (Lyons *et al.*, 1979; Wang, 1982), often followed by lateral phase separation of the components of the membrane (Platt-Aloia and Thomson, 1987). Diagnostic for such phenomena are leakage of cytoplasmic solutes from the cells and the dysfunction of membrane proteins (Yamawaki *et al.*, 1983; Yoshida *et al.*, 1986). The transition temperature ( $T_m$ ) of membranes in chilling-sensitive tropical plants has been estimated at approximately 10°C (Crowe *et al.*, 1989c), which means that membranes are in the liquid crystalline phase under ambient conditions. In neem seeds, storage below 10°C was considerably less successful than above; 10 and 15°C being optimal (Figs. 3.6 and 3.7). This suggests that neem seeds have a similar  $T_m$  of membranes at about 10°C.

When the MC in anhydrobiotic organisms falls below the level at which membranes are fully hydrated (0.20 g H<sub>2</sub>O/g DW = 25% MC), the  $T_m$  of membranes tends to increase (Hoekstra *et al.*, 1989; Crowe *et al.*, 1997b; Hoekstra *et al.*, 1997). If the  $T_m$  *per se* were the limiting factor for survival on drying, then successful storage of neem seed lots having MCs of less than 10% would be possible only at elevated temperatures. However, Figure 3.8 shows that under dry conditions the seeds survive even at subzero temperatures. We suggest that when membranes are in the gel phase, additional deteriorative processes can occur in seeds having MCs  $\geq 10\%$ , which do not take place in seeds having MCs  $\leq 8\%$ . In this respect, internal leakage or phase separations may be involved. In the drier seeds (MCs  $\leq 8\%$ ), internal leakage will be impeded due to the high viscosity of the cytoplasm.

One consequence of elevated  $T_m$  in dry anhydrobiotes is the increased chance of damage during imbibition, when plasma membranes turn from the gel phase into the liquid crystalline phase (Crowe *et al.*, 1989b; Hoekstra *et al.*, 1992). It is just this sensitivity to imbibition that was found in the dry neem seeds (6% MC; Fig. 3.9). Even imbibition at 22°C was suboptimal, which may explain the fusion of oil bodies seen after rehydration of artificially dried seeds (Leprince *et al.*, 1998). We therefore conclude that dry storage between 4 and 8% MC maintains a considerable level of viability, if imbibition is performed with caution, i.e. at least at 25°C or above. Storage of dry seeds can be performed safely at subzero temperatures (Fig. 3.8). The sensitivity to imbibitional stress may explain the conflicting reports in the literature on longevity during dry storage. The seeds may have been killed during imbibition rather than during the storage period. This also may be the cause of the biphasic pattern of viability in dry storage as we observed in Figures 3.3 and 3.4.

Figures 3.6 and 3.7 concern storage experiments with seeds that had elevated MCs ( $\geq 10\%$ ). Under these conditions, survival is limited by active metabolism, particularly at the higher end of the temperature range. It should be noted that in neem seeds, the percentage MC underestimates the water activity in the cytoplasm, due to the high oil content of approximately 50% (Leprince *et al.*, 1998; National Research Council, 1992; Chapter 5).

Taking all of these results together, it seems that the difficult storage behaviour of neem seed stems from: (i) an underestimation of metabolic rates at ambient temperatures when storage is performed at MCs in the range of 10-12%, due to the relatively high water activity in this oily seed (50% of the DW); (ii) the sensitivity to chilling when seeds are stored above 10% MC; and from (iii) the extreme sensitivity to imbibitional stress after storage at MCs of 8% and below.

### Acknowledgements

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## Is oxidative stress involved in the loss of neem seed viability? \*

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### Summary

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 their rapid loss of viability were investigated on different seed lots during exposure to 32% and 75% RH at 20°C. Within six 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 turgid as visualized by low temperature scanning electron microscopy images of fractured axes. The glutathione oxidation status was used to estimate oxidative stress during storage. The 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 oligosaccharide to sucrose was 0.19 on average in dehydrated neem seeds. The data suggest that the storage behaviour of neem seed has features that characterize it as orthodox.

## Introduction

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. 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). Intermediate seeds survive drying to moderately low MCs, and are often injured by low temperatures (Ellis *et al.* 1990, 1991). 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 the use of its seeds for propagation purposes is hampered by its reputedly intermediate type of storage behaviour. 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, it has been demonstrated that 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. 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 stressors like chilling, freezing, or exposure to environmental pollution (Bowler *et al.*, 1992). Seeds have several antioxidant systems that may help to prevent oxidative damage. Those 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, high oxidation may 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 intramolecular disulfide bridges (S-S) (Navari-Izzo *et al.*, 1997). During dry storage of plant systems, GSH can be oxidised 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 versus 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 have been suggested to depend on the ability of seeds to produce protecting sugars that stabilize biomembranes (Crowe *et al.*, 1992) and proteins (Carpenter and Crowe, 1989; Wolkers *et al.*, 1998). The sugars have been hypothesized to replace the disappearing water by hydrogen bonding to the membrane lipid polar headgroups and the proteins, thus keeping the native structure intact. 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 analysed 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 two 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 ( $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  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 removed endocarp) were weighed, dried at 103°C for 17 hours 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 and then placed on wet filter paper in germination boxes that were placed in the light during 8 hours/day at 30°C and in

the dark during 16 hours/day at 20°C. The germinated 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 hours 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. 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 operating at maximum speed. 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 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<sub>2</sub>HPO<sub>4</sub> (giving a pH of approximately 7.0) and 25 µl of the DTNB solution, the absorbance was read after one min at 412 nm (absorption coefficient = 13,600). 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 the 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  $\mu$ l 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  $\mu$ l of supernatant was mixed with 4  $\mu$ l of 2-vinylpyridine and 20  $\mu$ l 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. In duplicate, 100 mg of the granulated sample was homogenized with mortar and pestle and a little sand in 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  $\text{Na}_2\text{SO}_4$  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, Mass. 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  $\text{Na}_2\text{SO}_4$  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.

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 as 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 comparing 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 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 milli-Q water. After centrifugation in an Eppendorf centrifuge, the supernatants were diluted 50 times 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 Carbowax PA-1 column with guard column was used, operated at 30°C. Identification of carbohydrate peaks was by comparison with retention times of standard solutions in two different elution programmes. 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).

*Low temperature scanning electron microscopy (LTSEM)*

After 4 hours of soaking in water at 30°C, seeds were incubated on wet filter paper for 20 hours 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 in liquid nitrogen (-196°C). The stubs with the frozen axes were transferred into 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 two minutes to sublimate the contaminating water vapour, and then cross-sectioned with a cold sharp knife. After sputter-coating with 3 nm platinum, the coated specimen was placed inside the LTSEM and observed at 1-5 kV. The temperature of the specimens inside the LTSEM was kept at -180°C. An LTSEM (JEOL, model 6300 F, Tokyo, Japan) was used to examine the material.

**Results***Equilibrium moisture contents and germination capacity of seeds*

The equilibrium MCs that were reached after 3 weeks of exposure (Sacandé *et al.*, 1998) at 20°C were 5.5 and 11.7% (FW basis) for the RHs of 32% and 75%, respectively. Seeds from both sources initially germinated >90%. Seed viability was followed over the entire 26 weeks of exposure to the two different RHs (Fig. 4.1). High viability was maintained during the first 6 weeks of exposure to 75% RH, followed by a decrease thereafter to low viability at 26 weeks. At 32% RH, viability had dropped to approximately 70-80% germination after the first 3-6 weeks, and then

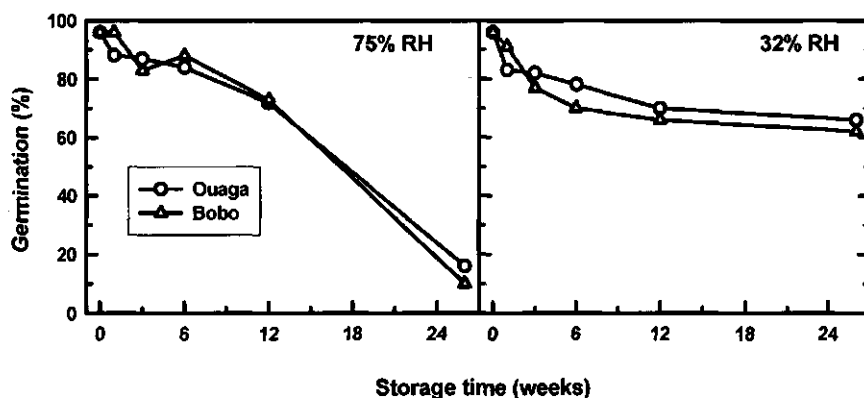
slowly further decreased to 60-70% germination over the next 20 weeks of storage. Both seed sources behaved similarly under the respective RH conditions.

#### *Non-protein thiols during dehydration and storage*

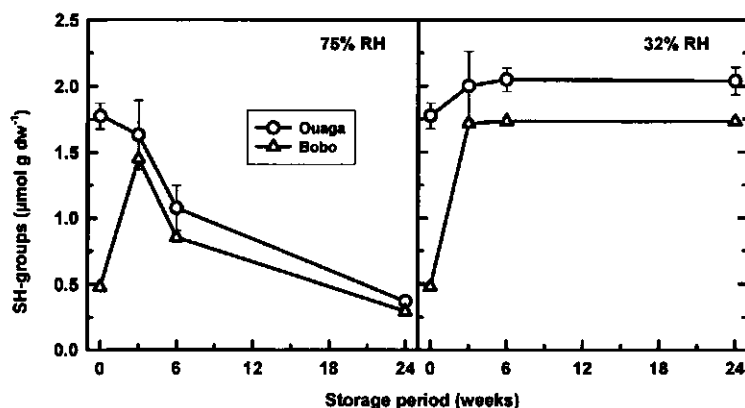
Because thiols are sensitive to oxidation, it is possible that they may become oxidized

during extraction, resulting in erroneous thiol-disulfide values (De Vos *et al.*, 1994). To determine whether such artifactual 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 indicates 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 75% RH at 20°C (Fig. 4.2). Analyses showed significant initial differences in 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 in the same order as those in the initially drier (Ouaga) samples (Fig. 4.2). 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) 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. 4.2.



**Figure 4.1.** Viability of neem seeds from Ouaga and Bobo, Burkina Faso, over 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 x 50 seeds). Data are significantly ( $P \leq 0.05$ ) different when they diverge by 15% or more ( $\chi^2$ -test).



**Figure 4.2.** Effect of storage on total non-protein SH-contents 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.

#### *Glutathione redox status*

A very similar pattern of changes in GSH content with drying and further dry storage was observed (Fig. 4.3) as found for the total non-protein thiols shown in Fig. 4.2. After an initial increase in 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 75% RH, whereas the seed lots stored at 32% RH had stable amounts during the entire storage period. 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 (data not shown).

The amounts of GSSG increased with the water loss of the samples during the first 3-6 weeks of exposure (Fig. 4.4). After 6 weeks this increase levelled off, or the GSSG contents slightly decreased (in the case of storage at 75% RH). Taken together, the increase in GSH in most of the samples and the simultaneous increase in GSSG during the first 3-6 weeks led to a net increase in the total glutathione levels. However, the subsequent decrease of the GSH and the more or less stable level of GSSG at 75% RH led to a loss of total glutathione during storage. At 32% RH storage, the amount of total glutathione did not change much. The amount of GSH during storage under both

RH conditions was slightly less than the total of non-protein SH-compounds in all

extracts, which means that GSH comprised most of the non-protein thiols present in the seeds.

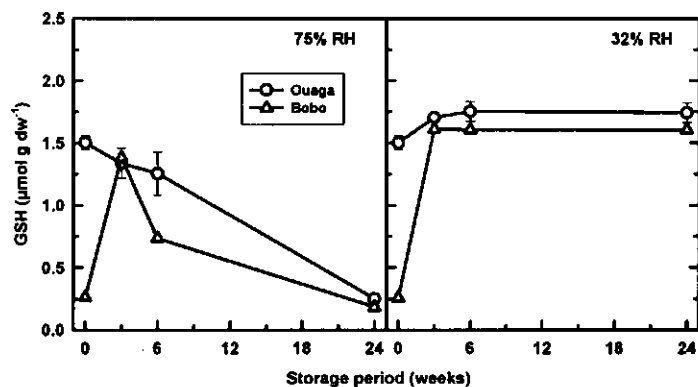


Figure 4.3. Change in GSH contents in embryos during storage of neem seeds from Ouaga and Bobo at 20°C at 32% and 75% RH. Each data point is the mean of three replicates from extracts of five embryos. SD is represented when larger than the symbols.

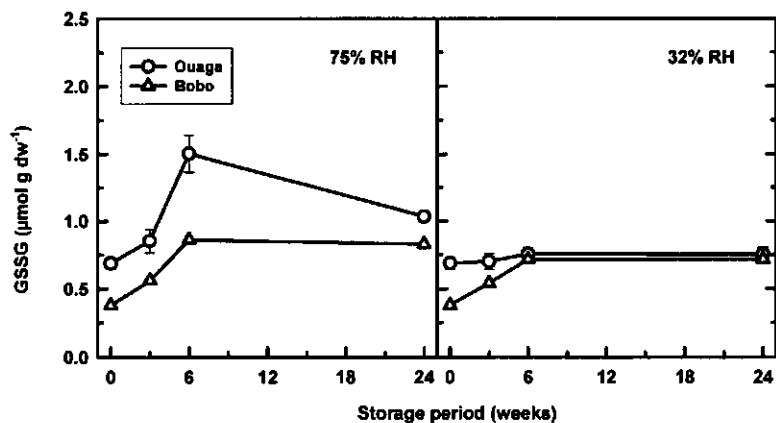
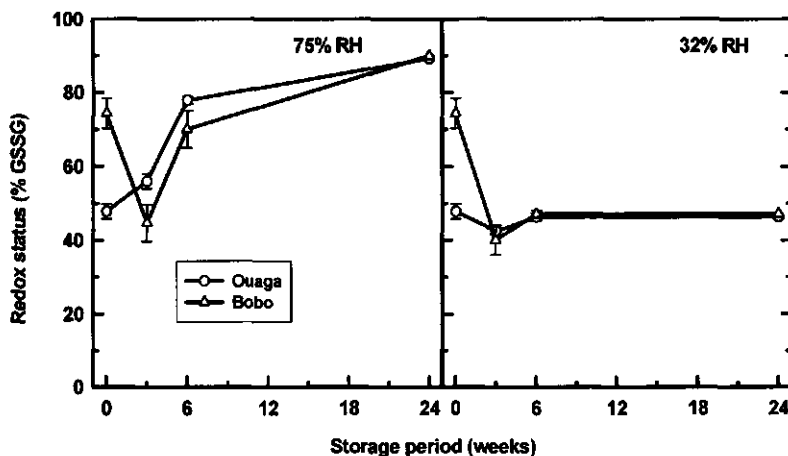


Figure 4.4. Effect of storage on the GSSG content in neem embryos during storage of neem seeds from Ouaga and Bobo at 20°C at 32% and 75% RH. Each data point is the mean

of three replicates from extracts of five embryos. SD is represented when larger than the symbols.



**Figure 4.5.** 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 symbol.

The ratio of GSSG to total glutathione can be considered as a measure of the cellular redox status (De Vos *et al.*, 1994; Kranner and Grill, 1996). Figure 4.5 shows that in the seeds stored at 75% RH the proportion of GSSG increased up to 90%, 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 higher rate than the formation of GSSG. Under conditions of 32% RH, the redox status remained stable over the entire period of storage. We conclude that the seeds 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 provide 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 4.1 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% RHs, and 20°C. During drying (first 6 weeks of storage), the 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. The 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 decrease in palmitic (16:0) and linolenic acid, and a slight increase in oleic acid when compared with the composition of fresh seeds, whereas at 32% RH storage 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 4.1). Neutral lipids comprised almost 50% of the DW, with a considerable similarity in acyl chain composition compared to that found for the phospholipids (data not shown). They had a low percentage (1-2%) of eicosenoic acid (20:1) and erucic acid (22:1) that were not found in the phospholipids. The free fatty acid content did not increase at 32% RH storage, but increased considerably at 75% RH storage.

**Table 4.1.** 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.

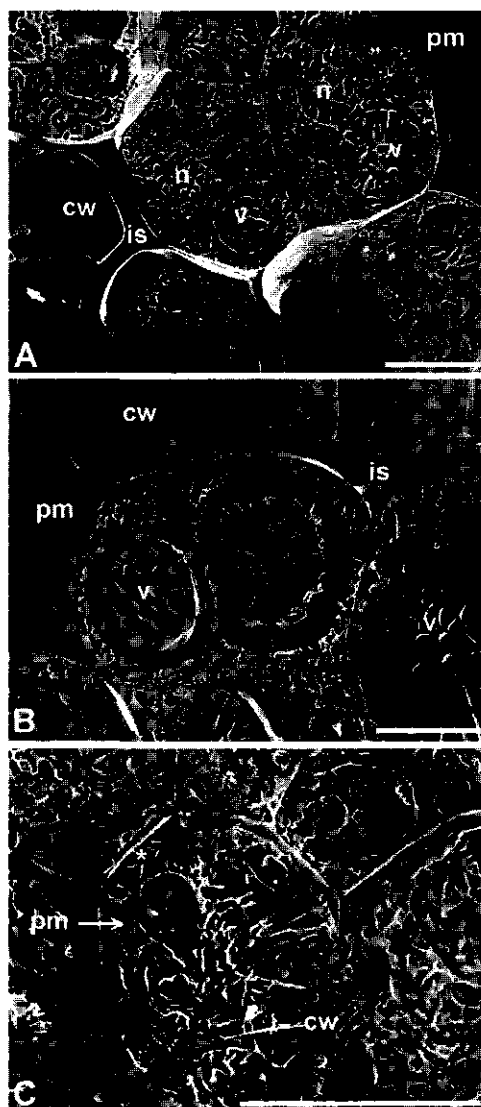
Treatment	FFA-content mg/g DW	PL-content Mg/g DW	Mol % fatty acids in PL					DB index
			16:0	18:0	18:1	18:2	18:3	
Fresh, no drying	0.4	5.1	21.2	8.1	50.7	17.0	2.9	0.94
32% RH								
6 weeks	0.3	10.0	20.5	8.1	51.4	18.4	1.6	0.93
24 weeks	0.3	10.4	21.4	6.8	54.3	16.1	1.3	0.91
75% RH								
6 weeks	2.1	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	-



### *Ultrastructural changes of fresh and stored axes*

After 24 hours of water uptake in Petri dishes, fractured cells of isolated axes were inspected by LTSEM. Figure 4.6A shows that the axis cells from fresh seeds had a turgid 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 from the fresh seeds (Fig. 4.6B). However, axis cells from the seeds stored for 6 months at 75% RH had the typical appearance of debilitated cells that had lost turgor (Fig. 4.6C).

**Figure 4.6.** LTSEM micrographs of cryo-fractured axes from neem seeds that were soaked for 4 hours in water at 30°C, followed by incubation on moist filter paper for 20 hours 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  $\mu$ m). 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.



*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. Figures 4.7 and 4.8 show the changes in sugar content 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 stabilized thereafter (Fig. 4.7). 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 increase in oligosaccharides and glucose content was confounded by a decrease that continued over the entire storage period. The amount of sucrose under both conditions of RH varied somewhat, with that at 75% RH being always below that at 32% RH (Fig. 4.8). The seeds from Bobo had oligosaccharide contents that were low compared to those from other sources. The average sugar contents in seed lots from four sources after drying at 32% RH (6 weeks storage) gave the following results. Raffinose,  $5.6 \pm 2.6$  (SD) mg/g DW; stachyose,  $7.2 \pm 4.0$  mg/g DW; sucrose,  $69.0 \pm 19.4$  mg/g DW; glucose,  $3.4 \pm 0.7$  mg/g DW; and negligible amounts of less than 0.3 mg/g DW of fructose. These sugar contents from the four sources gave an average oligosaccharide/sucrose mass ratio of 0.19.

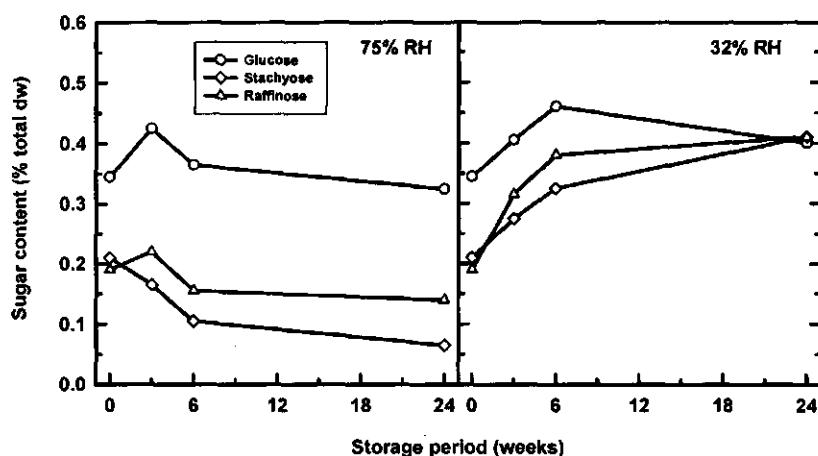
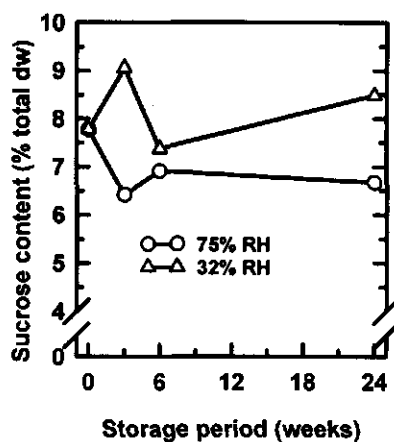


Figure 4.7. Composition and changes in sugars - glucose, stachyose and raffinose, in embryos of neem seeds from Bobo stored at 32% and 75% RH at 20°C. Each data point is the mean of two determinations on five embryos each.



**Figure 4.8.** Change in sucrose content in embryos of neem seeds from Bobo stored at 32% and 75% RHs, at 20°C. Each data point is the mean of two determinations with five embryos. SD is smaller than the symbols.

## Discussion

Oxidative stress and compositional changes in a number of neem seed lots from different 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. 4.1). These data are consistent with survival data of neem seeds during dehydration and storage as reported in the scanty literature (reviewed by Hong *et al.*, 1996; Poulsen, 1996). Seed storage behaviour of neem is controversial. It has been characterised 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 also have been categorised 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 more or less agrees with the levels of survival observed in our storage experiments at 32% RH (5.5% equilibrium MC). However, orthodox behaviour has been claimed as well, 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 neem seeds contain 50% of their DW as oil (Table 4.1; Sacandé *et al.*, 2000; Chapter 5), the actual water content in the cytoplasm outside the oil will be

twice as high as might be expected at first sight, i.e., 20.9% on a FW basis. Under these conditions the cytoplasm is not in the glassy state (Sacandé *et al.*, 2000; Chapter 5). It has been suggested that 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 phase (Sun, 1997; Buitink *et al.*, 1998). By contrast, at 32% RH the cytoplasm in neem seeds has been shown to be in the glassy state (Sacandé *et al.*, 2000; Chapter 5).

Our data on the extent of oxidative stress during drying and subsequent storage at 32 and 75% RH (Fig. 4.5) concur with the presence and absence of cytoplasmic glasses, respectively. The seeds that were exposed to high RH clearly suffered from oxidative stress, whereas the 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 will be reduced immediately back to GSH by the action of glutathione reductase. However, desiccated tissues accumulate GSSG (De Vos *et al.*, 1994; Kranner and Grill, 1996) dependent on oxidative stress, because glutathione reductase cannot presumably be active in seeds in the dry state.

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 loss of other cytoplasmic compounds. We thus observed decreases in oligosaccharides and phospholipids contents during storage, which did not occur in storage at 32% RH.

An interesting observation was made when comparing seed samples of initially different MCs with respect to their glutathione, oligosaccharides and phospholipids 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 prepare themselves to better 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 polyunsaturated 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 4.1) 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 to the loss in phospholipid content may be attributed to 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. 4.6C) 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 in 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. 4.7), in amounts comparable with those found in orthodox seeds. The average mass ratio of oligosaccharide to sucrose of 0.19 for four neem seed sources 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 oligosaccharide 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; Chapter 3) may have contributed to their reputation as being difficult to store. In addition different dehydration histories that may lead to variations in the amounts of protective substances in the dehydrated seeds might explain the variation in the extent to which neem seeds can be stored successfully.

## A study of water relations in neem seed that is characterised by complex storage behaviour\*

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### Summary

Neem (*Azadirachta indica*) seed is reputed to have limited tolerance to desiccation, to be sensitive to chilling and imbibitional stress, and to display intermediate storage behaviour. To understand this behaviour the properties of water in seed tissues were studied. Water sorption isotherms showed that at similar relative humidity (RH), the water content was consistently higher in axes than in cotyledons, mainly due to elevated lipid contents (51%) in the cotyledons. Melting transitions of water were observed at water contents higher than 0.14 g H<sub>2</sub>O g dw<sup>-1</sup> in the cotyledons and 0.23 g H<sub>2</sub>O g dw<sup>-1</sup> in the axes using differential scanning calorimetry. Beside melting transitions of lipids, as verified by infrared spectroscopy, changes in heat capacity were observed which shifted with water content, indicative of glass-to-liquid transitions. State diagrams on the basis of the water content of seed tissues, and also on the basis of the RH at 20°C are given. Longevity was considerably improved, and sensitivity to chilling/subzero temperatures was reduced when axis and cotyledons were dehydrated to moisture contents  $\leq$  approx. 0.05 g H<sub>2</sub>O g dw<sup>-1</sup>. However, longevity during storage at very low water contents was limited. A possible mechanism for the loss of sensitivity to chilling/subzero temperatures at low water contents is discussed. The results suggest that dry neem seeds in the glassy state have great potential for extended storability, also at subzero temperatures.

## Introduction

Seed storage longevity depends on the intrinsic properties of the species and on external factors such as temperature, relative humidity (RH), and, to a lesser extent, the composition of the gaseous atmosphere during storage (Priestley, 1986). To survive long-term storage in the dehydrated state, seeds have to be able to withstand desiccation to low water contents. A large group of so-called orthodox seeds have this ability, whereas another group of mainly tropical seeds, designated recalcitrant, are damaged during drying, mostly in the range of 0.4 - 1.0 g H<sub>2</sub>O g dw<sup>-1</sup> (Hong *et al.*, 1996). Empirical models have been constructed for the storage behaviour of orthodox seeds on the basis of moisture content and temperature, which can predict the viability of a seed lot over time at a broad range of different water contents and storage temperatures (Roberts, 1972; Justice and Bass, 1978; Ellis and Roberts, 1980).

Deterioration of orthodox seeds during storage is largely determined by the physical state and properties of water (see Walters, 1998, for a review). The physical state of water in seeds has been characterised on the basis of thermodynamic considerations by the motional and thermal properties of intracellular water. Degradative reactions may be controlled by the chemical potential of water and the availability of water for chemical reactions, which can be described by equilibrium thermodynamics (Vertucci and Roos, 1990, 1993). Water also can be regarded in terms of a viscous glass. A glass is defined as a meta-stable state that resembles a solid, brittle material, but retains the disorder and physical properties of the liquid phase (Franks *et al.*, 1991). The slow molecular diffusion in glasses renders chemical reactions improbable, which is considered to substantially enhance long-term stability (Slade and Levine, 1991). There is considerable support for the existence of glasses in dry orthodox seeds (Williams and Leopold, 1989; Bruni and Leopold, 1991; Williams *et al.*, 1993; Leopold *et al.*, 1994; Sun *et al.*, 1994). Seed metabolism is minimised in such a glass, and storage longevity is maximised, particularly at low temperatures. In contrast, the non-glassy, liquid phase allows accelerated physical and chemical deterioration of seeds (Sun and Leopold, 1993; Sun *et al.*, 1994), probably as a result of increased molecular mobility (Buitink *et al.*, 1998).

For the recalcitrant seeds in which water properties appear not to be linked with desiccation tolerance (Pammenter *et al.*, 1991; 1993) no predictive models have been proposed. The relatively high water content at which recalcitrant seeds start to lose viability is far above that at which a glassy state can exist at room temperature and non-frozen water is lost. Many recalcitrant tropical seeds are chilling-sensitive and rapidly lose viability even if stored at relatively high moisture contents. Viable recalcitrant seeds cannot be stored for long-term periods due to the active metabolism in the hydrated state. The exact causes of recalcitrant seed storage behaviour remain to be ascertained (Vertucci and Farrant, 1995), although orthodox seeds feature typical changes in key compounds during the acquisition of desiccation tolerance that do not occur to the same extent in recalcitrant seeds, e.g. the increase in oligosaccharide content (Horbowicz and Obendorf, 1994; Steadman *et al.*, 1996).

A third, intermediate class of seed behaviour has been recognised, which is characterised by an intermediate moisture limit below which the seeds cannot survive

dehydration (Ellis *et al.*, 1990; 1991). Seeds of neem, an economically important multipurpose tropical tree species, have limited tolerance to desiccation and do not store well for extended periods of time, e.g. years. Consequently, these seeds were considered to display intermediate behaviour (Gamené *et al.*, 1996; review by Hong *et al.*, 1996; Poulsen, 1996; Sacandé *et al.*, 1996; 1997; Hong and Ellis, 1998), although their behaviour has also been categorised as orthodox (Tompsett and Kemp, 1996). In addition, the seeds are chilling-sensitive above  $0.09 \text{ g H}_2\text{O g seed dw}^{-1}$  (Sacandé *et al.*, 1998). At and below  $0.09 \text{ g H}_2\text{O g dw}^{-1}$ , the seeds lose this chilling sensitivity, but become sensitive to imbibitional stress, which may partly explain their reduced tolerance to dehydration. The water contents at which neem seeds experience the above mentioned problems is well within the range of water contents at which equilibrium thermodynamics of water and glasses can play a role. A study of seed-water relations in terms of cytoplasmic glassy state and water properties is therefore expected to give more insight in the unusual behaviour of intermediate neem seeds during dehydration and storage.

The present paper explores the properties of water in dehydrating neem seeds. Water sorption isotherms for excised embryonic axes and cotyledons were constructed, and glass transitions were studied from thermal events recorded by differential scanning calorimetry (DSC). The results are discussed in relation to desiccation tolerance and storage longevity.

## Materials and methods

### *Plant material*

Fully mature neem (*Azadirachta indica* A. Juss.) seeds were harvested from yellow fruits in July 1997 (see Chapter 3; Sacandé *et al.* 1997 for the staging of seed development). Fruits were picked by hand from more than 25 trees in the Ouagadougou region (Burkina Faso). The preparation of the seeds was carried out locally on the day of harvesting. The fruits were soaked in water and rubbed with sand to depulp. The seeds were then cleaned with water and dried in the shade on a grid for two days. Seeds surrounded by an intact endocarp were selected and sent by air cargo to Wageningen, the Netherlands, in cotton bags, arriving a week after harvest.

### *Determination of moisture content and viability*

Excised embryonic axes (5) and cubes (3 of approximately  $3 \text{ mm}^3$ ) of cotyledons were exposed to the atmospheres above different saturated salt solutions or  $\text{P}_2\text{O}_5$  ( $<1\% \text{ RH}$ ) in closed containers at  $20^\circ\text{C}$  for at least one week (Vertucci and Roos, 1993) ( $\text{ZnCl}_2$  [5.5% RH], KOH [8% RH], LiCl [13% RH], K-acetate [25% RH],  $\text{MgCl}_2$  [32% RH],  $\text{Ca}(\text{NO}_3)_2$  [53% RH],  $\text{NaNO}_2$  [64% RH], NaCl [75% RH], KCl [85% RH] and  $\text{KNO}_3$  [91% RH]). Samples taken from the containers were rapidly sealed in Perkin Elmer aluminium DSC pans that were weighed, then punctured and oven-dried at  $96^\circ\text{C}$  for 48 hours, and weighed again, allowing determination of the water content. Water contents were expressed on a dry weight basis.



Seeds were stored in open storage conditions at 4 different RHs - 10% (established in a commercial cabinet), and 32%, 53% and 75% RH (established above saturated salt solutions) - for 1 month or 1 year at 20°C. Seeds also were dehydrated above the saturated salt solutions for approximately three weeks to obtain a range of water contents. They were then packed into laminated aluminium foil packets that were hermetically sealed, and stored for an additional three months at a range of temperatures. Seeds were allowed to germinate at 30°C/20°C (day/night) according to the method described in Sacandé *et al.* (1998). Final germination was scored on the basis of radicle protrusion (2 cm; ISTA, 1993).

#### *Total lipid analysis*

Total lipids were extracted from the seed tissues with chloroform-methanol using the modified Folch's method described in Hamilton *et al.* (1992), and the amounts were determined gravimetrically.

#### *Fourier transform infrared spectroscopy (FTIR)*

FTIR spectra were recorded on a Perkin-Elmer 1725 IR-spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK), equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin-Elmer microscope as described previously (Wolkers and Hoekstra, 1995). Oil pressed out of seed axes or cotyledons was placed between two diamond windows that were tightly mounted into a temperature-controlled brass cell. Temperature control of the sample in the instrument was with a computer-controlled device that activated a liquid nitrogen pump, in conjunction with a power supply for heating of the cell. The temperature of the sample was recorded using two PT-100 elements that were located close to the sample windows. The temperature dependence of the FTIR spectra was studied in the range between -60°C and 60°C, starting with the lowest temperature. Spectra were recorded every minute at temperature increments of 1.5°C min<sup>-1</sup>. The instrument was purged of water vapour with a Balston dry air generator (Balston, Maidstone, Kent, England).

The spectral region between 3000 and 2800 cm<sup>-1</sup> was selected and second derivative spectra were calculated [19 points smoothing factor; calculated using the Infrared Data Manager Analytical Software, version 3.5 (Perkin Elmer)]. The position of the symmetric CH<sub>2</sub> stretching vibration band of the oil around 2852 cm<sup>-1</sup> was determined from these second derivative spectra.

#### *Differential scanning calorimetry*

Melting transitions and changes in heat capacity of the samples were determined using a DSC (Pyris 1 DSC, Perkin-Elmer, Norwalk, CT, USA), calibrated for temperature with Indium (156.6°C) and methylene chloride (-95°C) standards and for energy with Indium (28.54 J g<sup>-1</sup>). Axes and cotyledons (5 to 10 mg) with different water contents were hermetically sealed in Perkin Elmer aluminium pans. After cooling the sample at 10°C min<sup>-1</sup> to -100°C and keeping this temperature for 10 min, scans were recorded from -100°C to 120°C at a rate of 10°C min<sup>-1</sup>. The enthalpy ( $\Delta H$ ) of the melting

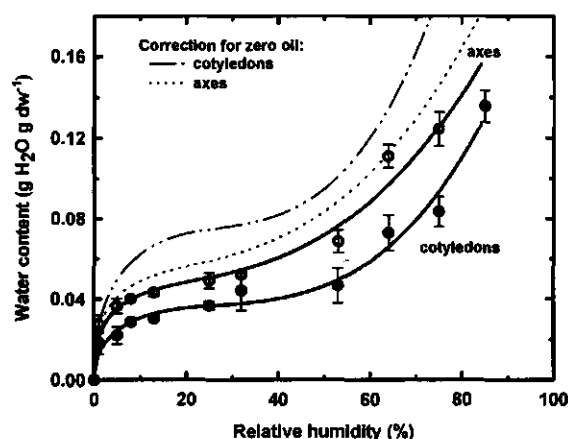
transition of water was determined from the area under the peak. Second order (glass)

transitions were recognised as a sudden, stepwise increase in specific heat. The midpoint of the temperature range over which the change in specific heat occurred was used to determine the glass-to-liquid transition temperature ( $T_g$ ). All thermograms were analysed using Pyris Software for Windows (Perkin-Elmer Thermal Analysis). Baselines were determined using an empty pan, and all thermograms were baseline-corrected. After recording the thermograms, water contents of the samples were determined as described above.

## Results

### Water sorption isotherms

Water sorption isotherms of excised axes and cotyledon cubes at 20°C are shown in Figure 5.1. Water contents were consistently higher in the axes than in the cotyledons for the same RH. Because lipids are inaccessible to water but nevertheless contribute to the dry weight, the difference in water absorption between axis and cotyledonary tissue might be explained by a difference in lipid content. The amount of lipids in both tissues was therefore analysed. We found 51% and 14% for the cotyledons and the axes, respectively, indicating that the cotyledons contained 3.7 times more lipids than the axes. Recalculating the water content for zero amount of lipids showed that the cotyledons absorbed more water from the vapour phase under similar conditions of RH than did the axes (dotted curves in Fig. 5.1).

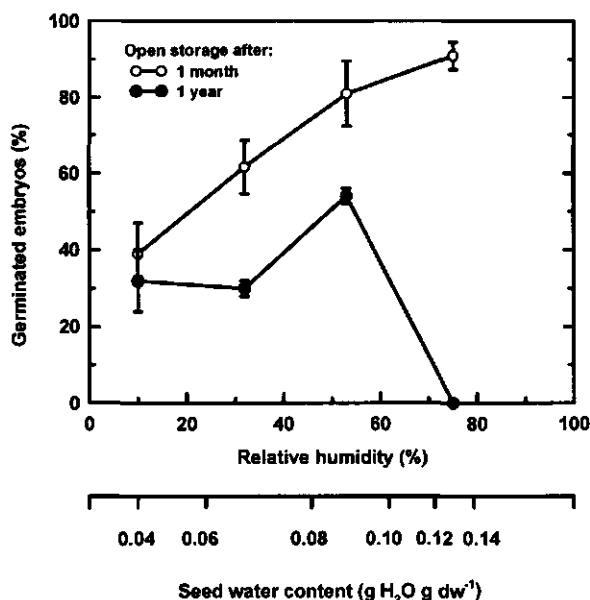


**Figure 5.1.** Water sorption isotherms of embryonic axes and cotyledon cubes of neem, showing the relationship between RH and water content. Tissues were exposed to a range of RH conditions at 20°C and sampled after one week of equilibration. Each data

point is the average of 4 samples  $\pm$ SD. The curves were fitted with Table Curve (Jandel Scientific, MS 3.0) as an aid to the eye.

#### *Desiccation tolerance and storage longevity*

Fresh seeds ( $0.37 \text{ g H}_2\text{O g dw}^{-1}$ ; 98% germination) were exposed to a range of RH conditions at  $20^\circ\text{C}$ . After one month of storage the seeds were allowed to germinate (Fig. 5.2). Germination decreased with decreasing RH, to reach a value of 40% at 10% RH ( $0.038 \text{ g H}_2\text{O g seed dw}^{-1}$ ). After one year of storage, the seeds exposed to 75% RH lost their germination capacity completely, and the germination percentages of the seeds exposed to 53%, 32% and 10% RH decreased further, the 53% RH conditions giving the best survival.



**Figure 5.2.** The effect of storage on the percentage of germinated neem embryos (original germination percentage = 98%). Seeds were exposed to 10, 32, 53 and 75% RH at  $20^\circ\text{C}$  and allowed to germinate after one month or one year of storage. An additional ordinate shows the equilibrium water content of the seeds at  $20^\circ\text{C}$  after 3 weeks at the different RHs. For each data point three samples of 25 embryos each were used. SDs are shown when they exceed symbol size.

The effect of a range of temperatures on the germination of seeds having different water contents during 3 months of hermetic storage is shown in Figure 5.3. It is clear that the seeds are chilling sensitive at water contents above  $0.048 \text{ g H}_2\text{O g embryo dw}^{-1}$ . In addition, a few seeds of  $\leq 0.048 \text{ g H}_2\text{O g embryo dw}^{-1}$  were able to resist temperatures as low as  $-20^\circ\text{C}$ . Fresh seeds ( $0.493 \text{ g H}_2\text{O g embryo dw}^{-1}$ ) lost viability at all the temperatures tested. After one month under these conditions the fresh seeds had higher viability at  $15$  and  $10^\circ\text{C}$  than at  $5^\circ\text{C}$  (data not shown). After one year of storage, the seeds having embryo moisture contents of  $0.074$  or  $0.099 \text{ g H}_2\text{O g dw}^{-1}$  lost viability at  $5^\circ\text{C}$  and  $20^\circ\text{C}$  completely, but there was some survival at  $10^\circ\text{C}$  and  $15^\circ\text{C}$  (see Fig. 3.6, Chapter 3). Note that the moisture content of the seed is greatly influenced by the endocarp (45% of the seed dry weight), that has a high sorption of water. Thus, under conditions of equilibrium RH, the water content of the total seed is considerably higher than that of the embryo.

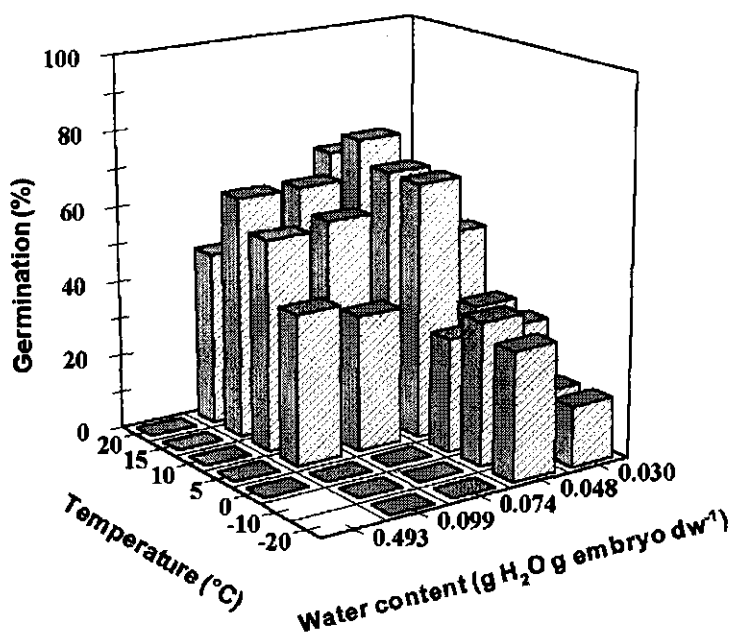


Figure 5.3. Effect of storage temperature and embryo water content on the percentage of germinated seeds after hermetic storage in laminated aluminium packets for three months. The different water contents were obtained by equilibration over different saturated salts for approximately three weeks before packing the

seeds. For each data point 100 seeds each were tested. Data are significantly different ( $P \leq 0.05$ ) when they diverge by 15% or more ( $\chi^2$ -test).

### *Thermal transitions*

Thermal transitions in neem axes and cotyledons of different water contents were determined by DSC from heating thermograms. Figure 5.4 shows a selection of endotherms of axes and cotyledons in which several transitions are noticeable. At high water contents, a broad endothermic peak was observed. The area and the temperature of this peak decreased with decreasing water contents, indicating that these peaks were water-melting transitions. At high water contents a sharp peak was observed superimposed on the broad peak. This pattern indicates the melting transition of pure water probably arising from water vapour condensing inside the DSC pans. The onset temperatures of the broad melting transitions were considerably lower in the seed tissues than in pure water or dilute solutions, as previously found for other biological systems (Vertucci, 1990).

The amount of unfrozen water can be calculated from the  $\Delta H$  of the melting endotherms versus the water content relationships for the dehydrating axes and cotyledons (Figs. 5.5A and 5.5B). The areas under the peaks were determined from thermograms similar to those shown in Figure 5.4. Two linear parts were apparent in the plots. The intersection with the horizontal line indicates the water content below which there is only unfrozen water left. These water contents were 0.23 and 0.14 g  $\text{H}_2\text{O g dw}^{-1}$  for axes and cotyledons, respectively. Correction for zero oil revealed that the critical water content was almost the same in both tissues (0.27-0.29 g  $\text{H}_2\text{O g dw}^{-1}$ ). The  $\Delta H$  per g  $\text{H}_2\text{O}$  of the melting transitions, calculated from the slopes, was approximately 300 J g  $\text{H}_2\text{O}^{-1}$  for both axes and cotyledons. The lines drawn represent the least-squares best fit ( $r^2 = 0.998$  for all regressions). The transition enthalpies of the axes and cotyledons below these critical water contents remained approximately 15 J g  $\text{H}_2\text{O}^{-1}$  (Fig. 5.5A) and 30 J g  $\text{H}_2\text{O}^{-1}$  (Fig. 5.5B), respectively. These enthalpies are due to the melting of lipids as outlined in the next section.

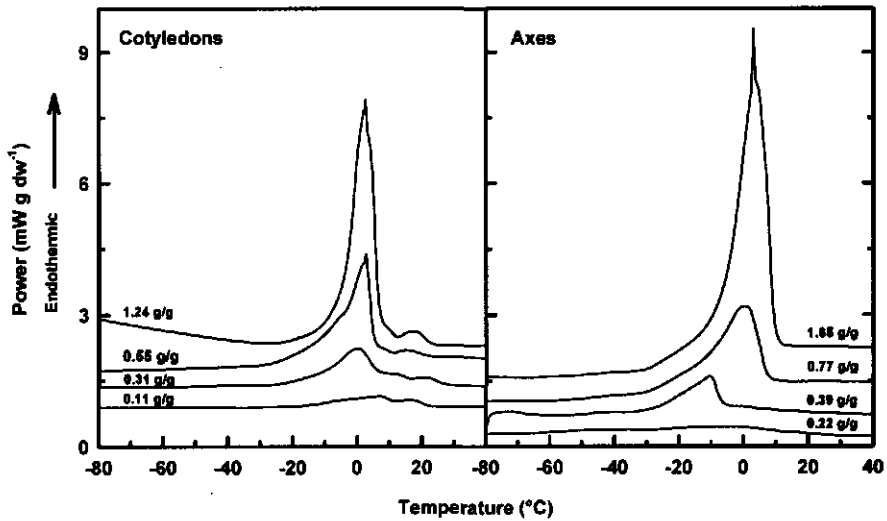


Figure 5.4. DSC heating thermograms of neem cotyledon cubes and axes equilibrated to different water contents using saturated salt solutions. Scans were recorded from  $-100^{\circ}\text{C}$  to  $120^{\circ}\text{C}$  at  $10^{\circ}\text{C min}^{-1}$ . Water contents (g/g) are expressed on the basis of dry weight.

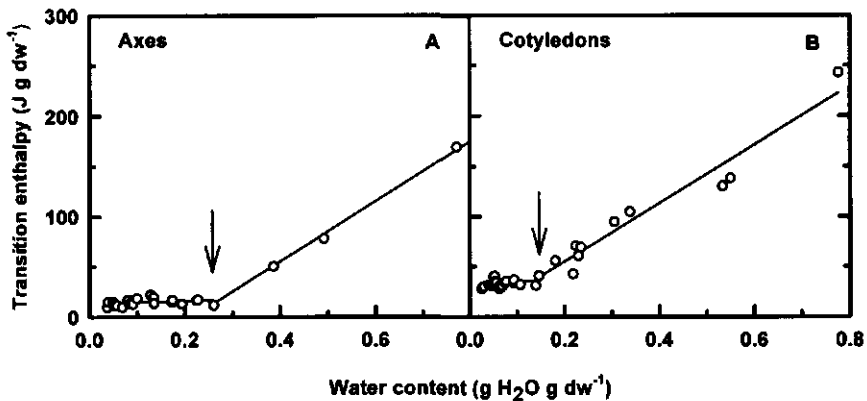


Figure 5.5. Enthalpies ( $\Delta H$ ,  $\text{J g dw}^{-1}$ ) of the melting transitions of water plotted as a function of the water contents of desiccating axes (A) and cotyledons (B). The arrows show the water content below which only non-frozen water was present.

Some first order thermal events were still visible below the water content at which freezable water could not be observed (Fig. 5.4). These events occurred at the same temperature irrespective of how far the tissues were dehydrated. A detailed thermogram is shown in Figure 5.6A for axes and in Figure 5.7A for cotyledons. The nature of the compounds causing these thermal events was verified by FTIR analysis of the symmetric  $\text{CH}_2$  stretching band of the oil that was pressed out of the specimens. Wavenumber versus temperature plots are presented in Figures 5.6B and 5.7B for axes and cotyledons, respectively. The peaks in the thermograms corresponded well with the discrete shifts in these plots, indicating that the thermal events seen in the thermograms of the dry specimens are indeed due to lipid melting.

Beside first order lipid transitions, other features are visible in thermograms of cotyledons having different water contents (Fig. 5.8). At low water contents, a shift in the baseline was observed above  $40^\circ\text{C}$ , indicating a second order transition. The temperature at which the midpoint of this transition occurred shifted to lower values with increasing water contents. Such a shift indicates that the transition observed is caused by melting of a glass (Leprince and Walters-Vertucci, 1995). In samples with elevated water contents the glass-to-liquid transition ( $T_g$ ) was detected at below  $-50^\circ\text{C}$ . Similar shifts were observed in scans of the axes (data not shown). In the thermograms of cotyledons with water contents below  $0.04 \text{ g H}_2\text{O g dw}^{-1}$ , a small first order transition of unknown origin was present around  $30^\circ\text{C}$ , possibly due to the melting of a lipid (marked with an asterisk in Fig. 5.8). The heating thermogram of cotyledons containing  $0.146 \text{ g H}_2\text{O g dw}^{-1}$  shows some additional features that follow the melting of the glassy state. The exothermic event, marked d, is indicative of a devitrification event (Leprince and Walters-Vertucci, 1995). Just after this devitrification event, a first order transition due to the melting of water (w) is apparent.

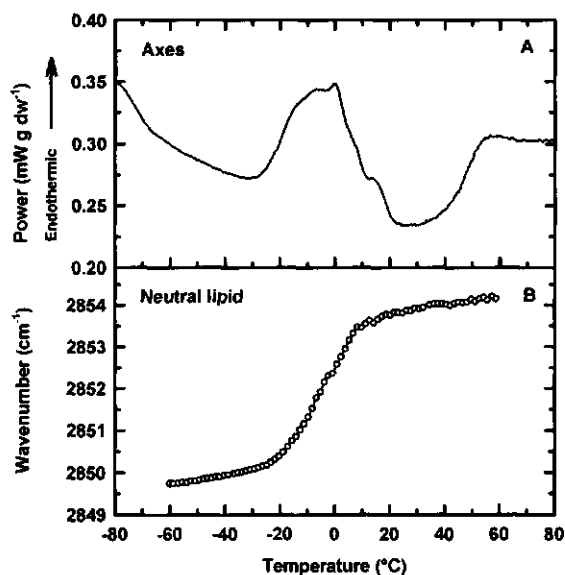


Figure 5.6. (A) Heating thermograms of dried neem axes at 0.04 g H<sub>2</sub>O g dw<sup>-1</sup>. (B) Wavenumber-temperature plot (FTIR) of the symmetric CH<sub>2</sub> stretching vibration band of oil pressed from the dried axes, representing melting behaviour during heating. The plot derived from FTIR spectra confirms oil-melting transitions between -20 and 20°C.

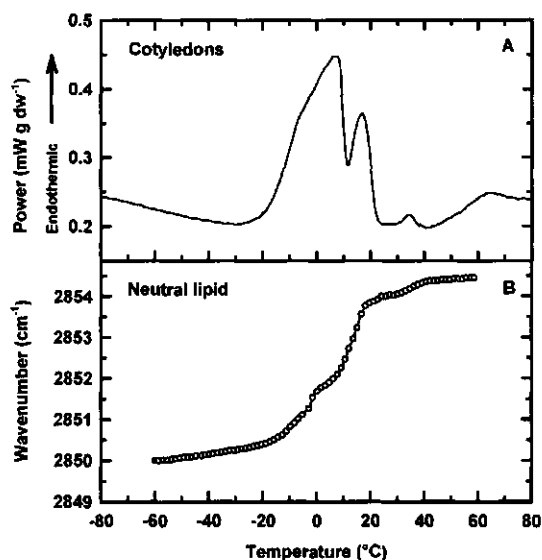
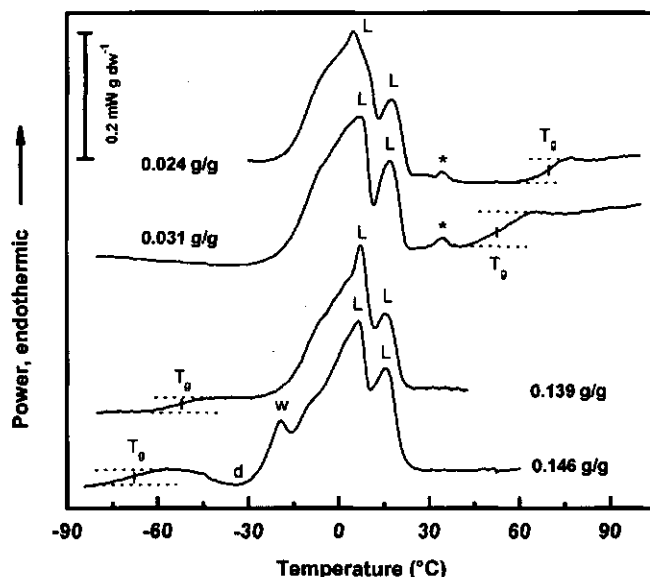


Figure 5.7. (A) Heating thermograms of dried neem cotyledon cubes at 0.03 g H<sub>2</sub>O g dw<sup>-1</sup>. (B) Wavenumber-temperature plot (FTIR) of the symmetric CH<sub>2</sub> stretching vibration band of oil pressed from the dried cotyledons, representing melting behaviour during heating. The plot derived from FTIR spectra confirms oil-melting transitions between -20 and 40°C.

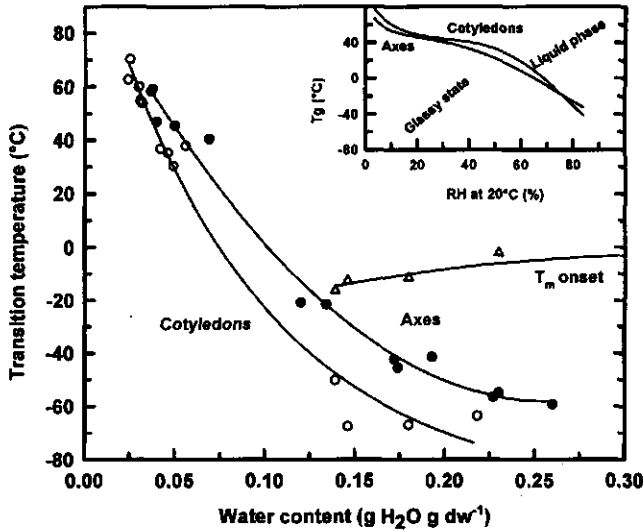


The midpoints of the temperature range over which the change in specific heat occurred in both axes and cotyledons for a range of water contents are presented in Figure 5.9.  $T_g$  increased with decreasing water content. The values of the state diagram for the axes were higher than those of the cotyledons. Between approximately  $-20^\circ\text{C}$  and  $+30^\circ\text{C}$ ,  $T_g$  was undetectable because of interference with the lipid melting. With drying,  $T_g$  reached values as high as  $70^\circ\text{C}$  at a moisture content of approximately  $0.025 \text{ g H}_2\text{O g dw}^{-1}$ . In the case of samples having water contents of  $> 0.3 \text{ g H}_2\text{O g dw}^{-1}$ , a second glass transition was observed at a constant temperature of  $-45^\circ\text{C}$  (thermograms not shown), following a devitrification event, representing  $T_g'$  (melting of the maximally freeze-concentrated cytoplasmic solute; Leprince and Walters-Vertucci, 1995). The phase diagram of water in the cotyledons is also presented in Figure 5.9. The relationship of  $T_g$  versus RH at  $20^\circ\text{C}$  is shown in the inset to Figure 5.9. To this end, the fitted state diagrams of Figure 5.9 were combined, with the fitted sorption curves of Figure 5.1, with the curves shown as the result of the calculations.



**Figure 5.8.** A selection of heating thermograms of neem cotyledons showing the glass-to-liquid transitions ( $T_g$ ) and lipid transitions (L) at four water contents (0.024, 0.031, 0.139, and  $0.146 \text{ g H}_2\text{O g dw}^{-1}$ ). The (\*) shows first order transitions of unknown origin, which only occur in the drier samples. In the

sample with the highest water content a devitrification event (d) and the transition of water (w) are indicated.



**Figure 5.9.** State diagram for neem seed axes and cotyledons derived from the mid temperatures of glass transitions in the thermograms. The phase diagram of water ( $T_m$ ) for cotyledons is also indicated. Tissues are in the glassy state below the curves and in the liquid phase above the curves. Inset: State diagram for neem seed axes and cotyledons on the basis of the RH to which the tissues were equilibrated at 20°C. The curves were constructed from the curves fitted to Figures 5.1 and 5.9.

## Discussion

Neem seeds are reputed to have limited desiccation tolerance and a relatively short storage longevity (Gamené *et al.*, 1996; Hong and Ellis, 1998; Sacandé *et al.*, 1996; 1998). Because they can tolerate an equilibrium RH of 10% (Fig. 5.2) and storage at -20°C (Fig. 5.3), albeit with a considerable decrease in germination percentage, their behaviour has orthodox features rather than typically recalcitrant or intermediate characteristics. Although the general opinion has it that neem seed behaviour is intermediate, it has also been categorised as orthodox (Tompsett and Kemp, 1996). Nevertheless, stored seeds are difficult to handle, because they are sensitive to chilling/subzero temperatures above 0.048 g H<sub>2</sub>O g embryo dw<sup>-1</sup> (Fig. 5.3) and are extremely sensitive to imbibitional damage when dry (Sacandé *et al.*, 1998). These characteristics may have contributed to their complex storage behaviour.

The water sorption isotherms of cotyledons and embryonic axes of neem (Fig. 5.1) have the characteristic sigmoidal shape as found previously for other seeds by Vertucci and Leopold (1984). The shoulder of the isotherms has been associated with the presence of structural water (Chung and Pfost, 1967; Mahama and Silvy, 1982; Vertucci and Leopold, 1987). The lower equilibrium moisture contents of the cotyledons compared with those of the axes cannot solely be explained by differences in lipid content. After correction to zero oil content, the cotyledons had a higher equilibrium moisture content than the axes (Fig. 5.1, dashed curves).

The thermograms (made by DSC) that were obtained with neem axes and cotyledons were comparable to those found for other seeds or seed tissues (Vertucci, 1990; Pammenter *et al.*, 1991; Leprince and Walters-Vertucci, 1995). The non-frozen water limit in axes and cotyledons was  $0.23 \text{ g H}_2\text{O g dw}^{-1}$  and  $0.14 \text{ g H}_2\text{O g dw}^{-1}$  respectively (Fig. 5.4). Correction to zero lipid contents elevated these values to  $0.27$  and  $0.29 \text{ g H}_2\text{O g dw}^{-1}$ , which are in the range reported for other seed tissues, both desiccation tolerant and intolerant (Vertucci, 1990; Pammenter *et al.*, 1993).

Neem seeds have been successfully cryopreserved at the temperature of liquid nitrogen when they had first been dried to  $0.09 \text{ g H}_2\text{O g dw}^{-1}$  for the entire seed and  $0.23 \text{ g H}_2\text{O g dw}^{-1}$  for isolated axes (Berjak and Dumet, 1996). Chaudhury and Chandel (1991) showed that isolated embryos with a water content  $\geq 0.37 \text{ g H}_2\text{O g dw}^{-1}$  died when exposed to liquid nitrogen, but that they mostly survived when desiccated to  $0.13$  or  $0.04 \text{ g H}_2\text{O g dw}^{-1}$ . These water contents are in the range at which no freezable water is expected (see Fig. 5.5), given the fact that the intact embryo consists mainly of cotyledon tissue (99.5%). Liquid nitrogen storage may be successful if freezing rates are fast enough to avoid intercellular ice formation even when there is some freezable water present in the tissue (Vertucci, 1989; Wesley-Smith *et al.*, 1992).

Apart from the melting transitions of water, the thermograms showed first order transitions that could be attributed to different classes of lipids, as evidenced by the temperature-dependent FTIR data of the symmetric  $\text{CH}_2$  stretch of the neutral lipids pressed out of the specimens (Figs. 5.6 and 5.7). Inspection of the thermograms (Fig. 5.8) further revealed second order transitions that shifted with water content, suggestive of the presence of cytoplasmic glasses (Williams and Leopold, 1989; Leopold *et al.*, 1994; Leprince and Walters-Vertucci, 1995). The presence of glasses has been suggested to reduce ageing rates of stored seeds (Leopold *et al.*, 1994; Sun, 1997), and this suggestion has been verified for pollen (Buitink *et al.*, 1998).

State diagrams were constructed from the mid- $T_g$  values obtained at different water contents (Fig. 5.9). The curve separating the glassy state from the liquid phase for the axes was higher than that for the cotyledons. With such curves at hand, one could speculate about the possible significance of water relations with respect to long-term storage and chilling/subzero sensitivity of neem and other intermediate seeds. It was interesting to note the loss of sensitivity to chilling and subzero temperatures for seeds that were stored at embryo water contents of  $\leq 0.048 \text{ g H}_2\text{O g dw}^{-1}$  (Fig. 5.3). An indication that dehydration can alleviate chilling stress can be inferred from the storage data on neem seeds in Hong and Ellis (1998). Inspection of the state/phase diagrams in Figure 5.9 revealed that both the cotyledons and the axes were in the glassy state under these conditions. The question is whether the presence of the glass might be involved in this loss of chilling sensitivity. To answer such a question, the possible cause of chilling sensitivity must first be considered. The phase transition temperature of membranes in tropical plant species has been shown to occur around 10–15°C, which is considerably above that of chilling-tolerant plant species (Crowe *et al.* 1989c). Cooling below the phase transition temperature leads to formation of the gel phase, often followed by phase separations (Platt-Aloia and Thomson, 1987). Particularly phase separations are not easily restored upon return to temperatures above the phase transition and may lead to extensive leakage. The membranes in neem seedlings also have an elevated transition temperature (10°C; Chapter 6). An explanation for the loss of sensitivity to chilling/subzero temperatures  $\leq 0.048 \text{ g H}_2\text{O g embryo dw}^{-1}$  could be that the transition temperature was depressed to below that of the hydrated control, or that phase separation did not occur. The first possibility is not very likely, since this has never been observed *in vivo* in other dry systems (Hoekstra and Golovina, 1999). However, when, at low water contents, molecules other than water (e.g. sugars) are hydrogen bonded to the membrane components and at the same time to the glass, lateral diffusion of membrane components might be restricted. Phase separations may thus be prevented, abolishing chilling/subzero sensitivity.

Curves were constructed of  $T_g$  versus RH at 20°C to enable us to make predictions about favourable storage conditions when seeds are equilibrated at certain RH (Fig. 5.9, inset). 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; Fig. 5.2). A considerable extension of the storage longevity (to at least 2 years) is reached when both tissues are in the glassy state, at around 50% RH at 20°C. This also applies to seeds stored at even lower RHs (Fig. 5.2). The amount of water in these samples keeps the cytoplasm below  $T_g$ , but above the level at which the structural stability of the cells would be compromised. The critical water content below which the seeds will not germinate is difficult to determine, because such dry seeds are extremely sensitive to imbibitional stress. This stress can be at least partly alleviated by imbibition at elevated temperatures (25°C–35°C) (Sacandé *et al.*, 1998).

In conclusion, the significant loss of the seed viability upon drying and cold storage (this paper; Sacandé *et al.*, 1998) indicates that neem seed still poses more problems than an orthodox seed type. The state/phase diagrams as shown in Figure 5.9 gave insight into the peculiar behaviour of neem seeds. Correlating the germination results in Figures 5.2 and 5.3 with the state diagrams in Figure 5.9 suggests that cautious manipulation may enable seed lots of neem to survive for a number of years when the seeds are sufficiently dehydrated and in the glassy state. The characterisation of water properties in neem seeds illustrates that there is a potential for the development of efficient methods for storage of tropical seeds displaying intermediate storage behaviour.

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## Viability loss of neem seeds associated with membrane phase behaviour\*

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### Summary

Storage of seeds from neem (*Azadirachta indica*), a multipurpose tropical tree, is difficult because of the sensitivity of the seeds to chilling and imbibitional stresses. We tested the hypothesis that an elevated phase transition temperature ( $T_m$ ) of membranes is responsible for this storage behaviour, using a spin probe technique, Fourier transform infrared spectroscopy, and electron microscopy. The critical minimum temperature for germination was between 10 and 15°C, the same temperature range at which the *in situ*  $T_m$  of hydrated membranes was established. Viability of fresh embryos was lost within two weeks of humid storage at 5°C, but remained high at 25°C. The loss of viability coincided with an increased leakage of  $K^+$  from the embryos upon imbibition and an increased plasma membrane permeability as detected with the spin probe technique. Freeze-fracture replicas of plasma membranes from hydrated axes chilled for 14 days showed lateral phase separation and signs of the inverted hexagonal phase ( $H_{II}$ ). Dehydrated embryos were sensitive to soaking (4 h), particularly when this was performed at low temperatures, but the fresh embryos were not. Low temperature scanning electron microscopy revealed that after soaking at 5°C axis cells had suffered an apparent disorganization of their internal cytoplasmic structure and did not become turgid. After soaking at 35°C, the cells were turgid and had a healthy appearance with clearly discernible organelles. Freeze fracturing occurred across the core of the plasma membranes after the 35°C soaking, but not after the 5°C treatment, suggesting that the membranes had deteriorated after the 5°C imbibition. We suggest that the high intrinsic  $T_m$  of 10–15°C in tropical seeds leads to chilling injury below that temperature range. Imbibitional injury is associated with extensive plasma membrane damage and occurs when dry seeds are rehydrated below a critical temperature.

## 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 (ISTA, 1993), standard germination tests do not reveal how, where and when damage occurs in seeds. There are a number of reasons why seeds lose viability. Physical, chemical and metabolic processes occurring during dehydration, storage, or rehydration 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). The use of vital stains, and measurements of solute leakage and membrane permeability can give indications of the state of the seed's plasma membranes. Free radical accumulation, oxidative stress and rate of respiration have all been shown to be associated with the proper functioning of plasma membranes and the viability of seeds (Leprince *et al.*, 1990; 1994; 1999).

The objective of storing seeds is to maintain their viability for extended periods of time so as to use them when needed. In desiccation-tolerant seeds, the removal of water during drying does not cause irreversible damage that compromises germination when these seeds are later incubated under favourable conditions. They can usually be stored for periods of many years. However, some seeds, often of tropical origin, cannot withstand dehydration. They are also often chilling sensitive (Corbineau and Côme, 1988; Chin *et al.*, 1989; Tompsett, 1994). As a result, such seeds cannot be preserved for long periods of time (Roberts, 1973). A further group of seeds that demonstrate behaviour intermediate between the afore-mentioned categories of desiccation tolerance and storage behaviour has been described (Ellis *et al.*, 1990, 1991). When they originate from tropical climates, such intermediate seeds are often chilling sensitive.

Neem (*Azadirachta indica*) is an important tropical tree species with many uses, whose seeds have been categorised as having intermediate storage longevity (Sacandé *et al.*, 1996; 1998; Hong and Ellis, 1998). Neem seeds are chilling sensitive at MC  $\geq 10\%$ . Their limited desiccation tolerance has been partially attributed to their sensitivity to imbibitional stress below 10% MC (Sacandé *et al.*, 1998). Survival after dehydration was improved by rehydrating the dry seeds at elevated temperatures of around 35°C. This sensitivity to both chilling and imbibitional stress has contributed to neem seed's reputation as being difficult to store.

Many plants of tropical origin suffer injury when they are kept below 10-15°C for some time. Apart from the dysfunction of some membrane enzymes (Lyons *et al.*, 1979a,b; Yamawaki *et al.*, 1983; Yoshida *et al.*, 1986), increased leakage of cytoplasmic solutes from the cells has been observed (Bergevin *et al.*, 1993; Bertin *et al.*, 1996). This chilling sensitivity has been attributed to a conformational transition in cell membranes from the liquid crystalline to the gel phase (Lyons *et al.*, 1979a,b; Wang, 1982), 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 transition temperature of membranes as measured by FTIR in intact tomato hypocotyls was around 10–15°C (Crowe *et al.*, 1989c). In contrast, the *in situ*  $T_m$ s of plants from temperate zones are usually around or below 0°C (Crowe *et al.*, 1989c; Hoekstra *et al.*, 1991). Leakage can be attributed to transiently increased permeability during the phase transition (Hammoudah *et al.*, 1981).

During imbibition (rehydration), dry seeds leak intracellular solutes into the surrounding medium. If the leakage persists, it is a sign that plasma membranes are damaged. The causes of imbibitional leakage have been extensively studied in pollens and model lipid systems (Crowe *et al.*, 1989b; Hoekstra *et al.*, 1992). The  $T_m$  of membranes increases during dehydration, and the gel phase may be formed at ambient conditions, depending on the phospholipid composition (reviewed in Hoekstra and Golovina, 1999). Imbibitional damage has been attributed to plasma membranes being in the gel phase at imbibition (Crowe *et al.*, 1989b). Melting these membranes prior to imbibition by preheating or prehydration from the vapour phase can alleviate the stress (Hoekstra and Van der Wal, 1988). It has recently been hypothesized that the rigidity of the plasma membrane at imbibition is the critical factor determining whether the organ(ism) survives rehydration or not (Hoekstra *et al.*, 1999). Increasing the elasticity of the plasma membranes would prevent rupture during imbibition.

It has also been suggested that the sensitivity to chilling of neem seed is associated with a high  $T_m$  of plasma membranes in the fresh seed (Sacandé *et al.*, 1998). High  $T_m$  values have been found in membranes of other tropical plant species. Hence, lowering the temperature below  $T_m$  would lead to gel phase formation and lateral phase separation, followed by leakage. The extreme sensitivity to imbibitional stress of dry neem seed has also been hypothesized to depend on an intrinsically high  $T_m$  of the plasma membranes (Sacandé *et al.*, 1998).

These hypotheses were tested in this paper. The effects of chilling temperatures on leakage of solutes and membrane barrier properties as measures of the functional state of the plasma membranes were investigated using a nitroxyl spin probe technique. This technique has been used recently to study the *in situ* membrane integrity and permeability of very small amounts of plant material (Golovina and Tikhonov, 1994; Golovina *et al.*, 1997). Freeze fracturing and TEM were used to investigate changes in membrane structure associated with chilling. The *in situ*  $T_m$  of membranes was determined by FTIR microspectroscopy. Imbibitional damage was investigated in relation to the intactness of the plasma membranes. The behaviour of membranes in this tropical tree seed species is discussed in the light of our results, and improved protocols for the storage of tropical seeds with difficult storage behaviour are proposed.



## Material and methods

### *Plant material*

Experiments were carried out using seeds from yellow fruits of neem (*Azadirachta indica* A. Juss.), which were harvested from plantations at Ouagadougou, Burkina Faso. The seeds were prepared using the method described by Sacandé *et al.* (1996, 1997). The seed covers (endocarp) of the fresh seeds were removed to utilise embryos for the determination of initial water content and germination capacity. Three replicates of five embryos were weighed, then dried at 96°C for 48 hours and again weighed to determine the moisture content (MC) calculated as a percentage of the fresh weight (FW) (ISTA, 1993).

### *Determination of viability*

The embryos were soaked in tap-water for four hours at different temperatures (2 to 40°C) and thereafter transferred to a constant temperature of 25°C for germination on wetted filter paper in Petri dishes (10 cm diameter). Other seeds were incubated directly in the Petri dishes for germination at a range of different temperatures. Replicates of 10-15 embryos per Petri dish were used for the determination of germinability. The seeds were inspected regularly during 4 weeks and the germinated individuals were removed. Embryos were considered as germinated when radicles had elongated by 2-3 cm (ISTA, 1993). Statistical analysis of the germination data was carried out using the  $\chi^2$ -test, and differences were regarded as significant at  $P \leq 0.05$ .

### *Measurements of $K^+$ -leakage*

$K^+$ -levels were measured in eluates from four replicates of 10 embryos each, using a flame photometer (PFP 7, Jenway, Felsted, UK). Fresh embryos which had been maintained in humid conditions at 5 or 25°C for different durations (up to 20 days) were used. After weighing, they were incubated in 40 ml of Milli-Q (Millipore) water in Petri-dishes, and after 6 h, the  $K^+$  leached from the embryos was determined in 4 ml of incubation solution using the flame photometer.  $K^+$ -leakage from the embryos was expressed in  $\mu\text{moles/g FW}$  over the first 6 h of incubation. The same embryos were tested for their germination capacity.

### *Electron paramagnetic resonance spin probe measurements*

A nitroxyl spin probe technique was used to estimate the integrity (or barrier properties) of plasma membranes in neem seed tissues. EPR spectra were obtained at room temperature using an X-band EPR spectrometer (model 300E, Bruker Analytik, Rheinstetten, Germany) as described by Golovina and Tikhonov (1994) and Golovina *et al.* (1997). Briefly, embryonic axes and cotyledon cubes from fresh neem seeds were excised after different periods of storage at 5°C. Samples were put into capillaries (2 mm diameter), then 1-2  $\mu\text{l}$  of a solution containing 1 or 2 mM perdeuterated TEMPONE (4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy) and 120 mM potassium ferricyanide was added. The spectra were recorded as soon as possible (time for tuning was about 1-2 min) to avoid the disappearance of the signal. Spectra were recorded at a

power of 2 mW (20 dB) and a modulation amplitude of 0.5 Gauss, conditions that excluded saturation and overmodulation of the EPR spectra. They were normalized to the same concentration of TEMPONE and the same number of scans, so as to be able to compare spectra. Each sample contained approximately the same amount of cotyledon tissue or one axis.

The EPR spin probe technique is based on the differential permeability of membranes to the stable TEMPONE radical and to ferricyanide ions. Adding TEMPONE to cells results in an EPR spectrum that is the sum of signals from all environments where TEMPONE is present. To selectively remove the extracellular spin probe signal, plasma membrane-impermeable transition metal ion complexes (ferricyanide ions) were added. When spin probe molecules contact transition metal ions, the unpaired electron of the spin probe relaxes more quickly, which results in the spectrum broadening up to invisibility. When viable cells are placed in this broadened solution, the TEMPONE signal will reappear because only the spin probe molecules can penetrate into the cells, whereas the charged  $\text{Fe}(\text{CN})_6^{3-}$  ions cannot, which excludes broadening inside these cells (Smirnov *et al.*, 1992).

Within the cell, TEMPONE molecules partition between the lipid fraction (oil and membranes) and the aqueous cytoplasm. The intracellular EPR signal from TEMPONE consequently originates from two different surroundings - lipid and water -, which are resolved in the high field (right-side peaks) region of the spectrum. The signal from TEMPONE molecules outside of the cells will be broadened. Thus, the triplet signal is exclusively derived from TEMPONE molecules inside the cells. When ferricyanide penetrates the cytoplasm through deteriorated plasma membranes, the aqueous signal disappears, but the lipid signal remains because the lipid phase is impermeable to ferricyanide.

## Electron microscopy

### *Transmission electron microscopy*

Samples from seed tissues were glued with TBS (Electron Microscopy Sciences, Washington, USA) onto golden specimen carriers (3 mm, Bal-Tec AG, Liechtenstein) that were placed on a polished brass block cooled by melting ice. Immediately after fixing the specimens, the carriers were plunge-frozen in liquid propane at  $-180^\circ\text{C}$  and stored in liquid nitrogen ( $-196^\circ\text{C}$ ). Freeze-fracture replicas were prepared in a BAF 400 freeze-fracture apparatus (Bal-Tec AG, Liechtenstein) at  $-120^\circ\text{C}$  and  $10^{-7}$  Torr. Platinum was evaporated at an angle of  $40^\circ$ , and carbon was evaporated to support the replicas. The replicas were immersed in 60% glycerol to alleviate cracking, then cleaned with a 50%  $\text{CrO}_3$  solution for 4 h and soaked overnight in bleach solution. The replicas were rinsed in distilled water and collected on EM grids. Electron micrographs were made with a TEM (JEM-1200 EX II, Jeol, Japan) at 80 kV. The membrane fracture face nomenclature of Branton *et al.* (1975) was used for the description of the freeze-fracture images.

*Low temperature scanning electron microscopy*

Whole axes and cotyledon tissue (from 3 seeds for each treatment) were fixed onto a stub with colloidal carbon adhesive (LeitC Neubauer Chemikalien, Münster, Germany). The stubs were placed on a specimen holder and immediately plunged into LN<sub>2</sub> (-196°C). The frozen samples were transferred into a cryo-transfer unit (CT 1500-HF Oxford Instruments, UK). The unit consisted of a cryo-preparation chamber at high vacuum ( $10^{-6}$  Pa) dedicated to an LTSEM (JEOL, model 6300 F, Japan) and a cryo-stage inside the microscope. The specimens were placed inside the cryo-chamber at -85°C, immediately freeze-fractured with a cold scalpel, and freeze-dried for 2 min at -85°C and  $10^{-7}$  Torr. The samples were then sputter-coated with 10 nm platinum. The LTSEM was used to examine the coated seed tissue at 5-10 kV, keeping the temperature of the specimens at -180°C.

**Determination of the  $T_m$  of membranes by FTIR**

We used *in situ* Fourier transform infrared microspectroscopy (FTIR) to determine the  $T_m$  of membranes in hydrated 10-day old seedling roots. FTIR spectra were recorded on a Perkin-Elmer 1725 IR-spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK), equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin-Elmer microscope using the method described by Wolkers and Hoekstra (1995). A thin slice from the root tip was rapidly transferred to a cooled sample holder. The slice was placed between two CaF<sub>2</sub> windows that were tightly mounted into a temperature-controlled brass cell. The temperature of the sample in the instrument was regulated with a computer-controlled device that activated a liquid nitrogen pump, in conjunction with a power supply for heating of the cell. The temperature of the sample was recorded using two PT-100 elements that were located close to the sample windows. The temperature dependence of the FTIR spectra was studied in the range between -60°C and 70°C, starting with the lowest temperature. Spectra were recorded every minute at temperature increments of 1.5°C min<sup>-1</sup>. The instrument was purged of water vapour with a Balston dry air generator (Balston, Maidstone, Kent, UK).

The spectral region between 3000 and 2800 cm<sup>-1</sup> was selected and second derivative spectra were calculated [19 points smoothing factor; calculated using the Infrared Data Manager Analytical Software, version 3.5 (Perkin Elmer)]. The position of the symmetric CH<sub>2</sub> stretching vibration band of the hydrated membranes around 2852 cm<sup>-1</sup> was determined from these second derivative spectra.

## Results

### Chilling stress in hydrated fresh seeds

#### *Effect of temperature on germination*

When highly viable (96% germinative capacity) fresh seeds (37.8 % MC) were incubated in water at a range of constant temperatures, none germinated at  $\leq 10^{\circ}\text{C}$  (Fig. 6.1A). Maximum germination occurred when seeds were incubated at  $\geq 15^{\circ}\text{C}$ , indicating that the threshold temperature for germination lies between 10 and  $15^{\circ}\text{C}$ . However, embryos took two weeks to complete their germination at  $15^{\circ}\text{C}$  (Fig. 6.1B). Incubation at  $25^{\circ}\text{C}$  or  $30^{\circ}\text{C}$  reduced this period to six and four days, respectively. Final germination percentages obtained at temperatures ranging from 15 to  $30^{\circ}\text{C}$  did not differ significantly from each other. A temperature of  $25^{\circ}\text{C}$  was therefore used for germination of neem seeds in all further experiments.

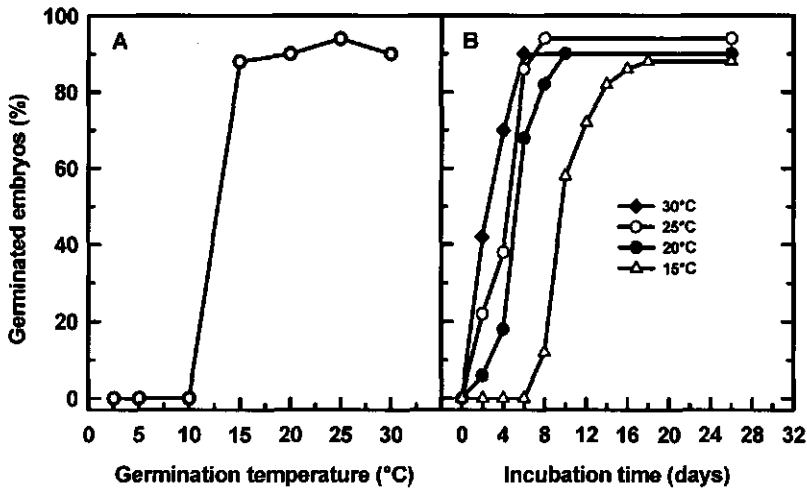
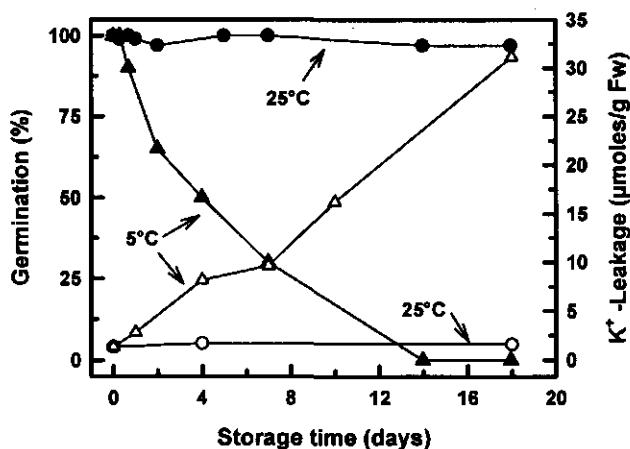


Figure 6.1. Germination capacity (A) and rates of germination (B) of fresh neem embryos (37.8 % MC) incubated at constant temperatures from 2 to  $30^{\circ}\text{C}$ . No germination occurred  $\leq 10^{\circ}\text{C}$ , whereas maximum germination was obtained at  $\geq 15^{\circ}\text{C}$ . Data points are means of three replicates of 15 embryos each. Data are significantly different ( $P \leq 0.05$ ) when they differ by 25% or more ( $\chi^2$ -test).

*Storage at chilling temperatures: effects on germination and K<sup>+</sup>-leakage*

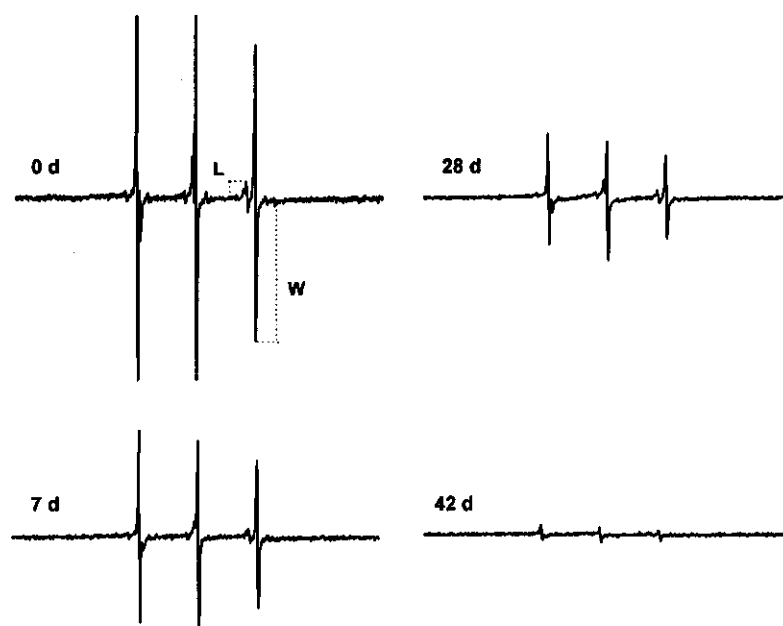
Besides their inability to germinate at and below 10°C, neem seeds (≥10% MC) have been reported to be chilling sensitive in storage (Sacandé *et al.*, 1998; Chapter 3). If the cause of this chilling sensitivity was damage to plasma membranes and not dysfunction of cytoplasmic enzymes, one would expect the chilled embryos to become leaky. Upon incubation in water for germination at 25°C, the leakage of K<sup>+</sup>-ions from fresh embryos chilled at 5°C for variable periods of time was measured, so as to investigate the functional state of the plasma membranes. Figure 6.2 shows that storage at 5°C led to a considerable increase in leakage, which coincided with a decrease in germination percentage. None of these chilled embryos germinated after 14 days of storage at 5°C. Control embryos kept at 25°C showed high germination over the entire storage period, and leakage remained low.



**Figure 6.2.** Effect of storage of fresh neem embryos (37.8% MC) under high humidity conditions at 5°C and 25°C on K<sup>+</sup>-leakage (open symbols) and germination (filled symbols) at 25°C. The leakage is expressed in µmoles/g FW over the first 6 h of incubation in water. The same embryos from which K<sup>+</sup>-leakage was measured were also used in the germination test. Germination data are from 40 embryos; Leakage data are from 4 replicates of 10 embryos.

### Storage at chilling temperatures: effect on membrane barrier properties

Plasma membrane barrier properties can be studied with a nitroxyl spin probe method (Golovina *et al.*, 1997). A selection of typical EPR spectra of TEMPONE from embryonic axes of fresh neem embryos after storage at 5°C for different durations is shown in Fig. 6.3. All spectra contained cytoplasmic and lipid components, discernible at the high field region of the spectrum (right side). Because each spectrum was recorded from only one axis at the same instrumental conditions (power and modulation amplitude), the amplitudes of the different spectra can be compared. The spectra show a gradual decrease in the amplitudes of both the lipid and cytoplasmic components with time of storage. Amplitudes of these components in the axes of seeds stored at 25°C did not show such a decrease (data not shown).



**Figure 6.3.** Representative EPR spectra of perdeuterated TEMPONE in fresh axes in the course of storage of neem embryos under humid conditions at 5°C. Each spectrum was recorded using one axis. The lipid (L) and aqueous (W) cytoplasmic components are indicated in the high-field region (right side) of the spectrum. Ferricyanide (120 mM) was always present as the broadening agent.

From similar EPR spectra of TEMPONE from chilled axes, the amplitudes of the lipid and cytoplasmic peaks were determined (Figs. 6.4A,B). The amplitude of the aqueous cytoplasmic peak did not change during first 4 days of cold storage, but thereafter it decreased. The (W/L) ratio of the line heights of water to lipid is regarded as a good measure of the barrier properties of plasma membranes (Golovina *et al.*, 1997). When ferricyanide starts penetrating through a deteriorating membrane, the amplitude of the aqueous component decreases, which causes the W/L ratio (R-ratio) to decrease. A high ratio thus represents low permeability. An R-value of 9.5 was measured for the control axes at the onset of the cold storage (Fig. 6.4C). Unexpectedly, the ratios increased during further storage at 5°C, to reach a maximum at approximately 17 days, and decreased thereafter. After 42 days, the R-value had decreased to 6. However, R-values can be used as a measure of plasma membrane integrity only when the lipid component does not change. Therefore, we cannot use R-values for characterising membrane integrity in this case. The amplitudes of the aqueous cytoplasmic component are more informative in this respect, suggesting that there is a gradual loss of plasma membrane integrity from 4 days of storage at 5°C onward.

There was a discrepancy between the already reduced germination capacity at 4 days of storage at 5°C (Fig. 6.2) and the still high amplitude of the aqueous component in the EPR spectrum (Fig. 6.4B) indicative of a lack of membrane damage. It may be that injuries that developed during incubation on wetted filter paper in Petri dishes at 25°C reduced germination. We therefore also recorded EPR spectra of TEMPONE from axes after several days of incubation of the 4-day chilled embryos in the Petri dishes at 25°C. Only non-germinating embryos were selected for this analysis of plasma membrane integrity. The amplitude of the aqueous peaks decreased from  $205 \pm 26$  for the control 4-day chilled axes to  $125 \pm 13$  after two days of incubation, and to  $88 \pm 53$  after 5 days. This decrease in amplitude with days of incubation might be interpreted to mean that some slight membrane damage, which existed already after 4 days of cold storage, developed further during incubation at 25°C, which may explain the failure of the embryos to germinate.

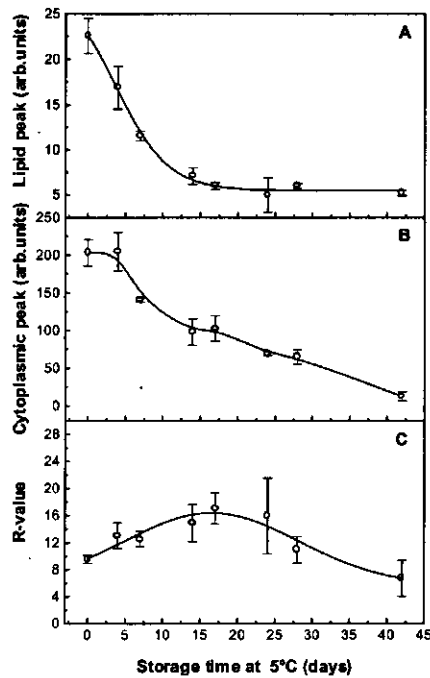
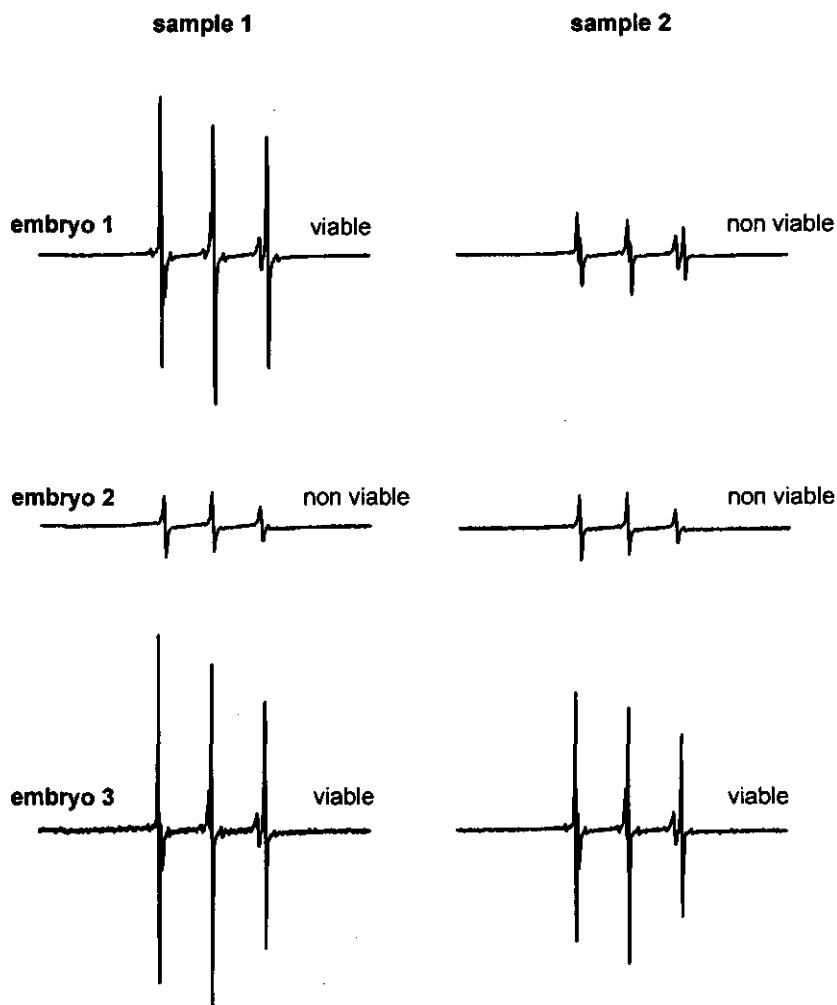


Figure 6.4. Quantitative aspects derived from EPR spectra of perdeuterated TEMPONE in fresh neem axes during storage at 5°C. Amplitude of (A) the lipid component and of (B) the aqueous component; (C) R-ratio (ratio W/L) as a measure of plasma membrane barrier properties. Amplitudes are average of spectra from 6 different axes  $\pm$ SE.

Cotyledons from the same embryos, which had provided the axes (Fig. 6.4), were probed for membrane intactness with the spin probe technique. The EPR spectra from cotyledons varied considerably, even within parts of the same cotyledon, and were difficult to quantify with respect to membrane integrity. Figure 6.5 shows a selection of these spectra obtained from cotyledons of three individual embryos after 14 days of storage at 5°C. Specimens from two different locations in the cotyledon were assayed, sometimes showing large differences in the amplitude ratio of the lipid to aqueous components (cf sample 1 and 2 in embryo 1, Fig. 6.5). The ratio (W/L) can be used here to roughly discriminate viable (intact membranes) from non-viable (deteriorated membranes) tissues. Whereas sample 1 from embryo 1 appears to represent viable tissue, sample 2 from another location in the same cotyledon appears to represent non-viable tissue. Such spectra of TEMPONE characterizing viable tissue were also observed in cotyledons of fresh neem seeds (viable) before storage at 5°C (data not shown). The spectra from embryo 2 (both samples 1 and 2) lacked the cytoplasmic component, indicative of complete membrane disruption (tissues non-





**Figure 6.5.** Examples of EPR spectra of perdeuterated TEMPONE in fresh cotyledon cubes from 3 different embryos after 14 days of storage of neem embryos in humid air at 5°C. Sample 1 and 2 represent cubes from the same cotyledon. Ferricyanide (120 mM) was always present as the broadening agent. The cubes were marked nonviable or viable on the basis of the ratio W/L.

viable), whereas the spectra from embryo 3 indicate viability of the tissues from both locations. Apparently, damage in cotyledons does not occur simultaneously in all seeds or even inside one cotyledon. These data are supported by the results of tetrazolium staining, which showed patches, which were unstained among stained tissue (data not shown).

The EPR spectra from cotyledon tissue in embryos after 4 days of storage at 5°C did not show any damage (spectra not shown) and resembled those in Figure 6.5 (embryo 1, sample 1) characterizing viable tissue. It was found that damage in cotyledon tissue appeared to develop during subsequent incubation for germination at 25°C as in the case of axes (spectra not shown).

#### *Storage at chilling temperatures: effect on plasma membrane ultra-structure*

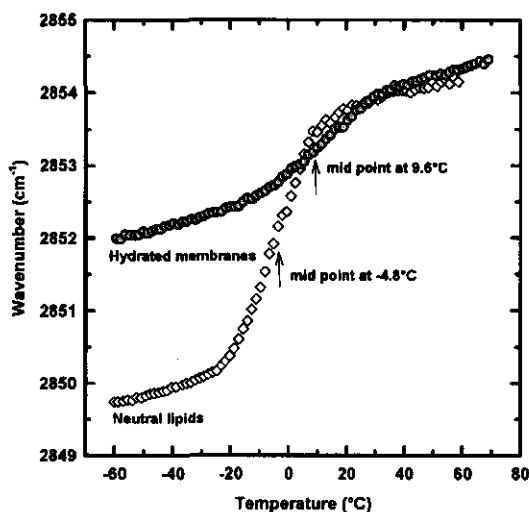
The possible causes of leakage and increased permeability of the plasma membranes of fresh embryos stored at 5°C were studied using freeze-fracture and TEM. Figure 6.6A shows a representative freeze fracture image of the PF side of a plasma membrane in a fresh neem embryonic axis, displaying randomly distributed IMPs. Some plasmodesmata (white arrows) are clearly visible. After 14 days of storage of fresh embryos at 5°C, a number of features can be observed in the axes (Figs. 6.6B-D). Figure 6.6B shows an image of a replica of the PF side of a plasma membrane having a reasonably random distribution of IMPs, with the imprints of cellulose fibrils from the cell wall clearly visible. Patterns of phase separation are evident in Fig. 6.6C, with the IMPs accumulated in particular areas of the replica and IMP-free zones in between. The image shows a depression that resembles a fracture jump lesion, similar to the lesions observed in freeze-injured rye leaves (Webb and Steponkus, 1993). A more detailed inspection of this replica (Fig. 6.6C) revealed bands of tubular structures that we preliminarily identify as inverted hexagonal phase (Fig. 6.6D).

#### **Phase transition of membranes *in situ* ( $T_m$ )**

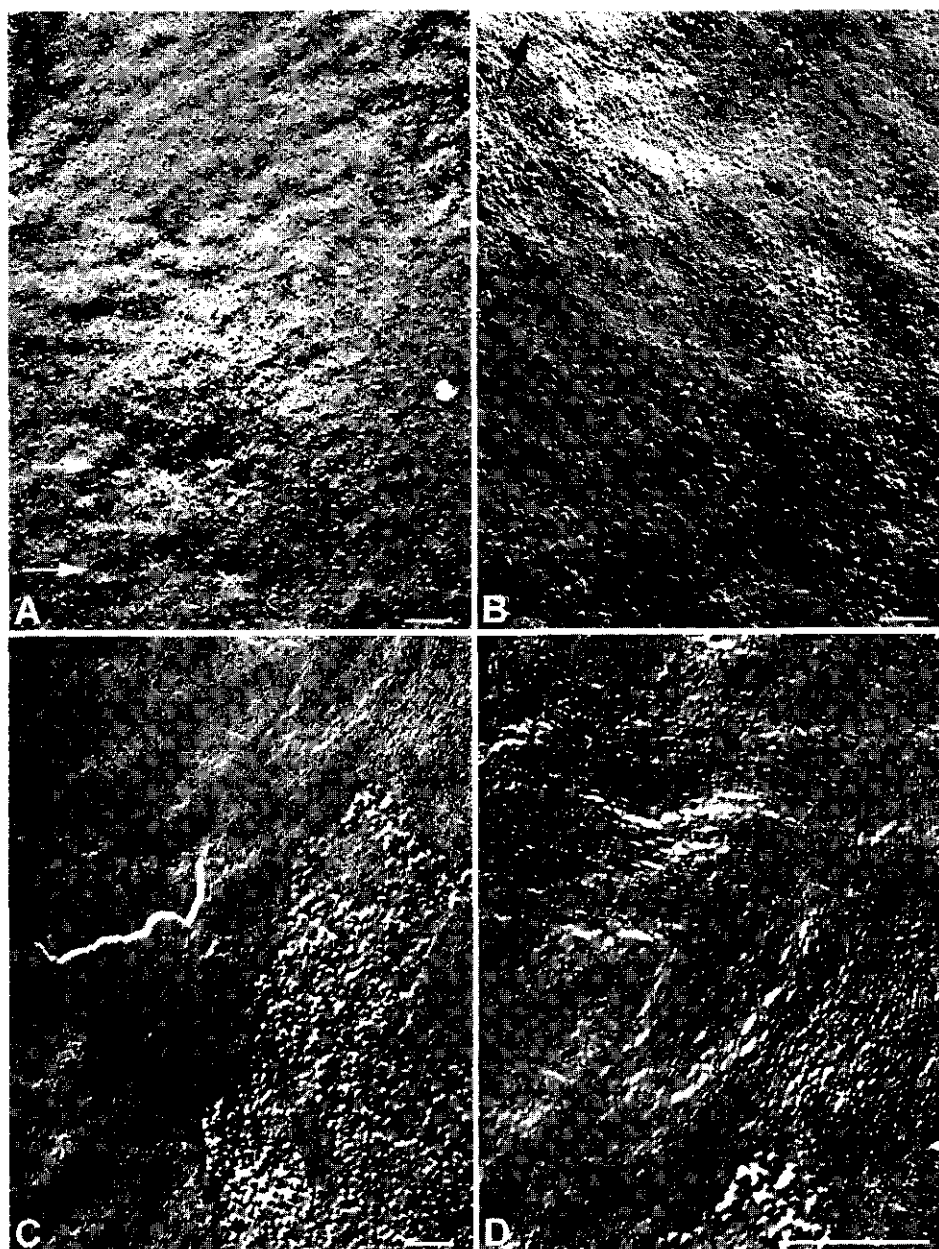
In an attempt to understand the mechanisms involved in the sensitivity to chilling, the *in situ*  $T_m$  of membranes was probed using FTIR microspectroscopy. By following the wavenumber of the symmetric  $\text{CH}_2$  stretching vibration band (at  $2852\text{ cm}^{-1}$ ) during heating of a sample, one can obtain information about the relative packing density of the acyl chains of lipids (Casal and Mantsch, 1984). However, the abundant oil in fresh seeds and, even in the axes, confounded the spectra too much to be able to determine thermal events attributable to membranes. We therefore had to resort to roots of germinating 10-day old seedlings as the experimental material, which had consumed most of their oil. Figure 6.7 shows a representative wavenumber versus temperature plot for a slice of an axial root tip. The mid value during the cooperative phase transition from the gel to liquid crystalline phase lies at approximately 9.6°C. To verify that oil did not contribute too much to this plot, a wavenumber vs. temperature plot of oil pressed out of a non-germinated axis is also presented. From this plot a  $T_m$  of -4.8°C was derived. Even if the root tip slice had residual amounts of oil, it is clear that the estimated  $T_m$  of the lipids in the root tip is higher than that of pure oil, suggesting

that there was a considerable contribution of membrane lipids to the spectrum. If correction for traces of neutral lipid in the root tip plot were possible, it would probably increase the estimated  $T_m$  of membranes by at most a few °C. This value nevertheless demonstrates that the  $T_m$  of membranes in fresh neem tissue ranges in the order of what has been found for other tropical plant species.

**Figure 6.6.** TEM micrographs of replicas from freeze-fractured axes that were excised from fresh neem embryos after 14 days of storage in humid air at 25°C, or 5°C. (A) 25°C; the IMPs on the PF side are randomly distributed and plasmodesmata are visible (white arrows) (bar = 100 nm). (B) 5°C; the IMPs on the EF side have a reasonably random distribution; imprints of cellulose microfibrils are visible (bar = 100 nm). (C) 5°C, the IMPs on the PF side show distinct clustering and there are IMP-free zones (bar = 100 nm). (D) Details of the IMP-free zone from micrograph (C) in which arrays consisting of inverted hexagonal phase can be distinguished (bar = 10 nm). The black arrows indicate the shadowing direction.



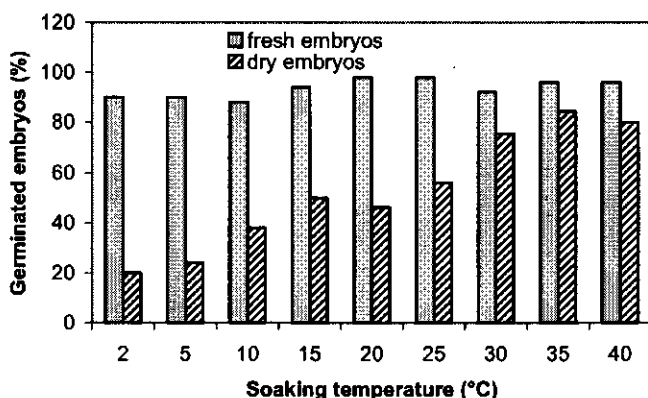
**Figure 6.7.** Wavenumber-temperature plot of the symmetric  $\text{CH}_2$  stretching vibration band (FTIR) of a (hydrated) root slice of a 10-day old neem seedling and of oil pressed out of a dry neem axis. The mid value of the melting transition, which we consider as  $T_m$ , was determined at 9.6°C and -4.8°C for the root and the oil, respectively.



## Imbibitional chilling stress in dry neem seeds

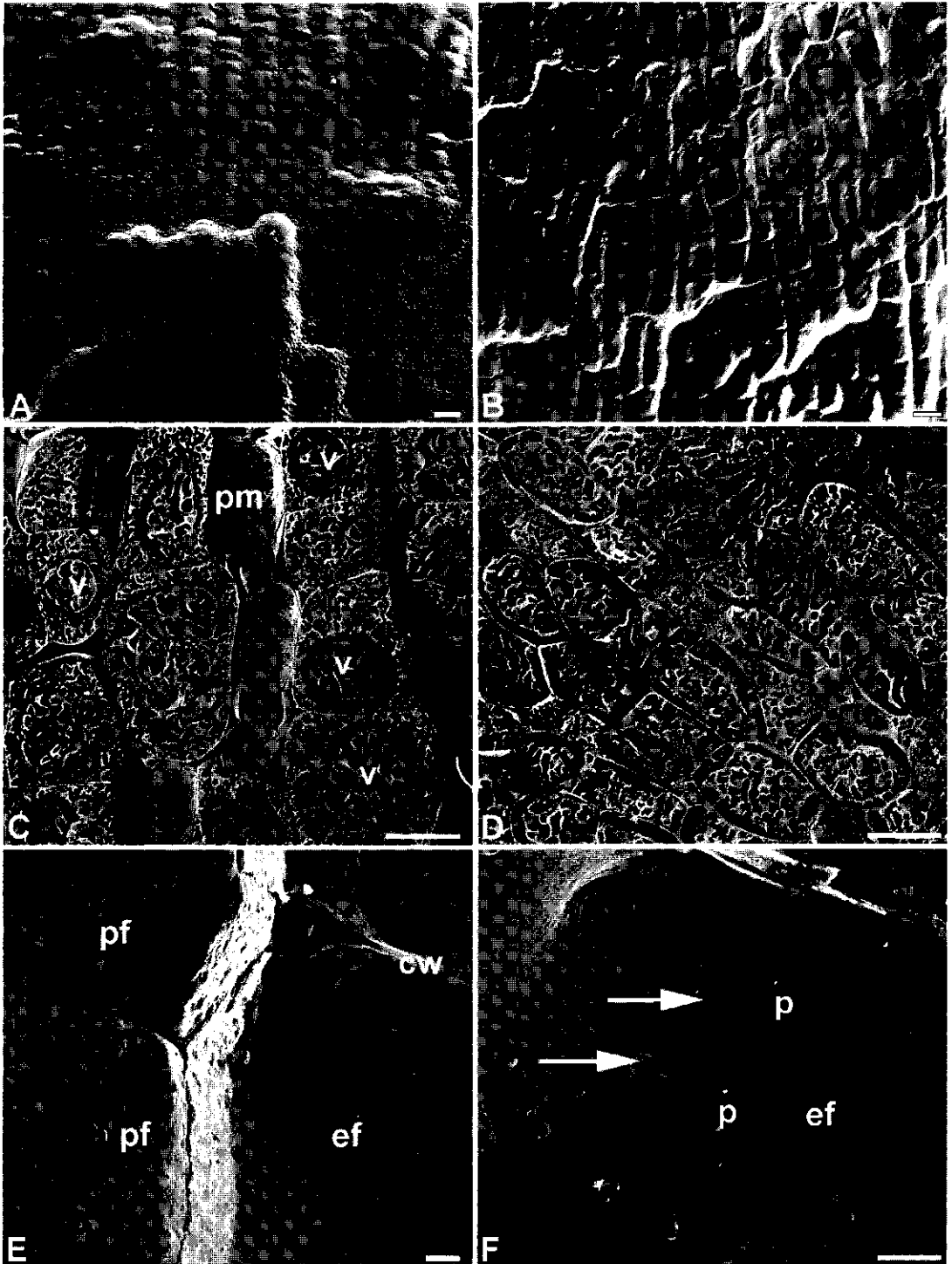
### *Effect of imbibition temperature on germination*

With dehydration, neem seeds become increasingly sensitive to rehydration in liquid water (Sacandé *et al.*, 1998; Chapter 3). Imbibitional damage is more extensive at lower soaking temperatures and increases with the age of the dry seeds. To investigate the sensitivity to imbibition, embryos (seeds without endocarp) from a fresh (37.8% MC) and a dried (4.3% MC; after 6 weeks at 32% RH, 20°C) neem seed lot were soaked for 4 h in water at a range of temperatures, followed by incubation in water (Petri dishes) at 25°C. Figure 6.8 shows the percentage of germinated embryos after these treatments. More than 90% of the fresh embryos germinated at all of the imbibition temperatures tested, indicating that the fresh seeds were not sensitive to soaking under chilling conditions. In contrast, germination of the dry embryos was low at low soaking temperatures and high at elevated soaking temperatures, 30–40°C being optimal.



**Figure 6.8.** Effect of the temperature of soaking (for 4 h) on the germination (at 25°C) of neem embryos. The embryos tested were fresh (37.8% MC) or dried (4.3% MC, after equilibration to 32% RH during 6 weeks at 20°C). The initial viability was >90%. Data points are means of three replicates of 15 embryos each. Data are significantly different ( $P \leq 0.05$ ) when they differ by 25% or more ( $\chi^2$ -test).


**Figure 6.9.** Cryo-SEM micrographs of axes excised from dry neem embryos (4.3% MC) that were soaked in water for 4 h, either at 35°C or 5°C, followed by an additional incubation in water (in Petri dishes) for 15 h at 25°C. Surface morphology after (A) the 35°C soaking treatment and (B) the 5°C soaking treatment. Cryo-SEM micrographs of fractured cells after (C) the 35°C soaking treatment and (D) the 5°C soaking treatment. (E,F) Details of the PF and EF sides of plasma membranes (35°C treatment) showing pits and plasmodesmata (white arrows) and cellulose microfibrils in the EF side (F). Bars are 10 µm in A–D, and 1 µm in E,F. The perforations in E are due to ice crystal growth during plunging in liquid nitrogen. cw = cell wall, cf = exoplasmic face, p = pit, pf = protoplasmic face, pm = plasma membrane, v = vacuole.



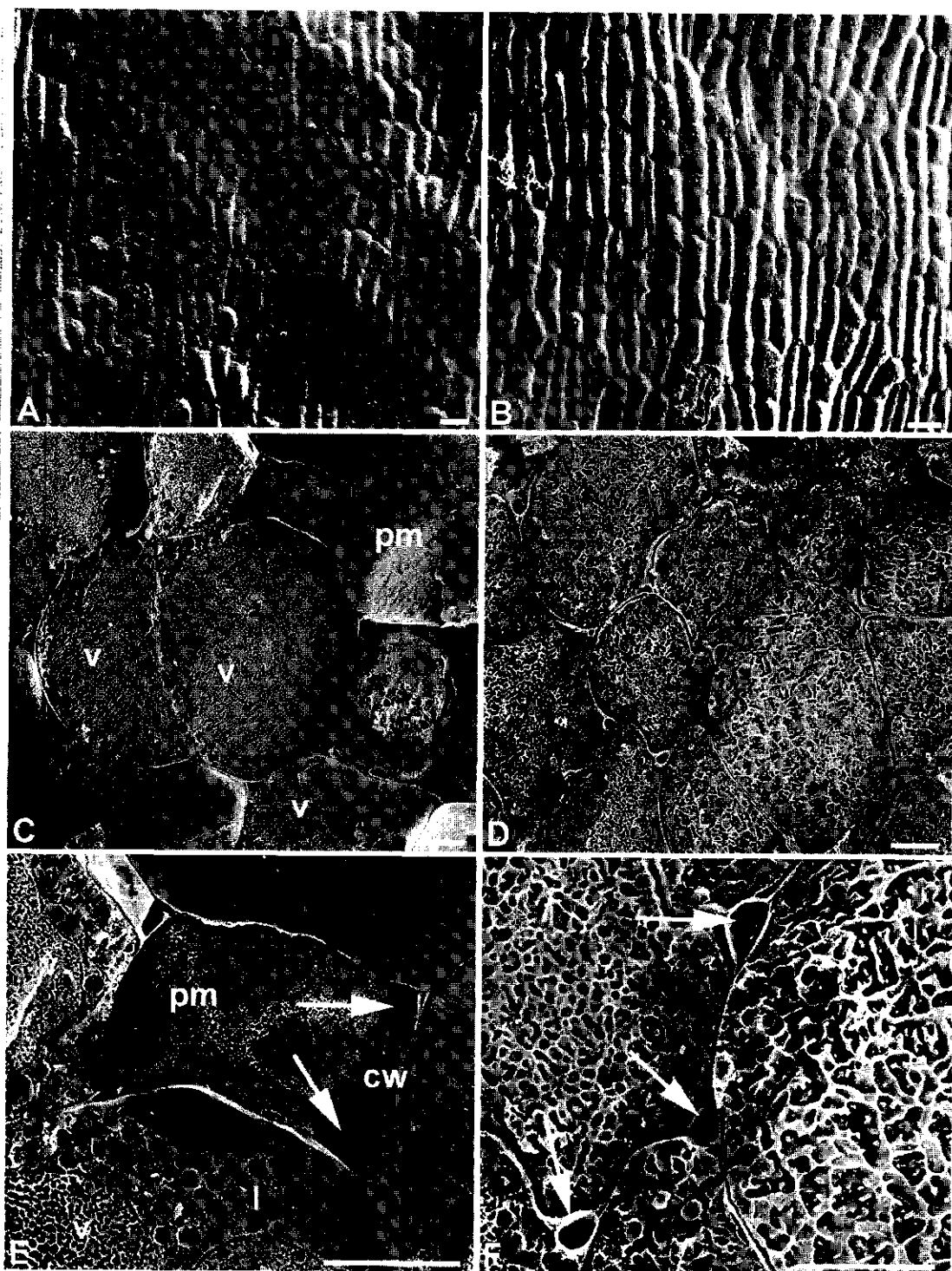
*LTSEM inspection of imbibitional injury*

In order to be able to explore the relationship between imbibitional injury of the dry embryo and membrane damage, LTSEM images were made from dry embryos that were soaked for 4 h in water at 5°C or 35°C, followed by 15 h of incubation on wetted filter paper in Petri dishes at 25°C. Soaking at 5°C killed the embryos, whereas embryos soaked at 35°C germinated well. Figure 6.9 shows representative LTSEM images from axes after the two treatments. Whereas after the 35°C-soaking treatment (Fig. 6.9A) the axes cells had a surface morphology typical of swollen turgescient cells, those after the 5°C soaking treatment were not turgescient and appeared wrinkled (Fig. 6.9B).

Cryofracturing revealed the internal structure of the axis cells. In the 35°C-treated axis cells the fracture often occurred across the core of the plasma membranes (Fig. 6.9C), but almost never in the 5°C-treated axes (Fig. 6.9D). The cytoplasm appeared to be appressed to the plasma membrane after the 35°-soaking treatment, and vacuoles were apparent. Cryo-fracture images of the 5°C-soaked axis cells show that there was space between the cell wall and the remainder of the plasma membrane enclosing the cytoplasm, which supports the suggestion that these cells were not turgescient. This lack of turgescence must be ascribed to the loss of the barrier properties of the plasma membrane after the soaking treatment followed by 15 h of further water uptake at 25°C. Figures 6.9E,F show details of the plasma membranes of axis cells after the 35°C treatment, with pits and plasmodesmata clearly visible (E,F), and imprints of cellulose microfibrils on the exoplasmic face (F). This indicates the firm appression of the plasma membrane to the cell wall, which is typical of a turgescient cell.



**Figure 6.10.** Cryo-SEM micrographs of cotyledons from dry neem embryos (4.3% MC) that were soaked in water for 4 h, either at 35°C or 5°C, followed by an additional incubation in water for 15 h at 25°C. Surface morphology after (A) the 35°C soaking treatment and (B) the 5°C soaking treatment. Cryo-SEM micrographs of fractured cells after (C) the 35°C soaking treatment and (D) the 5°C soaking treatment. Details of the cytoplasmic structure and intercellular space (white arrows) are presented in (E) (35°C treatment) and (F) (5°C treatment). Bars are 10 µm. cw = cell wall, l = lipid body, pm = plasma membrane, v = vacuole.





A similar picture emerged when the same LTSEM inspection of cotyledons was performed, except that the surface morphology of the 35°C and 5°C treatments appeared rather similar (Fig. 6.10A,B). As for the axes, cryofracturing caused the fracture plane to tend to follow the core of the plasma membranes after the 35°C-treatment (Fig. 6.10C), but not after the 5°C treatment (Fig. 6.10D). The cytoplasm appeared appressed to the plasma membrane after the 35°-soaking treatment, with organelles and oil bodies clearly visible. Cryo-images of the 5°C-soaked cotyledon cells show a space between the cytoplasm and the cell wall, and the cytoplasm has a disorganized appearance lacking clearly discernible organelles. The cell walls had an undulating appearance, indicating loss of turgor. Figures 6.10E and F show details of Figs. 6.10C and D, respectively. The intercellular space between cotyledon cells after the 35°C treatment had a triangular shape and no water, in contrast to that after the 5°C treatment, which had a spherical shape and was filled with water.

## Discussion

### *Chilling stress*

The viability of seeds of tropical origin is known to be adversely affected by chilling temperatures (Corbineau and Côme, 1988; Chin *et al.*, 1989; Tompsett, 1994). It has been shown that stored neem seeds are sensitive to chilling temperatures when the MC of the seed is  $\geq 10\%$  (Sacandé *et al.*, 1998). This sensitivity to chilling limits the potential for the long-term maintenance of neem seed viability during storage. We hypothesized that the causes of chilling sensitivity may lie in the elevated  $T_m$  of membranes in neem seeds, as it is for vegetative tissues of a few other tropical plants ( $T_m$  ranging from 10–15°C; Crowe *et al.*, 1989c; Mazliak, 1992). Although it was difficult to determine  $T_m$  in seeds because of the abundant oil, FTIR spectra of fresh root tips of neem seedlings indicate a  $T_m$  of at least 9.6°C (Fig. 6.7), and probably a few °C higher. This implies that below about 10°C, membranes tend to be in the rigid gel phase. As a result, it might be expected that solutes would leak from hydrated seeds. We indeed found that fresh embryos leaked more  $K^+$  the longer they were stored, and that this coincided with a decrease in germination capacity (Fig. 6.2). The spin probe method provided further evidence for reduced integrity of the plasma membranes with time at 5°C (Figs. 6.3, 6.4, 6.5). A study of the plasma membrane ultrastructure supports the view that a phase transition occurs below 10°C in fresh neem seed (Fig. 6.6). After 14 days at 5°C, IMP-free areas were found in the plasma membranes of axes adjacent to areas of clustered IMPs (Fig. 6.6C), although intact cells with random distribution were also apparent (Fig. 6.6B). This points to lateral phase separation.  $H_{II}$ -like structures, which may have formed upon phase separation, were observed. It is not entirely clear whether the lateral phase separation and  $H_{II}$  phase are primarily a result of the low temperature or whether they are a secondary effect of leaky membranes in the deteriorating cells. Besides the occurrence of the  $H_{II}$  phase, an interesting feature that was observed is the fracture jump lesion in the replica of the plasma membrane of the chilled seed (Fig. 6.6C). In freezing damaged rye leaves,

this lesion was ascribed to freeze induced dehydration (Webb and Steponkus, 1993). In our case no such dehydration took place because the temperature was not reduced below 0°C, so we attribute the appearance of this type of lesion to the effects of the transition of the plasma membrane to the gel phase. In rye, the temperature probably has to be lowered to far below 0°C to evoke a phase transition to the gel phase in the plasma membranes - a temperature that also allows the formation of ice crystals.

There are indications that further dehydration of neem seeds below 10% MC alleviates chilling/subzero-°C stress (Hong and Ellis, 1998; Sacandé *et al.*, 1998, 2000). This may be due to the absence of a phase transition or a phase separation. Because a depression of  $T_m$  with drying is very unlikely (actually the reverse is usually observed (reviewed in Hoekstra and Golovina, 1999)), we suggest that it is the lateral phase separation that does not occur, probably because of a decreased lateral mobility of the membrane components. It is likely that the lateral mobility is restricted at low water contents because of hydrogen bonding interactions with the glassy cytoplasmic matrix (Sacandé *et al.*, 2000).

Fresh neem embryos did not germinate when incubated at temperatures of  $\leq 10^\circ\text{C}$  (Fig. 6.1). The threshold temperature for germination thus appears to be between 10 and 15°C. Although not examined for membrane damage, it is expected that similar membrane phase behaviour is involved in the inability of the embryos to germinate.

#### *Imbibitional stress*

A number of seeds, particularly those of tropical origin, are damaged when they are plunged into water at low temperatures (Hobbs and Obendorf, 1972; Cohn and Obendorf, 1976; Woodstock *et al.*, 1985). The seeds of cotton, soybean, cowpea, maize, and sorghum are typical examples. These phenomena have also been observed in pollens and yeast (Van Steveninck and Ledebøer, 1974; Hoekstra, 1984). The decrease in germinability coincides with increased leakage of solutes from the tissue. The injury can be prevented by prehydration from the vapour phase or preheating before imbibition in water or germination medium (Cal and Obendorf, 1972; Hoekstra and Bruinsma, 1975; Hoekstra, 1984; Hoekstra and Van der Wal, 1988). It is thought that the conformational status of the plasma membranes just before imbibition is involved in imbibitional injury (Crowe *et al.*, 1989b). It has recently been hypothesized that the mechanical properties of the plasma membranes determine the ability to withstand imbibition (Hoekstra *et al.*, 1999). Plasma membranes exhibited holes within seconds after imbibition when they were in gel phase just before imbibition. The treatments that alleviate imbibitional stress such as prehydration from the vapour phase and preheating also melt the plasma membranes.

Because of the intrinsically high  $T_m$  of membranes in tropical plants and the fact that dehydration tends to increase this  $T_m$ , it seems logical that tropical seeds are particularly sensitive to imbibitional damage. We show in Fig. 6.7 that neem has a membrane  $T_m$  typical of tropical plants. We have previously demonstrated that neem seeds become sensitive to imbibitional stress after drying, a phenomenon which is augmented during ageing under dry conditions (Sacandé *et al.*, 1998). In addition, in Fig. 6.8 we show that dry embryos are sensitive to soaking in water during the first

4-h, particularly at chilling temperatures. Hydrated embryos were insensitive to the 4-h soaking at low temperatures. Apparently the four hours of chilling is too short to cause problems in the plasma membranes.

Support for a plasma membrane-related cause for the imbibitional injury comes from our LTSEM micrographs depicting axes and cotyledons after soaking of dry embryos at 5 and 35°C (Figs. 6.9, 6.10). The surface morphology of the axes clearly indicates loss of turgor after the soaking at 5°C, in contrast to the swollen appearance of cells after soaking at 35°C. The ultrastructural appearance of cryo-fractured cells supports this view. The strong appression of the plasma membrane to the cell wall, as evidenced by the imprints of cellulose microfibrils after the 35°C imbibitional treatment, is proof of turgescence, viable cells. The 15 h incubation following the 4-h soaking was meant to allow the embryo to fully rehydrate. In the case of soaking injury it also allowed further deterioration of the cells. The axis and cotyledon cells of the 5°C soaking treatment do indeed have a fully deteriorated appearance. The fracturing did not follow the core of the plasma membranes, which can be interpreted to mean that these membranes had become virtually nonexistent. We suggest that damage to the plasma membranes underlies imbibitional chilling injury.

Dry seeds of papaya, characterized as exhibiting intermediate storage behaviour (Ellis *et al.*, 1991), fail to germinate when they are incubated in water at 20°C, but do so at higher temperatures (Wood *et al.*, 2000). It has been shown that they remain viable for some time, even when they do not germinate. The seeds need an additional heat treatment to release the dormancy and allow them to germinate. This behaviour has been described as dehydration-imposed dormancy. The LTSEM micrographs (Figs. 6.9, 6.10) of neem embryos indicate that the reduced germination below the optimal soaking temperatures of 30–40°C is likely to be due to imbibitional damage and not to dehydration-imposed dormancy.

The results reported in this paper suggest that the elevated  $T_m$  of membranes in neem, a tree of tropical origin, is associated with the sensitivity of seeds to both chilling and imbibitional stress. A high  $T_m$  increases the likelihood of the occurrence of the rigid gel phase when membranes are dehydrated or chilled. Plasma membranes in the gel phase are assumed to be responsible for the injuries caused by chilling and imbibition. These sensitivities may explain the conflicting reports in the literature on desiccation tolerance and storage longevity of neem seeds. Seeds may have been killed during rehydration rather than during dehydration and storage. Protocols for handling and storage of neem seed and tropical seeds in general, which pay special attention to appropriate rehydration procedures, are recommended.

### Acknowledgements

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## General discussion

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The importance of neem (*Azadirachta indica*) in developing countries in the tropics is widely recognised. Neem has many uses and is eliciting interest from industry because it contains anti-malarial, antimicrobial, antifungal, insecticidal and insect-repellent substances (Dhar *et al.*, 1998, and references therein). However, the propagation of neem from seed is hampered by its short storage longevity. The present research project was aimed at improving the life span of neem seeds in storage, in order to facilitate and enhance planting programmes in the Sahelian and other arid and semi-arid zones. To this end, factors influencing desiccation tolerance and storage longevity of the seeds were investigated using a variety of techniques. These investigations contributed to a better understanding of the complex storage behaviour of neem seeds. The resultant recommendations for improving neem seed storage may be applicable to tropical seeds in general.

Conventional methods of storage involve keeping seeds at low temperatures and moisture contents. Seeds that cannot tolerate such conditions are difficult to manipulate and conserve (Ellis and Roberts, 1980). This makes their management in seed banks problematic and their availability for tree planting programmes erratic. Such intermediate or recalcitrant storage behaviour is common in seeds from tropical plant species (Hong *et al.*, 1996).

Little is known about the biology of tropical seeds in general. There is controversy in the literature concerning the characterisation of the storage behaviour of neem seed (review by Poulsen, 1996; Hong *et al.*, 1996; Tompsett and Kemp, 1996; Hong and Ellis, 1998). Different authors report being able to store neem seeds for periods ranging from a few months to a number of years and thus have characterised neem as recalcitrant, as intermediate, or even as orthodox. This variation in storage capability may be due to a number of factors including genetic differences (different accessions), the maturation stage of the fruit or seed, the temperature and rate of drying, the temperature and MC during storage, and the method of rehydration. The fact that seeds from some lots have been successfully stored for more than one year indicates that seed behaviour may potentially be orthodox and that long-term storage of neem seed may consequently be possible.

In the work described in this thesis, seeds from sources in Africa and Asia were desiccated and stored in a variety of different conditions, and several parameters were tested (Chapter 3). The results showed few differences in desiccation tolerance and storage behaviour between the seeds from these different sources. However, seeds originating from mature yellow fruits lived longer than the seeds from the younger green fruits, or the older brown fruits. Unless otherwise stated, seeds from yellow fruits were used throughout the experiments described in the thesis.

### **To which storage category does neem seed belong?**

#### *Evidence based on cell cycle activity*

Recalcitrant seeds have high MCs when they are shed, which allows them to maintain high levels of metabolism (reviewed in Pammenter and Berjak, 1999). Because DNA replication reflects high metabolic activity in seeds, the desiccation sensitivity of a seed species has been claimed to be linked to the amount of DNA in the nuclei. Mature seeds that have their nuclei in the quiescent G<sub>0</sub>/G<sub>1</sub> phase would be expected to be storable for long periods of time, whereas those that have their nuclei in G<sub>2</sub> phase would not. Data on cell cycle events collected using flow cytometry (Chapter 2) indicated that at harvest, mature neem seeds had their cells mostly in the G<sub>1</sub> phase, i.e., with a low proportion of 4C DNA and a high proportion of 2C DNA in their nuclei. In addition, the expression of  $\beta$ -tubulin, a protein required for passage through the cell cycle, was hardly detectable in the mature seeds. Similar results have been found with respect to the downregulation of nuclear divisions in dry tomato seeds, which are orthodox (De Castro *et al.*, 1995). From these facts we conclude that the difficult storage behaviour of neem seeds is not related to high DNA replication activity in the cells, as has been suggested for recalcitrant seeds (Pammenter and Berjak, 1999). However, there are limitations to the validity of this predictive criterion, because other factors also can influence cell cycle activity, for example, seed priming (Lanteri *et al.*, 1994). At present there is disagreement about the significance of the frequencies of 2C and 4C DNA in nuclei with regard to seed storage behaviour (Finch-Savage *et al.*, 1998; Gurusinghe *et al.*, 1999).

#### *Evidence based on sugar content and composition*

Desiccation tolerance may require a certain minimum amount of soluble sugars in seeds, as has been suggested for other anhydrobiotic systems (Hoekstra *et al.*, 1991; Leslie *et al.*, 1994, 1995). Oligosaccharides, in particular, have been linked with orthodox storage behaviour and extended periods of survival (Horbowicz and Obendorf, 1994; Steadman *et al.*, 1996). Our finding that a substantial amount of these protective oligosaccharides was present in dry neem seeds led us to conclude that the seeds could fall into the category of orthodox behaviour and may be able to acquire a considerable degree of desiccation tolerance and storage stability (Chapter 4).

*Evidence based on the ability to accumulate protective compounds during slow drying*

During slow drying of neem seeds from the fresh state to the equilibrium MC at 32% RH and 20°C, the amount of a number of compounds, such as reduced glutathione, a cytoplasmic antioxidant, the oligosaccharides, raffinose and stachyose, and phospholipids was found to increase (Chapter 4). These increases during slow drying suggest that neem seed may acquire a certain degree of tolerance to desiccation. The fact that seeds survive dehydration to 5% MC is further evidence that the storage behaviour of neem seed is orthodox. We have indications that enforced fast drying of fresh seed lots at high temperatures led to reduced levels of desiccation tolerance (Sacandé, unpublished results). We assume that during fast drying there was insufficient time to mobilise the antioxidant defence and macromolecule protective systems.

**Chilling sensitivity**

When seeds fail to tolerate dehydration, it may be necessary to cool them to low temperatures (above zero °C) to reduce metabolic activity for optimum storage longevity. However, many intermediate and recalcitrant tropical seeds are chilling sensitive, i.e., they do not tolerate temperatures below 10–15°C. This also holds for neem. Mature neem seeds having MCs of  $\geq 10\%$  were chilling sensitive and died within a few months (Chapter 3). At ambient temperatures storage longevity of neem seeds was also relatively short. The decline in viability coincided with oxidative stress, as shown by a change in the glutathione redox status, and with decreases in the amounts of oligosaccharides and phospholipids (Chapter 4). These factors are presumably linked to the loss of viability. It should be noted that water activity in the cytoplasm of neem seeds is generally underestimated because of the high oil content (50% of the DW), causing unexpected metabolic stress at the higher MC and temperature ranges.

Some authors reported similar results from comparable storage experiments at elevated MCs and concluded that neem seeds display recalcitrant storage behaviour (Webb *et al.*, 1984; Chaisurisri *et al.*, 1986; Nagaveni *et al.*, 1987; Maithany *et al.*, 1989; Ezumah, 1996; Msanga, 1996). Recalcitrant seeds from temperate climates are usually more tolerant to low temperatures than those 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; Chin *et al.*, 1988). Neem seeds dried further to 4–8% MC lost their chilling sensitivity, but became sensitive to damage on imbibition (for details, see below). It was found that dry neem seeds tolerated a 2-year storage period with an overall average germinability of 50% when kept at the temperature range of -20°C to +20°C (Chapter 3). In these conditions the redox status of glutathione was stable, suggesting that oxidative stress did not play an important role (Chapter 4). The few authors who designated neem seeds as orthodox reported successful storage at this particularly low MC range (4%-MC seeds: for 5–8 years at +4°C, Roederer and Bellefontaine, 1989; Bellefontaine and Audinet, 1993; and for 10 years at -20°C, Tompsett and Kemp, 1996).

### **Imbibitional stress**

The germination percentage of neem seeds decreased during equilibration to low constant MCs of  $\leq 8\%$ , and seeds surviving this drying treatment seemed not to deteriorate further during prolonged storage. The sample may have contained two populations of seeds: one that deteriorated fast, and another that deteriorated much more slowly. None of the seed lots completely lost germinability over the course of handling and storage. It later became clear that drying renders neem seeds extremely sensitive to imbibitional stress, which could be partially alleviated by soaking the seeds at elevated temperatures. The sensitivity to imbibitional stress increased with seed ageing (Chapter 3). It was necessary to soak seeds at higher temperatures (up to  $40^{\circ}\text{C}$ ) to compensate for the increased imbibitional stress, thus allowing seeds that had been stored for as much as two years to germinate. Because of this extreme sensitivity, dry seeds were probably often killed during imbibition rather than during storage. Although the ability to survive rehydration is a part of desiccation tolerance, the fact that careful rehydration at high temperatures (up to  $40^{\circ}\text{C}$ ) can considerably improve germination is evidence that seeds can withstand severe dehydration without loss of viability. We therefore believe that neem seeds are endowed with the competence to acquire desiccation tolerance and, are able consequently to survive storage for long periods. They can thus be considered to display orthodox storage behaviour.

### **Membrane properties as a cause of the complex storage behaviour of neem seeds**

The difficult storage behaviour of neem seed may stem from its sensitivity to (i) low temperatures at MCs  $\geq 10\%$  and (ii) imbibitional stress at MCs below  $10\%$  MC. Both factors may depend on the specific nature of the membranes in tropical plant species, as will be outlined next.

Many plants of tropical origin suffer injury when kept below  $10\text{--}15^{\circ}\text{C}$  for some time. The dysfunction of some membrane bound enzymes (Lyons *et al.*, 1979a) and increased leakage of cytoplasmic solutes from cells (Bergevin *et al.*, 1993; Bertin *et al.*, 1996) has been observed. The most probable cause of chilling injury are the structural changes that take place in membranes changing from the liquid crystalline to the gel phase. It has been shown that the *in situ* phase transition temperature ( $T_m$ ) in tomato hypocotyl membranes is around  $10\text{--}15^{\circ}\text{C}$  (Raison and Orr, 1986; Crowe *et al.*, 1989c). However, the *in situ*  $T_m$ s of membranes in plants from the temperate zones are usually around or below  $0^{\circ}\text{C}$ . Because model membranes tend to become transiently permeable to ions during phase transition (Hammoudah *et al.*, 1981), it is expected that in intact systems, leakage will also increase transiently during a phase transition. Furthermore, some membrane proteins, including ion pumps, may not function well when membranes are in the gel phase (Raison *et al.*, 1971). The  $T_m$  of  $10^{\circ}\text{C}$  of membranes in the root tips of neem seedlings confirms that neem has the typically high  $T_m$  of a plant from the tropics (Chapter 6). This high  $T_m$  may be due to the relatively high degree of saturation of the esterified acyl chains of the phospholipids (Chapter 4).

Although phase changes are reversible upon return to above- $T_m$  temperatures, extended periods of time under low temperature conditions may have drastic consequences (Chapter 6). It has been shown, for example, that lateral phase separations occur in the membranes of the tropical avocado fruit at chilling temperatures (Platt-Aloia and Thomson, 1987; Sharom *et al.*, 1994), i.e., membrane components sort according to their molecular species. Typical patterns can be observed in freeze fracture images, characterised by intra-membrane particle-free areas alternating with intra-membrane particle clusters. The components of a phase-separated system cannot easily resume their original position in the membrane upon return to above- $T_m$  temperatures. Phase-separated membranes cannot retain endogenous cellular solutes, which may result in death. In neem seeds kept at 5°C, increased leakage and decreased barrier properties were observed within a few days, which coincided with inability to germinate (Chapter 6). Phase separation and evidence of inverted hexagonal phase ( $H_{II}$ ) were detected in membranes of fresh neem seed axes that were maintained at 5°C for two weeks. It is unclear whether phase separation was a primary or secondary effect of storage at this low temperature. However, we suggest that a phase transition of membranes coinciding with a phase separation of membrane components may be responsible for cold sensitivity and the debilitation of neem seeds.

Although the high  $T_m$  (10°C) in hydrated neem tissues may explain the chilling sensitivity, it does not explain why this sensitivity is lost below 10% MC. Dehydration below the "magic" water content of 0.25 g H<sub>2</sub>O/g DW (below which biological molecules progressively lose their water shell) would be expected to increase  $T_m$ , as has been demonstrated in model lipid systems (Chapman *et al.*, 1967; Crowe *et al.*, 1985) and intact organ(ism)s (Hoekstra *et al.*, 1997). Due to the high oil content of neem seeds, the afore-mentioned 10% MC for the whole seed actually implies a cytoplasmic water content of approximately 0.22 g/g (= 18.2% on a fresh weight basis). With further drying one would expect an increase in  $T_m$  and, hence, an increase in the sensitivity to cold storage. Just the opposite was found. A possible reason for the loss of chilling sensitivity below 10% MC may be related to the increased viscosities in cytoplasm and membranes, as is addressed in the section on the glassy state.

It has been demonstrated for a number of anhydrobiotes that during drying the  $T_m$  of their membranes increases and even exceeds ambient temperature (Hoekstra *et al.*, 1997). This may not harm viability *per se*, as many such organ(ism)s can be stored successfully in the deep-freezer at -20°C - a condition that favours the existence of the gel phase in the membranes. However, at rehydration, extensive leakage may occur which leads to the death of the seed. It was initially thought that a change in membranes to the liquid crystalline phase during imbibition would cause transient leakage of endogenous solutes. However, it has recently been shown that the plasma membranes in such organ(ism)s completely and permanently lose their barrier properties, and that there are holes in the membranes immediately upon imbibition (Hoekstra *et al.*, 1999). On the basis of this information, it has been hypothesised that the rigidity of the plasma membrane at imbibition is the critical factor in whether the organ(ism) survives rehydration or not. Treatments that decrease the rigidity of the membrane, such as heating



or prehydration from the vapour phase, have been shown to prevent imbibitional stress and the associated leakage (Hoekstra and Van der Wal, 1988).

Similarly, soaking of dry seeds at elevated temperatures improved the survival of neem seeds (Chapters 3 and 6). Membranes become much less ordered (less rigid) after preheating, as measured by *in situ* EPR spectroscopy using membrane-specific spin probes (Sacandé, unpublished material). However, for the dry seeds to be rescued, soaking had to be carried out at unexpectedly high temperatures (35°C). This may stem from the fact that membranes in neem already have an elevated  $T_m$  that can be expected to be even higher in dry seeds. Unfortunately, we were unable to determine  $T_m$  in dry neem seeds from FTIR data because the abundant oil (50% of DW) obscured the melting of the phospholipids. However, by using membrane-specific spin probes we found that at the same temperature there was more solid phase in the membranes of dry neem seeds than in those of hydrated neem seeds, suggesting that  $T_m$  was increased in the dry state (unpublished data).

### **Glassy state**

When inspecting the storage survival data of neem seeds exposed to various RHs, it became clear that there was a break in the relationship between germination and MC. By lowering the MC of neem seeds at 20°C, a sudden improvement of storage longevity occurred at an equilibrium RH of 53% (<10% MC of the whole seed) (Fig. 7.1). To explain this characteristic, the properties of water in embryos were studied using differential scanning calorimetry, which allows detection of the presence of cytoplasmic glasses (Chapter 5). A glass is an extremely viscous liquid with solid state-like properties, which has been shown to occur in seeds and pollen (Buitink *et al.*, 1998). The molecular mobility has been shown to be considerably slower when the cytoplasm is in a glassy state than when it is in the liquid state. The slower molecular mobility in the glass has been linked to a slower rate of ageing (Buitink *et al.*, 2000).

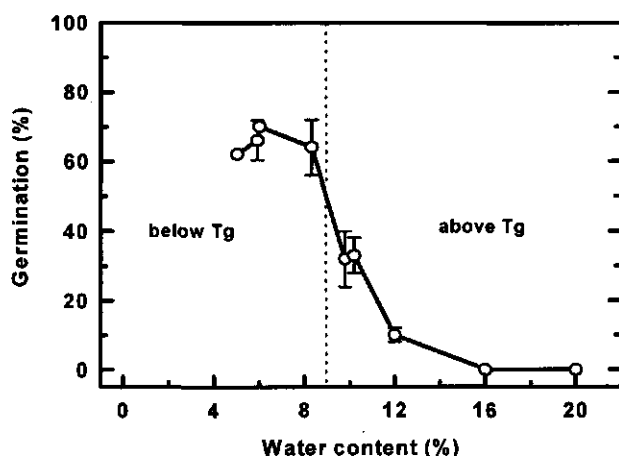


Figure 7.1. Germination percentage of neem seeds after 6 months storage at 20°C, showing better storage longevity at water contents below around 10% MC. Such an improved storage longevity coincided with the seeds being in the glassy state (below  $T_g$ ), whereas above 10% MC the seeds were in the liquid phase (above  $T_g$ ). The dotted line indicates the MC of the seeds at  $T_g$  (at 20°C).

In Chapter 5 it was shown by differential scanning calorimetry that during dehydration neem embryos became glassy, which is also noticeable by the fact that the embryos became hard. The transition to the glassy state occurred during drying just before the equilibrium RH of 53%, which is equivalent to 8.2% MC in the entire seed including the endocarp, or 5% MC in the embryo alone. Under these conditions longevity was more than 2 years at 20°C. State diagrams were produced to fully appreciate the effect of the cytoplasm being in the glassy state on storage longevity and chilling sensitivity. These state diagrams depict the temperatures at which the glassy state melts for all MCs of the embryos or equilibrium RHs to which the seeds were exposed (Chapter 5).

The state diagrams in Chapter 5 can be interpreted to mean that glasses are involved in the loss of chilling sensitivity in seeds having MCs of  $\leq 8\%$ . If the supposition that chilling sensitivity depends on phase transitions in membranes, eventually followed by phase separation, is correct, then the loss of sensitivity could be explained by a depression of  $T_m$  in the presence of a glass. However, it is not very likely that the  $T_m$  of membranes in dry neem embryos would be lower than that in fresh embryos. In other dry anhydrobiotes,  $T_m$  values have been measured that were always equal or higher than

those in the fresh controls (Hoekstra and Golovina, 1999). The only alternative supposition is that lateral phase separations in the membranes cannot occur in the

presence of a glassy state. This may be understood by considering that at low water contents, molecules other than water (sugars for example) are hydrogen bonded to the membrane components and at the same time to the glass, thus restricting lateral diffusion of membrane components (Lee *et al.*, 1986). We therefore believe that restricted lateral diffusion of membrane components is the cause of the loss of chilling sensitivity at low water contents.

### Predictive model for storage longevity of neem seeds

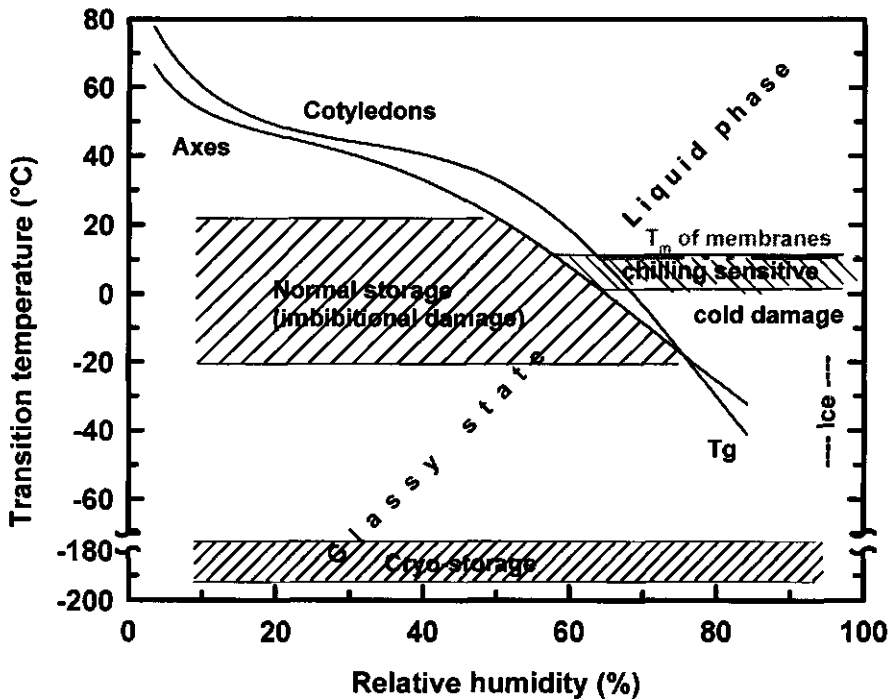
We have combined our experimental data concerning longevity and presence of a glassy state under conditions of different MC and temperature in a diagram (Fig. 7.2) which allows the prediction of efficient methods for the successful storage of neem seeds.

The diagram allows us to speculate about the possible significance of water relations with respect to long-term storage and chilling/subzero sensitivity of neem and tropical intermediate seeds. 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 (Chapter 3; Fig. 7.1). The sensitivity to chilling and subzero temperatures is lost in seeds that are stored at around 50% RH (embryo MCs  $\leq 0.048$  g H<sub>2</sub>O/g DW). Extension of the storage longevity for more than 2 years is possible when both axis and cotyledons are in the glassy state, i.e.,  $10\% \leq \text{RH} \leq 53\%$  and storage temperatures are between 20 and -20°C. This area of effective storage survival is illustrated in Figure 7.2. The low amount of water in all of these samples 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. Therefore, the box of the effective storage is left open at the low moisture side (Fig. 7.2). Other storage studies (Bellefontaine and Audinet, 1993; Tompsett and Kemp, 1996; Hong and Ellis, 1998) also give indications that dehydration can alleviate chilling stress in neem seeds. From our diagram it is clear that the reports of the successful storage of neem seeds were accomplished with seeds that were in the glassy state (see reviews Hong *et al.*, 1996; Poulsen, 1996; Tompsett and Kemp, 1996).

Successful cryogenic storage at liquid nitrogen temperatures has been reported for seeds that were first dried to 0.09 g H<sub>2</sub>O/g DW for the entire seed, or 0.23 g H<sub>2</sub>O/g DW for isolated axes (Berjak and Dumet, 1996). Seeds of higher MC do not tolerate cryogenic storage (Chaudhury and Chandel, 1991). Although safe MCs for cryogenic storage are in the range where no freezable water is expected (see Fig. 7.2; Chapter 5) liquid nitrogen storage may be successful when the freezing rates are fast enough to avoid intercellular ice formation, even with some freezable water present in the tissue (Vertucci, 1989; Wesley-Smith *et al.*, 1992). That no chilling damage is observed under such conditions is probably due to the fact that phase transitions and separations are relatively slow

processes compared with the time required to reach liquid nitrogen temperatures

during the cryopreservation procedure. The region of storage temperature and RH where chilling sensitivity in neem seed is observed is also illustrated in Figure 7.2.



**Figure 7.2.** Model depicting optimum conditions for neem seed storage. The curves indicate  $T_g$  in cotyledons and axes of neem seeds equilibrated at different RHs at 20°C. Above the curves the seed tissues occur in liquid phase, and below the curves in the glassy state. Extended longevity occurs in storage conditions (of RH and temperature) below  $T_g$ , when seeds are stored in a glassy state. The hatched area between 20 and -20°C illustrates good storage results over two years, which we left open at the low moisture side because of difficulties in determining the critical water content below which the seeds will not germinate. 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 (approximately 10°C) is also shown. Cryostorage is possible up to water contents at which ice formation occurs during the freezing procedure.

## Conclusions

This thesis offers new insights into the seed biology of neem. The research explored the causes of the loss of seed viability and provided explanations for the complex storage behaviour of neem seeds. Several mechanisms involved in the rapid loss of seed viability were investigated systematically and may also apply to other tropical seeds with storage behaviour similar to neem. Because applying the appropriate methods can prevent some of the afore-mentioned problems, there is a potential for better storage longevity of tropical seeds that are reputed to be difficult to store. We have shown that some of the storage problems for tropical seeds can be resolved. Many species may not be as recalcitrant as previously supposed. This may explain discrepancies in the published literature concerning the storage behaviour of seeds.

The results presented in this thesis can be used as a model for the handling of tropical seeds. It may assist projects such as the IPGRI/DANIDA project (1996) on storage and handling of recalcitrant and intermediate tropical tree seeds. Large-scale storage for seeds of neem and reputedly intermediate species (i.e., *Lannea microcarpa*, *Khaya senegalensis* (Desr.) A.Juss.) has been set up at the Centre National de Semences Forestières (CNSF) in Burkina Faso. However, because the literature on tropical seed physiology is limited, further experiments are needed to optimise seed handling and storage protocols.

In conclusion, the significant loss of viability upon drying and cold storage of neem seeds indicates that they still pose more problems than orthodox seed types. The state/phase diagrams shown in Fig. 7.2 helped to explain their difficult storage behaviour. Cautious manipulation may enable neem seed lots to maintain viability for a number of years when the seeds are sufficiently dehydrated and in the glassy state, and also at subzero temperatures. Understanding the role played by water in seeds can result in the development of efficient methods for long-term storage of tropical seeds displaying intermediate (and recalcitrant) storage behaviour. The results of the present research may assist projects and programmes that are aimed at improving handling and storage of such seeds.

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## Summary

Neem (*Azadirachta indica*) is an important multipurpose tropical tree species, frequently used in planting programmes in the arid tropics. However, its seeds are difficult to store for extended periods of time, as are those of many other tropical species which display intermediate or recalcitrant storage behaviour. This thesis describes the results of our investigations concerning factors involved in the rapid loss of germinability of neem seeds. Stress and survival under different conditions were studied in an attempt to understand the mechanisms associated with this loss of viability and to improve the storage longevity of neem seeds.

When neem seeds were stored at 75% RH at 20°C, they almost completely lost germinability within six months, whereas viability only decreased slightly at 32% RH. Early experiments showed that at 20°C, the best survival could be obtained when seeds were equilibrated to 50% RH. Later, it became clear that further drying below an equilibrium RH of 50% renders neem seeds sensitive to imbibitional stress, which could be partially alleviated by soaking the seeds at elevated temperature. The sensitivity to imbibitional stress increased with seed ageing. The fact that controlled rehydration at high temperatures (up to 40°C) could considerably improve the percentage of germination is evidence that seeds can withstand considerable dehydration. This indicates that neem seeds can be considered as orthodox, although the ability to easily survive rehydration is part of desiccation tolerance. Yet, the critical water content below which the seeds will not germinate was difficult to determine, because such dry seeds are extremely sensitive to imbibitional stress. Our data show that this water content is below 4%.

Results of storage in the cold indicate that neem seed is sensitive to temperatures of  $\leq 10^{\circ}\text{C}$ . At MCs of 4-8%, seeds were considerably more tolerant of low temperature storage and had an overall viability of 50% on average after 2 years of storage at temperatures between  $-20$  and  $20^{\circ}\text{C}$ .

The study of neem seed longevity under different conditions of water content and temperature revealed that there were hardly any differences in storage behaviour between seed batches and between seed lots, whatever their provenance (African Sahel or Asia). However, the seeds originating from mature yellow fruits lived longer than the seeds from younger green or older brown fruits.

High cell cycle activity has been associated with recalcitrance in seeds at maturity. This activity was reduced in neem seed at seed maturity, as it is in orthodox seeds. During dehydration of fresh seeds, total glutathione, oligosaccharides and phospholipids accumulated and remained stable, particularly in dry seeds. The ability to accumulate protective compounds during slow drying led us again to conclude that neem seed has features of orthodox rather than recalcitrant storage behaviour.

The fact that there was an apparent optimum moisture level for storage of neem seed led us to investigate the properties of water in the seed tissues. The water contents below which only non-frozen water was present and below which the cytoplasm was in the glassy state were determined. This information allowed the construction of state-phase diagrams, which showed that during drying at room temperature, the formation



of cytoplasmic glasses occurred at approximately 7% MC, - slightly higher in axes than in cotyledons. Lowering the MC improved storage longevity, coinciding with the seeds being in the glassy state (below  $T_g$ ). Seeds in the liquid phase (above  $T_g$ ) were shorter lived. All of the reported cases of successful storage of neem seeds may therefore have been with seeds in the glassy state. The state-phase diagrams of neem seeds suggest that glasses are involved in the prolonged storage stability. In addition, the loss of chilling sensitivity in seeds having MCs  $\leq 8\%$  can also be attributed to the presence of a glassy state.

Part of the storage problems of neem seeds could be ascribed to injuries to membranes during rehydration; part could be put down to chilling sensitivity. This led to the hypothesis that difficulties with storage stem from phenomena associated with the intrinsically elevated  $T_m$  ( $10^\circ\text{C}$ ) of membranes in neem seeds. Support for a role for the plasma membranes in chilling sensitivity came from results that indicated increased permeability at chilling temperatures. Freeze-fracture replicas of plasma membranes from chilled hydrated axes showed lateral phase separation and signs of inverted hexagonal phase. Imbibitional injury, which occurred when dry seeds were rehydrated below a critical temperature, is also associated with extensive plasma membrane damage. Low temperature scanning electron microscopy revealed that axis cells did not become turgid after soaking of dry seeds at  $5^\circ\text{C}$  and that the internal cytoplasmic structure was disorganised. Soaking at  $35^\circ\text{C}$  prevented this damage.

This work offers new insights into the biology of neem seed. It explored the causes of the loss of seed viability and provided explanations for the complex storage behaviour of neem seeds. The significant loss of viability upon drying and cold storage indicates that neem seeds still pose more problems than do orthodox seed types originating from temperate climates. Understanding the role played by water in seeds can help develop efficient methods for long term storage of tropical seeds displaying intermediate (or recalcitrant) storage behaviour. The results of the present research may assist projects and programmes aimed at improving the handling and storage of such seeds.

## Résumé

Le Neem (*Azadirachta indica*), une espèce pan-tropicale revêt une importance économique et écologique considérable de par ses usages multiples. Cependant ses graines ont une longévité très limitée, tout comme celles de beaucoup d'autres espèces tropicales récalcitrantes ou intermédiaires. Cette thèse met en évidence quelques facteurs déterminant la perte rapide de la capacité germinative des graines de neem. Le stress et la survie des graines sous différentes conditions ont été étudiés dans le but de comprendre les mécanismes associés à la perte de viabilité et de prolonger la longévité des graines.

Lorsque les graines de neem sont maintenues à 20°C et à une humidité relative de l'air de 75%, elles perdent presque totalement leur pouvoir germinatif au bout de six mois. Par contre, leur viabilité ne décroît que légèrement après stockage à 32% d'humidité relative pour la même période. Préliminairement, nos résultats ont montré qu'à 20°C, les graines équilibrées à 50% d'humidité relative présentaient les meilleurs taux de survie. Cependant, le séchage en deçà de 50% d'humidité relative s'est par la suite avéré rendre les graines de neem vulnérables au stress lié à l'imbibition. Ce stress peut être minimisé par le trempage des graines sèches à une température élevée. De plus, nous avons montré que la sensibilité au stress d'imbibition augmente avec le vieillissement des semences. Le fait que la réhydratation contrôlée à une température élevée (jusqu'à 40°C) puisse considérablement améliorer le pourcentage de germination, permet de conclure que la graine de neem peut subir une forte déshydratation sans perdre sa viabilité. Toutefois, la teneur en eau au-dessous de laquelle les graines ne germent plus est difficile à déterminer du fait que de telles graines séchées deviennent extrêmement sensibles au stress d'imbibition précédant la germination. Nos résultats montrent que cette teneur en eau est inférieure à 4%.

Nos expériences de conservation au froid montrent que la graine de neem est très sensible aux températures inférieures à 10°C. Mais, les lots de graines séchées entre 4 et 8% de matière fraîche sont considérablement tolérants aux basses températures de conservation et germent en moyenne 50% après deux ans de stockage aux différentes températures entre -20 et 20°C. Les lots de graines contenant plus de 8% d'eau, par contre, perdent leur viabilité pendant la première année de stockage.

L'étude de la longévité des graines de neem sous différentes conditions de contenu hydrique et de température, n'a pas pu révéler une différence quelconque de comportement entre des lots de différentes provenances (Sahel africain ou Asie). Au contraire, des différences de comportement ont été obtenues pour des lots de graines de différentes maturités. Les graines issues de fruits jaunes conservent mieux leur pouvoir germinatif que celles issues de jeunes fruits verts ou de fruits bruns plus âgés.

L'intense activité du cycle cellulaire a été associée à la récalcitrance des semences à leur maturité. Comme dans le cas des graines orthodoxes, cette activité décroît au cours du développement des graines de neem, et est fortement réduite à leur maturité. L'étude des composants protecteurs impliqués dans la résistance à la dessiccation a montré que les contenus en glutathione total, oligosaccharides et phospholipides augmentent pendant la déshydratation de graines fraîches. D'autre part ces contenus demeurent stables dans les graines sèches. Dès lors, l'ensemble de

nos résultats permet de conclure que la conservation de la graine de neem répond aux critères de type orthodoxe plutôt que récalcitrant.

Le seuil apparent de teneur optimum en eau pour la conservation prolongée des graines de neem, nous a conduit à étudier les propriétés physiques de l'eau dans les tissus embryonnaires. Les contenus hydriques au-dessous desquels il ne subsiste que seulement l'eau non congelable dans ces tissus ont été déterminés. D'autre part, nous avons montré que les embryons peuvent former un état vitreux (liquide figé) ou verre. En effet, le verre cytosolique se forme pendant le séchage à la température ambiante, lorsque la graine atteint 7% en contenu hydrique – légèrement plus élevé dans les axes que dans les cotylédons. Les données nous ont permis la construction d'un diagramme de phase-état. La réduction de la teneur en eau améliorant la longévité des graines coïncide avec la formation d'état vitreux dans les graines. Les graines dont le cytoplasme n'est pas en état vitreux, c'est à dire en état liquide, ont une longévité beaucoup réduite. Le diagramme de phase-état suggère donc que la formation de l'état vitreux est associée à la stabilité prolongée des graines en conservation. Il apparaît alors que les rares cas de conservation prolongée des graines de neem, rapportés dans la littérature, auraient été réalisés avec des graines dans l'état vitreux. La perte de sensibilité des graines sèches ( $\leq 8\%$  de teneur en eau) au froid pourrait également s'expliquer par la formation de l'état vitreux.

Une partie des difficultés de conservation des graines de neem est attribuable aux altérations des membranes pendant la réhydratation, une autre partie pourrait être liée à la sensibilité au froid non gelant. Nos résultats ont suggéré que les problèmes de conservation proviennent de phénomènes associés à une température intrinsèque de fusion élevée des membranes hydratées des graines de neem ( $T_m \sim 10^\circ\text{C}$ ). Ceci fut démontré par l'accroissement de la perméabilité des membranes aux températures froides. De plus, l'observation fine des cryo-fractures des axes hydratés et soumis au froid non gelant a révélé une séparation latérale de phase et des signes de phase hexagonale inversée dans les membranes plasmiques. Le stress d'imbibition qui se produit lorsque des graines sèches sont rehydratées en deçà d'une certaine température critique, est aussi associé à la détérioration extensive de la membrane plasmique. En observant des cryo-fractures au microscope à balayage électronique de faible champ, nous avons établi qu'après un trempage des graines sèches à  $5^\circ\text{C}$ , les cellules des axes embryonnaires n'étaient pas turgescentes. La structure interne de leur cytoplasme était désorganisée. A contrario, le trempage des graines à  $35^\circ\text{C}$  prévient ce type d'altérations.

Cette thèse offre de nouvelles perspectives dans la biologie de la graine de neem. Elle explore les facteurs induisant la perte de viabilité et fournit des explications au comportement complexe des graines de neem en conservation. La perte significative de viabilité pendant le séchage et pendant le stockage au froid non gelant, indique malgré tout, que les graines de neem posent toujours plus de problèmes que les graines de type orthodoxe de climats tempérés. La compréhension du rôle de l'eau et ses propriétés biophysiques dans les graines sont importantes pour le développement de méthodes permettant la préservation à long terme des semences tropicales intermédiaires (et récalcitrantes). Nous espérons que les résultats de la présente recherche assisteront les projets et itinéraires techniques qui visent l'amélioration de la manutention et la longévité de telles semences.

## Samenvatting

Neem (*Azadirachta indica*) is een voor veel doeleinden bruikbare tropische boomsoort, die met name in de droge tropen wordt aangeplant. Het zaad is echter moeilijk in leven te houden gedurende bewaring. Het lijkt in dit opzicht op zaad van veel andere planten uit de tropen dat wordt gekarakteriseerd door intermediair of recalcitrant bewaargedrag. Dit proefschrift beschrijft de resultaten van onderzoek naar factoren die betrokken zijn bij het snelle verlies van kiemkracht van neem zaden.

Stressomstandigheden en de reactie van neem zaden daarop werden bestudeerd in een poging om de mechanismen te doorgronden die met de korte levensduur samenhangen en om het succes van bewaring te verbeteren.

Neem zaden verloren hun kiemkracht binnen zes maanden onder bewaaromstandigheden van 20°C en 75% relatieve vochtigheid (RV). Bij lagere RV (32%) werd slechts een geringe daling in levensvatbaarheid waargenomen. Experimenten uit de beginperiode van het onderzoek lieten zien dat bij 20°C de beste bewaarbaarheid kon worden verkregen onder condities van rond de 50% RV. Later werd het duidelijk dat door verder te drogen bij een lagere RV, de zaden gevoelig werden voor imbibitiestress. Deze gevoeligheid kon gedeeltelijk worden opgeheven door de zaden te herbevochtigen bij verhoogde temperatuur. Bij veroudering van het zaad nam de gevoeligheid voor imbibitiestress toe. Het feit dat gecontroleerde herbevochtiging bij verhoogde temperaturen (tot 40°C) het percentage kieming kon verhogen is het bewijs dat de zaden sterke uitdroging kunnen weerstaan. Dit geeft aan dat neem zaad in feite kan worden beschouwd als orthodox, alhoewel het vermogen om herbevochtiging gemakkelijk te overleven deels uitmaakt van uitdroogtolerantie. Toch was het kritische watergehalte waaronder de zaden niet kiemen moeilijk vast te stellen, omdat zulke droge zaden extreem gevoelig zijn voor imbibitiestress. De gegevens laten zien dat dit watergehalte onder de 4% ligt.

De resultaten van bewaring in de kou geven aan dat neem zaden gevoelig zijn voor temperaturen van 10°C. Bij watergehalten van 4-8%, waren zaden aanzienlijk beter bestand tegen opslaan bij lage temperatuur en hadden een levensvatbaarheid van gemiddeld 50% na twee jaar bewaring bij temperaturen tussen -20 en 20°C.

Bij de bestudering van de levensduur van neem zaden onder verschillende condities van watergehalte en temperatuur bleek dat er nauwelijks verschillen waren in bewaargedrag tussen zaadpartijen, ongeacht hun herkomst (Afrikaanse Sahel of Azië). Echter, zaden afkomstig uit rijpe gele vruchten overleefden langer dan zaden uit jongere, groene, of oudere, bruine vruchten.

Recalcitrant gedrag van zaden in het rijpe stadium is normaliter geassocieerd met een aanzienlijke celcyclusactiviteit. Deze activiteit was laag bij rijp neem zaad, net als bij orthodoxe zaden. Gedurende uitdrogen van verse zaden, namen de gehalten aan totaal glutathion, oligosacchariden en fosfolipiden toe, en bleven daarna constant, met name in droge zaden. Het feit dat de zaden in staat bleken om beschermende stoffen te accumuleren tijdens langzaam drogen, leidde wederom tot de conclusie dat neem zaad eerder gelijkenis vertoont met orthodoxe dan met recalcitrante zaden.

Het eerder genoemde optimale watergehalte voor de bewaring van neem zaden heeft geleid tot het onderzoek naar de eigenschappen van water in de zaadonderdelen. De watergehalten werden bepaald waaronder alleen niet-bevroren water voorkwam en waaronder het cytoplasma in de glastoestand verkeerde. Met deze informatie konden "state-phase" diagrammen worden geconstrueerd waaruit kon worden afgeleid dat gedurende drogen bij kamertemperatuur een cytoplasmatisch glas gevormd wordt bij ongeveer 7% vochtgehalte, - in het geval van de zaadas wat hoger dan bij de cotylen. Reduktie van het vochtgehalte verbeterde de levensduur tijdens bewaring, wat samenviel met de aanwezigheid van glas in het zaad (beneden de  $T_g$ ). Zaden waarvan het cytoplasma in de vloeibare fase verkeerde (boven de  $T_g$ ) hadden een kortere levensduur. Alle gerapporteerde gevallen waarin neem zaden succesvol bewaard werden zullen daarom zaden hebben betroffen in de glastoestand. De state-diagrammen suggereren dat glassen betrokken zijn bij de vergrote opslagstabiliteit van neem zaden. Bovendien kan het verlies van koudegevoeligheid bij zaden die vochtgehalten hebben van 8% worden gerelateerd aan de aanwezigheid van een glastoestand.

De bewaarproblemen konden deels worden toegeschreven aan membraanschade opgelopen tijdens rehydratie, deels aan koudegevoeligheid. Dit heeft tot de hypothese geleid dat de moeilijkheden met de bewaarbaarheid voortkomen uit fenomenen die geassocieerd zijn met de specifiek hoge  $T_m$  (10°C) van membranen in neem zaad. De verhoogde permeabiliteit die ontstaat bij bewaring in de kou ondersteunt een rol voor de plasmamembraan in de koudegevoeligheid. Replica's van plasmamembranen uit gekoelde, gehydrateerde zaadassen, die gemaakt werden via vriesbreken, lieten laterale fasenscheiding zien en tekenen van de omgekeerde hexagonale fase. Imbibitieschade die optrad bij rehydratie onder een kritische temperatuur, werd ook geassocieerd met uitgebreide schade aan plasmamembranen. Lage-temperatuur-scanning-electronenmicroscopie onthulde dat cellen van de zaadas niet volledig kunnen zwellen na wateropname van de droge zaden bij 5°C en dat de cytoplasmatische structuur er wanordelijk uitzag. Wateropname bij 35°C voorkwam deze schade.

Dit werk verschaft nieuw inzicht in de biologie van neem zaad. Het verkende de oorzaken van het verlies van levensvatbaarheid van de zaden en poogde het complexe bewaargedrag te verklaren. Het aanzienlijke vitaliteitsverlies als gevolg van drogen en opslaan in de kou geeft aan dat de bewaring van neem zaad problematischer is dan dat van orthodoxe zaadtypen afkomstig uit gematigde klimaten. Het begrijpen van de rol van water in zaden kan bijdragen aan de ontwikkeling van efficiënte methoden voor de lange-termijnbewaring van tropische zaden die intermediair (of recalcitrant) bewaargedrag vertonen. The resultaten van dit onderzoek kan bijdragen aan projecten en programma's die tot doel hebben de verwerking en bewaring van zulke zaden te verbeteren.

## About the Author

Moctar Sacandé was born on the 12<sup>th</sup> of August 1962 in Bobo Dioulasso, Burkina Faso. He attended high school at the College Charles Lwanga, Nouna, and then at the College Joseph Moukassa, Koudougou in 1983, where he obtained a Bacalaureat - option D - in Mathematics and Natural Sciences with honour. He went to the Institut Supérieur Polytechnique (ISP) at the University of Ouagadougou, where in 1986 he completed his Engineer's degree in Rural Development, specialising in Water and Forests. As part of his training at the ISP, he conducted studies on forest management, nursery techniques and silviculture, and on tropical tree seed biology. Since his graduation, he has been working for the Centre National de Semences Forestières in Burkina Faso. In 1989 he set up and was in charge of the CNSF station in Bobo Dioulasso. He supervised university and vocational school students for their research. He has been a member of a number of committees and co-organised the IUFRO Seed symposium of the Group P.2.04.00, which took place in Ouagadougou in 1992.

He completed a MSc in Tropical Forestry specialising in Ecology and Silviculture at Wageningen University in 1995 and in the same year started a PhD programme on the storage of tropical seeds. The studies reported in this thesis result from collaboration between the CNSF in Burkina Faso, and the CPRO and Wageningen University, in the Netherlands. Apart from science, Moctar enjoys travelling, scouting and sport, but also family life.

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