Conservation of Tree Seeds from Tropical Dry-lands

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A mon père Gnadema Neya
A ma mère Ouégnoné Yao
A mon oncle Bokao Neya
Merci pour tout ce que
vous avez fait pour moi.

Contents

Chapter 1

General introduction

Urge for more knowledge about the biology of tropical tree seeds

Deforestation of tropical regions is a well-documented problem that raised international concerns since many decades (FAO, 1996, 2003). Despite increased awareness of the importance of tropical forests, deforestation rates have not slowed. Analysis of figures from the Food and Agriculture Organization of the United Nations (FAO) shows that tropical deforestation rates increased by 8.5 percent over the period 2000-2005 as compared with the 1990s, while the loss of primary forests may have expanded by 25 percent over the same period.

Environmental problems, e.g. global warming, flooding, loss of biological diversity etc. are usually the emphasised consequences of deforestation in the printed press and in visual media presentations. On the other hand, deforestation has an enormous impact on the 1.5-2 billion people (Bonner, 1992), who depend on trees for livestock fodder, fruits, habitat construction, cooking and heating fuel, medicines, and religious and cultural practices. An alternative to mitigate the disastrous effects of deforestation would be to plant more trees, particularly in tropical dry-lands, where trees play a central role in the combat against desertification.

Tropical forests are rich in species diversity, and the majority of trees propagate via seeds. Regrettably little is known about the biology of tropical tree species, and, in particular, information on the seed biology is very scanty. Until recently, indigenous tree species were rarely used in reforestation programmes of most sub-Saharan African countries, preference being given to fast growing exotic species like *Eucalyptus camaldulensis* and *Tectona grandis*. Lack of basic information about the potential of indigenous tree species and insufficiency of high quality seeds and seedlings have contributed to this situation. This constitutes a serious threat to people who depend in their daily life on forest resources as well as to the conservation of biological diversity of tropical forests. Moreover, savannah tree species, in particular, are considered endangered as a result of over-exploitation and repetitive severe drought spells in recent decades (IUCN, 2004).

Among the possibilities for forest genetic resources preservation, the *ex situ* conservation of seeds is probably the most effective and widely practiced means of species conservation, with ca. 6 million specimens stored globally (FAO, 1996; Linington and Pritchard, 2001; Engelmann and Engels, 2002). However, before full commitment to the *ex situ* conservation of many tropical tree species, more research is needed in several fields of seed biology. Typical problems of tropical forest tree seeds, which need more study, include the phenology of flowering and fruiting, collection, cleaning, storage, and pre-treatment for germination (Bonner, 1992). Since a decade some efforts have been made to screen priority tropical tree seed species for their biology, especially the survival after drying (IPGRI/DFSC project). However, unlike most temperate zone species, most tropical species flower over a period of many months, and this period may vary from year to year. Therefore, finding multiple stages of seed maturity at any one time on the same tree is common in several tropical tree species. This feature obviously introduces heterogeneity within and among seed accessions, which has the potential to affect seed behaviour and germination (reviewed in Matilla *et al*., 2005).

Some years ago, a physiological study was conducted on the causes of the difficult storage behaviour of tropical seeds, using neem tree seed as a model system (Sacandé, 2000). Originally, it was thought that neem seeds die because of intolerance of water loss. However, it has been found that the difficult storage behaviour could largely be explained by the sensitivity of the seeds to chilling /sub-zero (°C) temperatures and to the stress associated with rehydration in water (imbibition) (Sacandé *et al*., 1998). The drier the seeds, the more sensitive they become to imbibitional stress, particularly when rehydration occurs at chilling temperatures. Based on the results obtained with neem seeds, it can be expected that seeds of other tropical tree species, which display difficult storage behaviour may experience problems other than desiccation intolerance. In that perspective, we have chosen to investigate seeds of *Khaya senegalensis*, *Lannea microcarpa* and *Sclerocarya birrea* – species for which difficult seed storage behaviour has been reported (Ouédraogo and Verwey, 1987; Kamra, 1990; Hong *et al*., 1996; Hong and Ellis 1998; Were and Munjuga, 1999; Daws *et al*., 2004a).

Khaya senegalensis, *Lannea microcarpa* and *Sclerocarya birrea* are important multipurpose tree species, which are used to prevent soil erosion, improve soil microclimate and provide shade, boundaries, fuel wood, timber, foods and medicines. We suspected that the main problem of the seeds of these species is not so much the lack of desiccation tolerance, but inappropriate handling, rehydration techniques, and pretreatments after drying and/or dry storage. Our project was aimed at improving the survival of these tropical dry-land tree seeds upon drying and subsequent storage, in

order to facilitate and increase their use in agroforestry and reforestation programs in Burkina Faso and other Sahelian countries. Prior to investigating the seeds of these three species, we further extended the existing knowledge on neem seed physiology by studying especially the effect of seed ageing on the sensitivity to imbibitional stress.

Seed storage behaviour

The seeds of many tree 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 ability of seeds to withstand desiccation (Roberts, 1973; Roberts *et al*., 1984; Kraak, 1993). In that respect, desiccation-tolerant seeds, which survive in the dehydrated state for periods that are predictable depending on storage conditions (mainly humidity and temperature) have been designated as showing orthodox storage behaviour (Roberts, 1973). Low water contents and temperatures best extend survival of such seeds in storage, as these conditions create a glassy state in the cytoplasm, in which molecular motion is very slow and, consequently, ageing is slow (Buitink *et al*., 2000). At the other extreme, the so-called recalcitrant seeds that are damaged by dehydration and also may be chilling-sensitive, generally cannot be stored effectively for useful periods of time (Roberts, 1973; Chin and Roberts, 1980). A third category, seeds with intermediate postharvest behaviour, has been defined later (Ellis *et al*., 1990, 1991). Seeds in this third category are relatively desiccation-tolerant, but cannot withstand removal of water to levels as low as for orthodox seeds. Such seeds, particularly if they are of tropical origin, also may be chilling-sensitive even in the dehydrated state (Ellis *et al*., 1990; Hong and Ellis, 1996). Examples are the seeds of coffee and papaya.
In most cases, it is relatively straightforward to classify seeds into one of these broad storage categories. However, seed lot heterogeneity and a number of confounding factors can lead to uncertainty in the classification or contradictory reports on the storage behaviour of a given species. For example, the rehydration characteristics of seeds can be altered after desiccation, or the germination requirements may change subtly during dry storage, e.g. cassava (Roberts *et al*., 1984), *Carica papaya* (Wood *et al*., 2000). The categorization of seed storage behaviour was originally based on the seeds of a narrow spectrum of mostly economically important crop species (Pammenter and Berjak, 1999). Thus, in the past 15 years, further studies on a considerable number of species, especially those from the tropics and sub-tropics, have indicated that there is a wide range in the postharvest responses of seeds to desiccation and storage conditions. This would suggest an open-endedness to the three categories

such that postharvest physiology may be considered as constituting a continuum across species (reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak, 1999).

Desiccation tolerance

Mechanisms

Defined as the ability of organisms to resume normal function after drying to the air-dry state and subsequent rehydration (Golovina and Hoekstra, 2003), the acquisition of desiccation tolerance is an essential part of the maturation programme in most types of seeds. A number of processes or mechanisms (reviewed by Pammenter and Berjak, 1999; Bartels and Salamini, 2001; Hoekstra *et al.*, 2001; Buitink *et al.*, 2002) have been proposed to confer, or contribute to, the acquisition of desiccation tolerance. These mechanisms involve structural and biochemical changes in the cell, such as the substitution of large vacuoles by the much smaller plastids containing storage substances, the accumulation of di- and oligosaccharides, and the synthesis of dehydrins and late embryogenesis abundant (LEA) proteins (reviewed by Dure, 1997; Pammenter and Berjak, 1999, Berjak, 2006).

Signalling to switch on the desiccation program in developing seeds may occur as a result of dehydration or via abscisic acid (Ingram and Bartels, 1996; Bartels *et al.*, 1997) that also inhibits premature germination (Hoekstra *et al.*, 2001). Although all the changes preceding the acquisition of desiccation tolerance take place during the maturation phase, usually when morphogenesis is completed, it has been found that the competence to acquire cellular desiccation tolerance is independent of seed morphological development (Golovina *et al.*, 2001). These authors claim that desiccation tolerance in seeds is a developmental process rather than a response to maturation drying or environmental conditions. It is important to distinguish desiccation tolerance from drought tolerance. While the first phenomenon is based on low intracellular water concentrations, the latter one is defined as the survival to low environmental water availability, without reduction in internal water concentrations (Alpert, 2005).

In most, if not all, of the proposed mechanisms of desiccation tolerance, membranes are considered a primary target of desiccation injury. Therefore, membrane stabilisation is thought to be a key mechanism of desiccation tolerance (Crowe *et al.*, 1992, 1998). In line with that, it was recently shown that amphiphilic metabolites partition into membranes during dehydration and play a role in desiccation tolerance

(Hoekstra *et al.,* 1997; Golovina *et al*., 1998; Golovina and Hoekstra, 2002; Hoekstra and Golovina, 2002).
Although studies on desiccation tolerance in seeds usually have been focussed on the capability to control the deleterious effects of desiccation, it is important to bear in mind that the resumption of normal metabolism after rehydration is an integral part of desiccation tolerance (Hoekstra *et al*., 1999; Golovina and Hoekstra, 2003).

Imbibitional damage

Imbibitional damage or imbibitional injury may be defined as an unsuccessful transformation of an organism from the chemically and structurally intact, metabolically inactive, dry state to the metabolically active, hydrated state (Nijsse *et al*., 2004). When dehydrated, anhydrobiotic organ(ism)s are placed in water, they leak different substances, mostly of low molecular weight such as ions, amino acids, sugars, etc. (Hoekstra *et al*., 1999). Depending on the severity of such leakage a system or organism may encounter variable extents of damage. In desiccation-tolerant systems, such as many pollens and seeds, the leakage is transient and will stop as soon as membranes resume their barrier properties. In contrast, the leakage from desiccation-sensitive systems may continue until most of the solutes are lost, leading to cell death. The main factors promoting membrane injury upon imbibition are low temperature and low initial moisture content of the organism.
Reduced membrane integrity is widely accepted as the reason for the persistent leakage of cellular solutes. However, the exact mechanism leading to increased membrane permeability has been a matter of several hypotheses. We do not intend to go into detail on the early hypotheses (reviewed by Hoekstra *et al*., 1999), for the good reason that most of them were found to be unlikely to happen. The prevailing hypothesis attributes leakage from dry systems to a membrane phase change from the gel to the liquid crystalline phase upon rehydration (Crowe *et al*., 1989a; Hoekstra *et al*., 1992). For these authors, particularly structural defects at the boundary between the co-existing gel and liquid crystalline phases are responsible for the increased permeability. Later on it has been found that, more than an effect of phase separation, permanent leakage is likely the consequence of mechanical ruptures in membranes (Hoekstra *et al*., 1999). The hypothesis is based on the fact that membranes in the gel phase have a higher rigidity as compared to those in the liquid crystalline phase (reviewed in Hoekstra and Golovina, 1999). Therefore, the exposure of the initially dry and rigid membranes to the considerable forces of rehydration would lead to permanent rupture of membranes and subsequent increase in leakage. Support for this hypothesis comes from the observation that pre-humidification of the dry organism in

humid air and imbibition at elevated temperatures can largely prevent imbibitional damage and increase survival (Hoekstra and Van der Wal, 1988, Sacandé *et al.*, 1998). This prevention of imbibitional damage has been attributed to the effects of these treatments on membrane phase behaviour (Crowe *et al.*, 1989b).
While considerable leakage as the consequence of imbibitional stress certainly leads to cell death in unicellular organisms, it has been shown that in multicellular organisms damage of one or more cell layers might be overcome (Nijsse *et al.*, 2004). In the case of seeds, the cells are generally well protected against the damaging effect of a membrane phase change during imbibition, because of the regulatory role played by seed coats in the penetration of water (Duke and Kakefuda, 1981). However, seed coat damage inflicted during seed processing and scarification pretreatments may render dry seeds vulnerable to fast uptake of water. Particularly in tropical seeds, imbibitional damage may constitute a major problem with respect to survival, because of the elevated gel-to-liquid crystalline phase transition temperature (T_m) of their membranes (Crowe *et al.*, 1989b; Sacandé *et al.*, 1998).

Longevity and ageing

Desiccation tolerance is understood to include not only the ability of cells to become air-dry without loss of viability, but also to successfully rehydrate (Hoekstra, 2005). The period between dehydration and rehydration, even though not precise in its definition, is referred to as life span or longevity in the dry state. Water content and temperature are the crucial factors controlling seed longevity in general (Roberts, 1973; Roberts *et al.*, 1984; Kraak, 1993). At low water contents and temperatures the cytoplasm turns into a glassy state, in which molecular motion is slow and, consequently, ageing is slowed (Buitink *et al.*, 2000). However, anhydrobiotic organisms differ considerably in their life span under similar storage conditions. The average longevity of orthodox seeds for instance ranges from 2-10 years under laboratory conditions (Priestley, 1986). Apart from the external conditions, water content and temperature, longevity depends on the inherent properties of each dry organism. Because life span results from genetic controls of the seed maturation program as well as from environmental conditions during growth and after physiological maturity, there is great variability in seed longevity among accessions within a species (Walters *et al.*, 2005).
During the loss of seed viability many biochemical and biophysical changes occur, including the loss of enzyme activity and membrane integrity (Stewart and Bewley, 1980; Priestley and Leopold, 1983; Ferguson *et al.*, 1990). A long life in the dry state therefore depends on long-term structural and biochemical intactness (Hoekstra, 2005). It is common knowledge that dried anhydrobiotes die because they age, and

ageing is thought to be the result of free radical-induced damage, particularly to membranes (Kranner and Lutzoni, 1999; Kranner *et al.*, 2002). Several reactions lead to free radical formation in seeds, but the widely accepted hypothesis of the cause of seed deterioration is lipid peroxidation and the associated free radical oxidative stresses (Priestley, 1986; Wilson and McDonald, 1986; Hendry, 1993). Among the numerous types of peroxidative reactions in which lipids serve as substrates, the most commonly cited ones involve breakage of the ester linkage between the acyl chain and the glycerol backbone (McKersie *et al.*, 1988) and the attack of the unsaturated bonds of the acyl chain (Chan, 1987). This implies that seed lipid acyl chain composition and particularly the proportion of saturated to unsaturated fatty acid chains in the membrane polar lipids may play a critical role in the rate of seed deterioration and the associated longevity. In that respect, quantification and compositional analysis of membrane polar lipids during storage may be a useful tool to monitor ageing and predict survival of seeds.

Dormancy

Seed dormancy is the temporary failure of a mature viable seed to germinate under environmental conditions that would normally favour germination (Hilhorst, 1995; Li and Foley, 1997). Many flowering plants exhibit some level of primary seed dormancy that can be either coat-imposed or embryo dormancy, or both (Bewley and Black, 1982; Li and Foley, 1997). Coat-imposed dormancy is due to the impermeability of the seed (or fruit) coat to water, while embryo dormancy is induced by the presence of a germination inhibitor such as abscisic acid (ABA) or underdevelopment of the embryo at fruit maturity (Baskin and Baskin, 1998; 2004). Coat-imposed dormancy is usually referred to as physical dormancy, whereas the terms physiological and morphological dormancy are reserved for the presence of germination inhibitors and embryo immaturity at fruit maturity, respectively.

In some species, a combination of two or more of the above-mentioned types of dormancy may occur (Nikolaeva, 1977; Baskin and Baskin, 1998; 2004) and this is known as morphophysiological or combinational dormancy. Physiological and morphological dormancy can be alleviated by germination promoters such as gibberellic acid and/or by further maturation of the embryo. Physical dormancy is the most common type of dormancy of the majority of dry savannah tree species (Baskin and Baskin, 1998). Fire, high or fluctuating temperatures, or biological actions such as gut

passage or micro-organisms, are some of the means by which coat-imposed dormancy is overcome in nature (Bewley and Black, 1994; Baskin and Baskin, 1998). A variety of artificial pre-sowing treatments such as hot water, sulphuric acid and mechanical scarification has successfully been used to alleviate seed coat-imposed dormancy (Teketay, 1996; Tigabu and Odéon, 2001; Ren and Tao, 2004). However, the optimal level of the various scarification treatments varies among species and needs to be determined empirically for any given species.

Seed rehydration characteristics or germination requirements may change during drying and/or dry storage, particularly in tropical and sub-tropical species (Roberts *et al.*, 1984; Wood *et al.*, 2000). Therefore, the investigation of seed germination behaviour and the identification of appropriate pre-treatments to improve seed germinability are of great importance for an efficient preservation of tropical tree species.

Some information about the species used

***Azadirachta indica* A. Juss. Family: Meliaceae**

Commonly known as the neem tree, *A. indica* is native to the dry forest zones of the Asian sub-continent including India, Sri Lanka, Pakistan, Bangladesh, Malaysia, Myanmar and Thailand. Detailed descriptions of the species and its numerous utilisations have been the subject of several books and reports (Tewari, 1992; Vietmeyer, 1992; Schmutterer and Doll, 1993; Schmutterer, 1995). The species has been introduced on the African continent at the beginning of the twentieth century, likely first in Nigeria (Dewaulle, 1977) and in Burkina Faso about 70 years ago (Devernay, 1994). Because of its multiple uses (e.g. improvement of the soil micro-climate, providing of shade, fuel wood, medicines and timber, and insecticide, pesticide and fungicidal effects of its by-products), neem has become widely distributed into the dry, arid and semi arid tropical and subtropical zones. Being a drought-tolerant species, neem has no particular site requirements. The species is therefore highly suitable for the combat against desertification and the improvement of degraded lands and nutrient-poor soils, particularly in tropical dry-lands.

***Lannea microcarpa* Engl. & K. Krause. Family: Anacardiaceae**

L. microcarpa is an excellent shade tree, often protected in farms and compounds in rural areas. The tree reaches 16 m tall by 2 m girth. It seems to prefer friable deep soil and is often found on cultivated land (Von Maydell, 1986). The species is used to prevent soil erosion, improve soil microclimate and provide shade, boundaries, fuel wood and timber. The bark is fibrous, and is used to make cordage. It has a sweet smell and contains a gum, which is soluble in water and edible. The leaves have a medicinal use as those of *L. acida*, but are considered to be less active. The inflorescences are grapes, in which it is usual to find fruits of different maturity stages, as in neem inflorescences (Neya *et al.*, 2004a). The fruit is a drupe, and the colour goes from green to purple-black during ripening. It has an edible pulp said in general to be preferred over that of *L. acida*. The fruit is eaten raw or dried, and a number of fermented (wine) or soft (juices) drinks are made from it (Lockett and Grivetti, 2000).

***Sclerocarya birrea* (A. Rich.) Hochst. Family: Anacardiaceae**

S. birrea is a tree well protected by farmers. The tree is about 13 m high, the short bole sometimes reaching over 2.5 m in girth. Species of the drier Sahel savannah, its natural area goes from Senegal and across tropical Africa (Von Maydell, 1986). It is often planted around villages and is easily propagated either from seed or cuttings. When big enough it is used for mortars and to make bowls. The dark wood yields a strong fibre and, when injured, exudes a nearly colourless gum, which becomes brittle and friable on drying. Anti-bacterial activity (Eloff, 2001) as well as anti-inflammatory properties (Ojewole, 2003) have been reported in the bark. The gum, dissolved in water and mixed with soot, is used to make ink. In veterinary medicine, a decoction is given to stock to increase appetite. The flowers are reddish or greenish, with the male and female flowers occurring on separate trees (Coates-Palgrave, 1977; Pooley, 1993). The fruit often drops from the tree when it is still green and then ripens to pale yellow on the ground (Pooley, 1993; Nerd and Mizrahi, 2000). The obovoid fruit is nearly 3.7 cm long with a leathery rink like a mango and a fibrous soft pulp covering the stone. They are eaten in many parts of tropical Africa and are commonly sold in markets (Okole and Odhav, 2004). When fully ripe they are having an acid but pleasant taste. The expressed juice makes an agreeable drink and, in Burkina like in many other areas, is fermented into an alcoholic beverage. In eastern and southern Africa the species is known as the marula tree, name of an industrially produced creamy drink produced from the fruits. The nut contains two or three seeds with oily and edible kernels. The nutritive importance of the edible pit in animal nutrition (Aganga and Mosase, 2001) as well as for human alimentation (Glew *et al.*, 2004) has been well established.

***Khaya senegalensis* (Desr.) A. Juss. Family : Meliaceae**

K. senegalensis is a tree, to 30 m tall with a short bole to 3 m girth, unbuttressed, bearing a round evergreen crown of shining foliage. Its distribution goes from the Guinean savannah in damp locations and along river-banks into drier transition savannah; in Mauritania to N and S of Nigeria, and across Africa to Sudan and Uganda (Arbonnier, 2002). The tree is commonly grown in the sudano-sahelian zone as a source of supply of bark as well as to be avenue shade trees. It has been recommended for planting in the sahel since the colonial time, to provide shade and fodder, and improve pasture. It should be considered useful in the fight against desertification. The wood of the species was probably the first African mahogany to be exported from W Africa (Gambia) on a commercial scale in the early part of the nineteenth century. It has been (and still is) heavily exploited for this trade as also for its other local uses with a result that it has become scarce in places, e.g. Ivory Coast, Burkina Faso (W Africa in general).

Khaya senegalensis has a variety of uses. The straight bole is easily processed. The wood converts into good quality charcoal, and ash from the wood is used as a preservative dressing on millet seed in store for sowing the following year. The seed is oily. Parched by boiling, the released oil is used to anoint the body. Oil from the seed is used as an insectifuge (Govindachari and Krishna-Kumari, 1998; El-Aswad *et al.*, 2003; Bamaiyi and Bolanta, 2006). A number of khivorine and swietenine-derivatives is reported present in the seed. A number of chemicals have been reported from the bark and other parts (Khalid *et al.*, 1998; Kayser and Abreu, 2001). The bark is extremely bitter and has a widely held reputation as a fever-remedy. As a substitute for quinine it has become known as "quinine of Senegal". Hypertension treatment is also reported. Such is the medical demand for the bark, that natural stands of the trees become badly mutilated. Flowers are sometimes used medicinally for stomach-complaints, and as an ingredient of anti-syphilitic prescriptions. Honey derived from its flowers is bitter, and a bitter principle (cailcedrin) has been isolated from it.

The seed is not dormant and normally germinates freely, but regeneration is frequently poor due to depredation by insects. Nursery propagation via seeds and transplanting of seedlings is the usual practice. Despite the numerous reports on the phytopathological and phytopharmaceutical properties of the species, few investigations have been performed on its seed biology and conservation.

Aim and outline of the thesis

The low income per inhabitant in the tropics, particularly in rural areas, renders billons of people dependent on forest resources for their daily life. Beside the environmental problems (global warming, flooding, loss of biodiversity etc.) caused by forest resources degradation, it is also directly the life of 1.5-2 billion people, which is threatened by the diminution of forest resources. One way to lessen the effects of deforestation is to develop adequate reforestation programs and functional agroforestry systems. The maintenance of the equilibrium of natural ecosystems and the challenge to preserve biological diversity compel that indigenous species are used in such reforestation and agroforestry programs. Regrettably, until recently, indigenous trees species were not often used in reforestation programs of most sub-Saharan African countries, preference being given to fast growing exotic species like *Eucalyptus camaldulensis* and *Tectona grandis*. One of the main reasons for such inadequacies is the lack of baseline information about the potential of indigenous tree species and the insufficiency of high quality seeds. Most tropical tree species propagate via seeds; however, information on the seed biology of these species is almost non-existent. Therefore, this project aimed at increasing the life span of the seeds of tropical dry-land trees in storage, in particular those of *Lannea microcarpa, Sclerocarya birrea* and *Khaya senegalensis*, with the ultimate goal of facilitating and increasing their use in agroforestry and reforestation programs in Burkina Faso and other Sahelian countries.

Previous studies indicated that *L. microcarpa* seeds are sensitive to desiccation (Hong *et al*., 1996) and exhibit non-orthodox seed storage behaviour (Daws *et al*., 2004a). However, the seeds also have been claimed to display orthodox behaviour (Flynn *et al*., 2004; Pritchard *et al*., 2004a). Several reasons, among which fruit maturity stage, germination requirements and other environmental factors may have contributed to the controversy of the reports on the seed biology of this species.

Likewise, reports on desiccation tolerance and storage behaviour of *S. birrea* seeds are rather contradictory. According to Ouédraogo and Verwey (1987) and Kamra (1990), *S. birrea* seeds exhibit recalcitrant storage behaviour, losing viability after only one month of storage. However, Were and Munjuga (1999) have reported the seeds to display intermediate storage behaviour. Other studies point to orthodox storage behaviour because the seeds have been found to tolerate desiccation to 10% moisture content or even less, with retention of their initial viability for up to four years of storage (Msanga, 1998; Ouédraogo *et al*., 1999). Support for this contention comes from Gaméné *et al*. (2004), who found that well-dried seeds can survive 18 months of storage at -18°C. Several reasons, among them seed developmental stage, might have led to these conflicting reports.

Concerning *K. senegalensis*, Hong and Ellis (1998) have described the seeds as intermediate. The seeds can be dried to moisture contents (MC) as low as 5%, but are sensitive to cold temperatures, which limits the possibilities for long term storage. Recently, Gaméné and Eriksen (2004) reported orthodox storage behaviour. However, in practice some difficulties resulting in sporadic and fast losses of viability of the seeds occurred even upon dry storage at low temperatures (0-5°C) (CNSF, Technical reports 1999-2004). These divergent reports on the seed biology of the three species are compared to what has been reported for neem tree seeds. Neem seed is probably one of the most studied tropical species, but also with the most controversial storage behaviour ever reported for a species. It has been described to display all the three existent categories of storage behaviours: recalcitrant, intermediate or orthodox (reviewed by Poulsen, 1996; Hong *et al.*, 1996). More recently, a physiological study conducted on the causes of the difficult storage behaviour of neem tree seed (Sacandé 2000), revealed that the loss of viability could largely be explained by the sensitivity to chilling /sub-zero °C temperatures and to the stress associated with rehydration in water (imbibition). It has been found that the drier the seeds, the more sensitive they become to imbibitional stress, particularly when the rehydration occurs at chilling temperatures (Sacandé *et al.* 1998).

In this thesis, we investigated the sensitivity of neem seed to imbibition in more detail. The hypothesis that the optimum rehydration temperature will increase with ageing was tested to further improve the survival of the seed after dry storage (Chapter 2). In this chapter, apart from seed germination, we report on a study of the type and severity of damage as a consequence of rehydration at unfavourable temperatures of seeds differing in age. By combining high imbibition temperatures and short soaking durations prior to incubation, conditions for optimum survival of neem seeds after drying and dry storage were identified. Cryo-planing and Low Temperature Scanning Electron Microscopy (Cryo-SEM) were used to characterise and localise the damage induced by low imbibition temperature, while Electron Spin Resonance (ESR) was utilised to investigate the effect of imbibition temperatures on membrane barrier properties and to estimate the proportion of dead cells in embryo axes upon rehydration, which would lead to loss of germinability.

The importance of fruit maturity stage at harvest for seed desiccation tolerance and the associated storability has been demonstrated for neem seeds (Sacandé *et al.*, 1998). However, none of above-mentioned studies on *L. microcarpa* and *S. birrea* have clearly identified the type of fruits, from which the seeds had been processed. For that reason, we investigated several seed lots of both species extracted from fruits collected at different stages of maturity. In Chapter 3, the impact of fruit maturity stage on desiccation tolerance and the associated long-term storability of seeds of *L. microcarpa*

and *S. birrea*, is reported. Seeds of both species were found to be desiccation tolerant, but they were characterised by low germination percentages and variation in germination capability between and among seed accessions, particularly in *L. microcarpa*. However, the germination ability of *S. birrea* seeds increased substantially after both drying and dry storage at a wide range of temperatures, from which it is suggested that physiological dormancy may occur in this species.

To examine factors underlying the overall low and variable germination of both fresh and dried seeds of *L. microcarpa*, seeds collected in three successive years were used to investigate the factors influencing germination. In Chapter 4, we report on the efficiency of mechanical scarification as a pre-sowing treatment to break coat-imposed constraints of seed germination and the implication of such constraints for the variability of germination between and among seed lots of this species.

Because there are several mechanisms, by which the seed coat can impose dormancy, further experiments were carried out to identify the underlying mechanism of seed coat-imposed constraints of germination in *L. microcarpa* seeds. In Chapter 5, we present a possible mechanism and the ecophysiological consequences of the observed germination behaviour.

The sensitivity of tropical tree seeds to low temperatures is one of the major problems limiting long-term storability. Understanding the mechanisms of such sensitivity to particular storage temperatures will be of great interest for the preservation of many tropical species. To that end, seeds of *K. senegalensis* were investigated for their survival under different storage conditions and the biochemical and biophysical changes associated with viability loss (Chapter 6). We report in this chapter on the changes in membrane physical structure and in quantity and composition of polar lipids associated with ageing and viability loss, and discuss the possible role of phospholipases in membrane degradation at low water contents and low, above zero °C temperatures.

In the general discussion (Chapter 7), we present the main constraints for the survival of the seeds of the different species, which probably have caused the controversial seed biological reports. Finally we make some practical and general recommendations for a better handling and conservation of tropical tree seeds.

Chapter 2

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

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Abstract

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

Introduction

According to their storage behaviour, seeds are classified into three categories, namely orthodox, intermediate and recalcitrant (Hong *et al.*, 1996). Approximately 90% of plant species have seeds that fall into the first category. The remaining 10% exhibit problems with the maintenance of seed viability (Hong *et al.*, 1996), which would concern approximately 25,000 plant species, worldwide. Nearly 75% of these plant species have seeds that are characterized by recalcitrant storage behaviour, with a large proportion originating from the tropics. Recalcitrance is defined as the inability of seeds to withstand drying below 96–98% relative humidity at 20°C (Hong *et al.*, 1996). An additional problem with these seeds is that they are often chilling sensitive, which further limits the possibilities of keeping them alive in storage. Examples are the seeds of economically important tropical species, such as cacao, coconut, rubber, avocado and mango. The so-called intermediate seeds (Ellis *et al.*, 1990, 1991), forming a third group of seeds, store better in dry conditions than recalcitrant seeds, but have a much shorter life span than orthodox species. Examples are the seeds of coffee, papaya, neem, *Khaya*, *Lannea* and *Sclerocarya*. These seeds might be sensitive to low temperatures during rehydration. The majority of studies on desiccation tolerance are focused on changes in organisms during drying and/or dry storage, regrettably without much consideration of the stresses associated with rehydration. It has been argued that successful imbibition leading to the resumption of vital processes should be considered an essential part of the mechanism of desiccation tolerance (Hoekstra *et al.*, 2001). During imbibition, desiccation-tolerant organisms (anhydrobiotes) leak solutes into the surrounding medium. This leakage is generally transient and does not lead to severe damage (Senaratna and McKersie, 1983; Hoekstra and Van der Wal, 1988). Lately, this leakage has been attributed to the transiently increased membrane permeability associated with the presence, in plasma membranes, of amphiphilic metabolites that are in the process of partitioning back into the aqueous cytoplasm during rehydration (Golovina *et al.*, 1998). Complete loss of cell viability due to irreversible membrane damage may occur when dried anhydrobiotic cells are allowed to imbibe in relatively cold media (Hoekstra *et al.*, 1999). This phenomenon is called imbibitional damage, and may lead to reduced germination ability. Elevated initial moisture content and warm imbibition alleviate the stress of rehydration.

Neem is an important tropical tree species, the products of which have multiple uses in Asia and, since its introduction, in the Sahelian regions of Africa (Bellefontaine, 1992). Essentially, the species propagates via seeds. Conflicting results have been published on the physiology and storage of neem seed (reviewed by Hong *et al.*, 1996; Poulsen, 1996). Neem seeds have been reported to lose viability within 1 to 4 months during *ex-*

situ conservation (Ezumah, 1986; Berjak *et al.*, 1995; Gunasena and Marambe, 1995; Msanga, 1996). They have also been reported to display orthodox behaviour (Roederer and Bellefontaine, 1989; Bellefontaine and Audinet, 1993; Tompsett and Kemp, 1996; Sacandé *et al.*, 2000b). Other studies concluded that neem seeds belong to the intermediate category of storage behaviour (Gaméné *et al.*, 1996; Sacandé *et al.*, 1996; Hong and Ellis, 1998; Varghese and Naithani, 2002). Many factors, such as seed provenance, maturity at harvest and conditions of storage and sensitivity to imbibition, have been suggested to explain these apparent controversies. Neem seeds are chilling sensitive at moisture contents (MCs) above 10% (fresh weight basis), but when dried to MCs below 10%, they are sensitive to imbibitional stress, limiting the tolerance to desiccation (Sacandé *et al.*, 1998). Although there is evidence of an increase in sensitivity to imbibition with reduction of MC, the MC may not be the main or the only one reason for the sensitivity of neem seeds to imbibition. For example, seed age has been suggested to increase the sensitivity to imbibition (Priestley, 1986; Zeng *et al.*, 1998).

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

Materials and methods

Seed material

Neem (*Azadirachta indica* A. Juss.) seeds were collected at two localities in Burkina Faso – Tougan and Ouagadougou. Mature (yellow) fruits were harvested by hand from at least 20 mother trees in each stand. Previous studies on the developmental stages of neem fruits have indicated that seeds from yellow fruits are physiologically mature (Sacandé *et al.*, 1997; Yaméogo, 1997; Neya, 1999). The seeds were prepared locally on the day of collection at the Centre National de Semences Forestières (CNSF) in

Ouagadougou, according to the method described by Sacandé *et al*. (1998). After depulping, the seeds were cleaned with water and dried in the shade on a grid for 1–2 d. Seeds, surrounded by an intact endocarp, were then selected and sent by air to Wageningen in cotton bags, arriving 1 week after harvest.
All seeds used in the present experiments were 'naked' seeds, i.e. seeds with the seed coat (endocarp) removed. Endocarp removal provided a more homogeneous germination and enhanced the rate of germination, but had no effect on seedling quality.

Moisture content and germination

Freshly collected seed lots were used directly in the experiments, or first dried to various MCs by exposure, for different lengths of time, to relative humidities (RHs) established above saturated solutions of $MgCl_2$ or LiCl in containers with circulating air at 20°C. A Rotronic hygroscope (±2% RH, Rotronic AG, Zürich, Switzerland) was used to measure relative humidity. For the ageing experiments, initially high-quality (>90% germination) seeds were equilibrated in circulating air above a saturated solution of $Ca(NO_3)_2$ at 20°C (final MC = 7.0%). After 9 weeks, some of the seeds were dried further above saturated LiCl for a week (final MC = 3.7%). This procedure provided seeds that were 10 weeks old having two different MCs. The remaining 10-week-old, 7.0% MC seeds were then stored for 7.5 months at 15°C in a closed plastic bag (final MC 7.0%). Subsequently, half of this seed lot was after-dried above saturated LiCl for a week (final MC = 4.3%). For simplicity, these MCs are referred to as 7% and 4%. The endocarps were removed just before the imbibition tests.
Seeds (50) were allowed to imbibe by pre-soaking them for 1 h in tap water (100 ml) in beakers kept in incubators set at temperatures between 15 and 40°C. After the soaking treatment, the seeds were sown on moist filter paper in plastic boxes (25 seeds/box) and incubated at a constant temperature of 30°C in a 12h/12h light/dark regime. Germination was scored at least twice a week until all viable seeds had germinated. Seeds were scored as germinated when the radicle had emerged to at least 2 cm. Three replicates of five seeds were used to determine the MC of seeds during the different experiments. The seeds were weighed before and after drying at 103°C for 17 h. The MC of seeds was expressed on a fresh weight basis.

Electron spin resonance spectroscopy

Electron spin resonance (ESR) spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker, Rheinstetten, Germany, model 300E). The water-

soluble nitroxide radical, perdeuterated TEMPONE (PDT, where TEMPONE = 4- oxo-2,2,6,6-tetramethyl-1-piperidinyloxy; a kind gift of Professor I. Grigoriev, Institute of Organic Chemistry of the Russian Academy of Sciences, Novosibirsk, Russia), was used as the spin probe for testing the integrity of the plasma membrane, according to the method described by Golovina and Tikhonov (1994).

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

Cryo-planing and cryo-scanning electron microscopy

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

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

Results

Effects of seed MC and imbibition temperature on germination

Seed batches of different MC, which were obtained during the drying of a freshly collected seed lot, were allowed to imbibe for 1 h at a range of temperatures from 15–40°C, followed by incubation at 30°C. The percentages of germinated seeds are presented in Fig. 1. There were no significant differences in percentage germination for seeds having MCs in the range 27–6%. But at 5% MC, the germination percentage declined significantly when imbibition occurred at 15–20°C, indicating that the seeds had become sensitive to low imbibition temperatures. These results confirmed that dried neem seeds are sensitive to imbibitional stress, and demonstrate that high viability (>80%) can be retained upon drying to low MCs (5%) as long as the seeds are properly rehydrated, i.e. at 25°C and above. The range of relatively high imbibition temperatures shown in Fig. 1 was chosen because injury can occur even at an imbibition temperature of 20°C in some neem seed lots (Sacandé *et al.*, 1998).

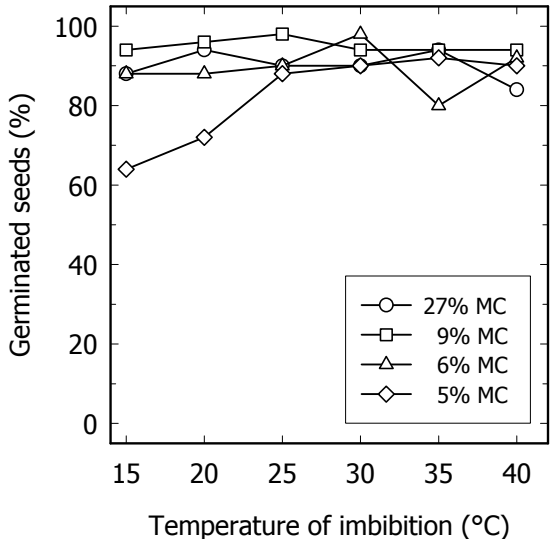

Figure 1. Effect of moisture content (MC) and imbibition temperature on neem seed germination (n = 50). After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C. Fresh seeds (27% MC) were dried at 20°C above saturated salts; the batches of 9% and 5% MC were taken during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

Effects of seed age and imbibition temperature on germination

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

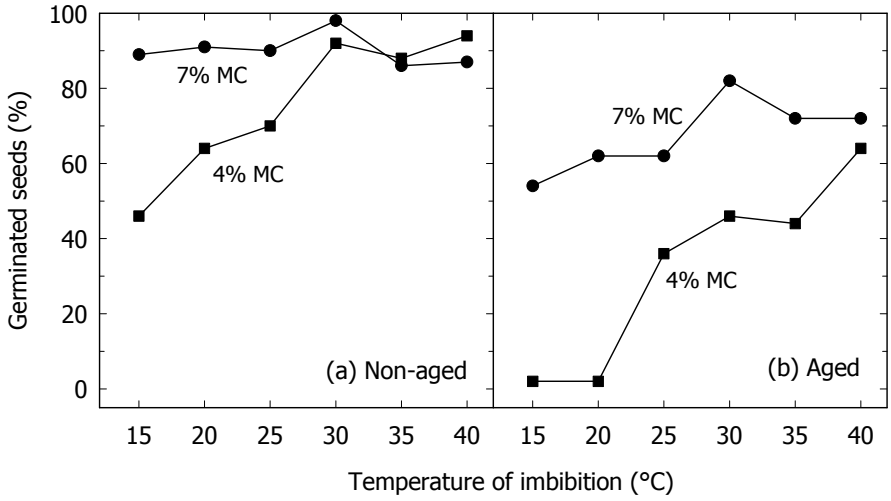

Figure 2. Effect of seed age and imbibition temperature on neem seed germination (n = 50) at two initial moisture contents (MCs) of the seeds (approximately 4% and 7%). After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C. High-quality (initial germination >90%)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

germination percentages also decreased further. These percentages were significantly lower than those of the non-aged, 7% and 4% MC seeds at all temperatures tested, except at 40°C ($P < 0.05$, same test). This dramatic increase of the effect of imbibition temperature on germination is caused by both factors – ageing and drying.

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

The aged seeds exhibited a slower start of radicle protrusion, as compared to the non-aged ones (Fig. 3). None of the aged, 4% MC seeds exhibited radicle protrusion after 2 d of incubation, whereas in all the non-aged seed lots, some germinated individuals

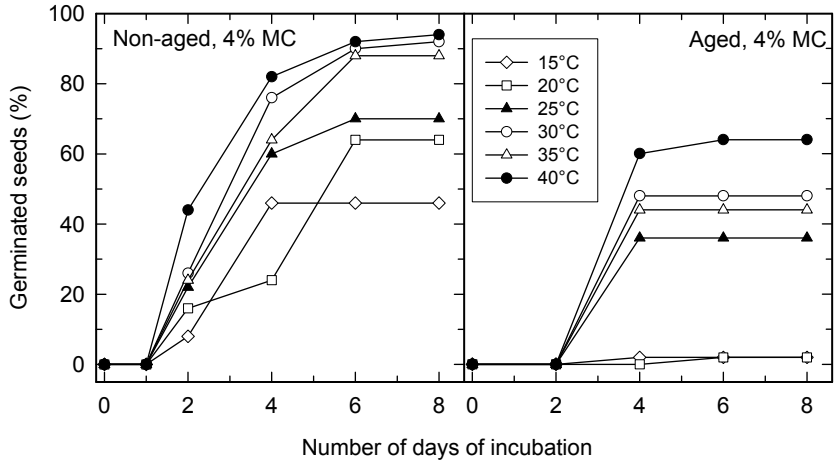

Figure 3. Effect of seed age and imbibition temperature on the rate of germination of the neem seeds [moisture content (MC) = 4%] from Fig. 2. After 1 h of imbibition at the indicated temperatures, seeds were further incubated at 30°C. Germination data are significantly different ($P < 0.05$) when they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

were noticed. The slow germination could be a sign of reduced vigour associated with ageing. There was no clear influence of the imbibition temperature on the rate of germination for either the aged or non-aged seeds.

Effects of imbibition temperature and seed age on membrane barrier properties

Figure 4 shows two contrasting ESR spectra of PDT. One is from the axis of a viable seed (non-aged; imbibition temperature 30°C) and one from the axis of a non-viable

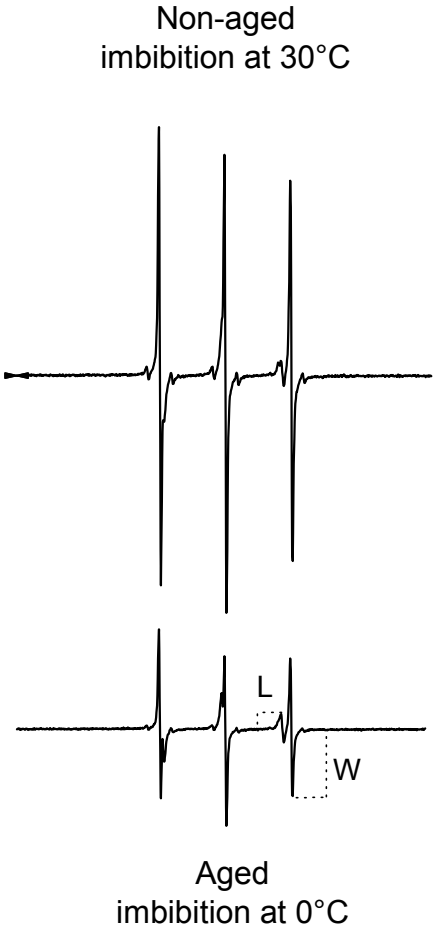

Figure 4. Representative electron spin resonance (ESR) spectra of perdeuterated TEMPONE (PDT) in axes of neem seeds after 1 d of rehydration. Imbibition was for 1 h at 30°C or 0°C, after which the seeds were further incubated at 30°C. Top spectrum: an axis from a non-aged seed (imbibition temperature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

seed (aged, imbibition temperature 0°C). Both spectra are the result of the superposition of two triplets. One triplet originates from PDT in aqueous cytoplasm surrounded by an intact membrane, the other from PDT in oil bodies. The lines of the two triplets are resolved at the right-hand side of the spectra (high-field lines). The amplitudes of the lines designated as W (water) and L (lipid) reflect the amount of spin-probe molecules in the cytoplasm and in lipid bodies, respectively. The ratio W/L, therefore, reports on the cytoplasmic volume of intact cells normalized to the amount of material (lipids) in the sample. On the basis of W/L values, the effects of imbibition temperature and seed age on the average integrity of cellular membranes in an embryo axis were estimated.

Figure 5 shows that the W/L values increased with increasing soaking temperature, for both the aged and the non-aged 4% MC seed lots. At each temperature tested, the W/L value for the aged seeds was significantly lower than that for the non-aged seeds. This means that the proportion of intact cells increased with increasing temperature of imbibition, and was higher in the non-aged seeds than in the aged seeds. Fresh neem

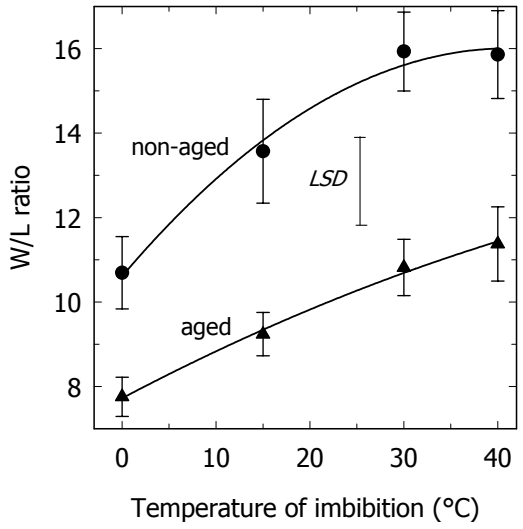

Figure 5. Effect of imbibition temperature on the water/lipid (W/L) ratio, as calculated from electron spin resonance (ESR) spectra of perdeuterated TEMPONE (PDT), in axes of neem seeds [moisture content (MC) = 4%] from the non-aged and the aged seed lots from Fig. 2. After 1 h of imbibition at the indicated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

axes (27% MC) had an average W/L value of 16.1, which is similar to the W/L values obtained after imbibition of the non-aged seeds at 30 or 40°C (Fig. 5).

By plotting the W/L values obtained for the axes versus the percentage germinated seeds (4% MC), straight lines could be drawn (Fig. 6), from which the average proportion of live cells at zero percent germination was approximated.

With an estimated W/L value of 9 for these dead axes and 16 for highly viable ones, it can be perceived that in non-germinating, imbibitionally injured axes, more than half of all the cells have intact plasma membranes and, thus, may be alive. The two seed lots in Fig. 6 differed in that the slope of the plot of the non-aged lot was steeper than that of the aged lot. This may be explained by the faster germination of the non-aged seeds (cf. Fig. 3). Because axes in these vigorous seeds showed some signs of swelling after 1 d of incubation in water (data not shown), W/L values may be higher than expected on account of the proportion of viable cells, because of cell extension. This could mean that the average proportion of dead cells in imbibitionally injured embryo axes is less than estimated above.

W/L values of single axes from one treatment give insight in the distribution of this parameter, allowing the threshold W/L value to be determined, below which the

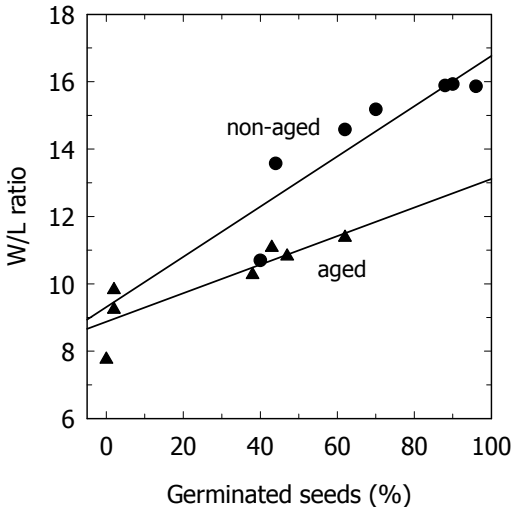

Figure 6. Plot of water/lipid (W/L) ratios in axes of non-aged and aged neem seeds (derived from Fig. 5) versus percentage germination. The different viabilities were obtained as a result of different imbibition temperatures (1 h, followed by incubation at 30°C).

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

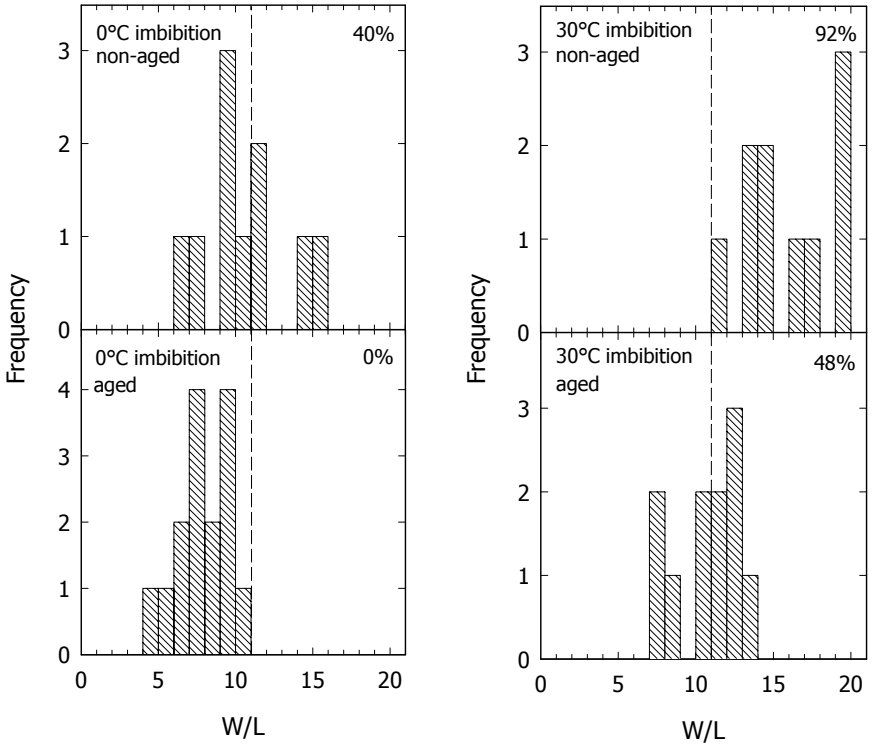

Figure 7. Distribution of the water/lipid (W/L) ratio in individual axes (10–15) of neem seeds from non-aged and aged seed lots [moisture content (MC) = 4%]. Imbibition temperatures were 0°C and 30°C (1 h). Percentage germination is indicated in the figures.

Effects of imbibition temperature and seed age on ultrastructure

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

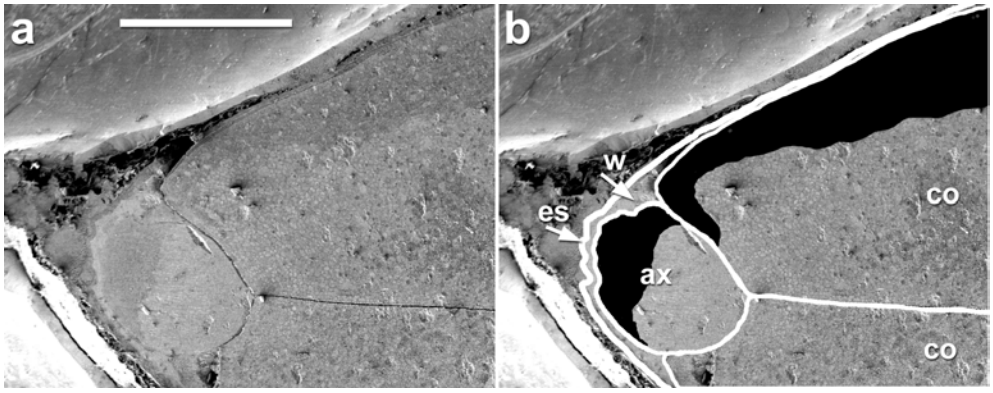

Figure 8. Micropylar end of a cryo-planed, aged (10-month-old) neem seed [moisture content (MC) = 4%], soaked in water at 0°C for 1 h, followed by incubation on moist filter paper at 30°C for 22 h. (a) Non-edited image; (b) edited image, highlighting the general anatomy (white lines) and areas of dead cells (black areas). The embryo consists of two large, oily cotyledons (co) and a small axis (ax). A very thin endosperm (es) surrounds the embryo, leaving spaces of free water (w; no solute remainder after etching) in between. Scale bar represents 1 mm; the total length of the seed is approximately 12 mm.

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

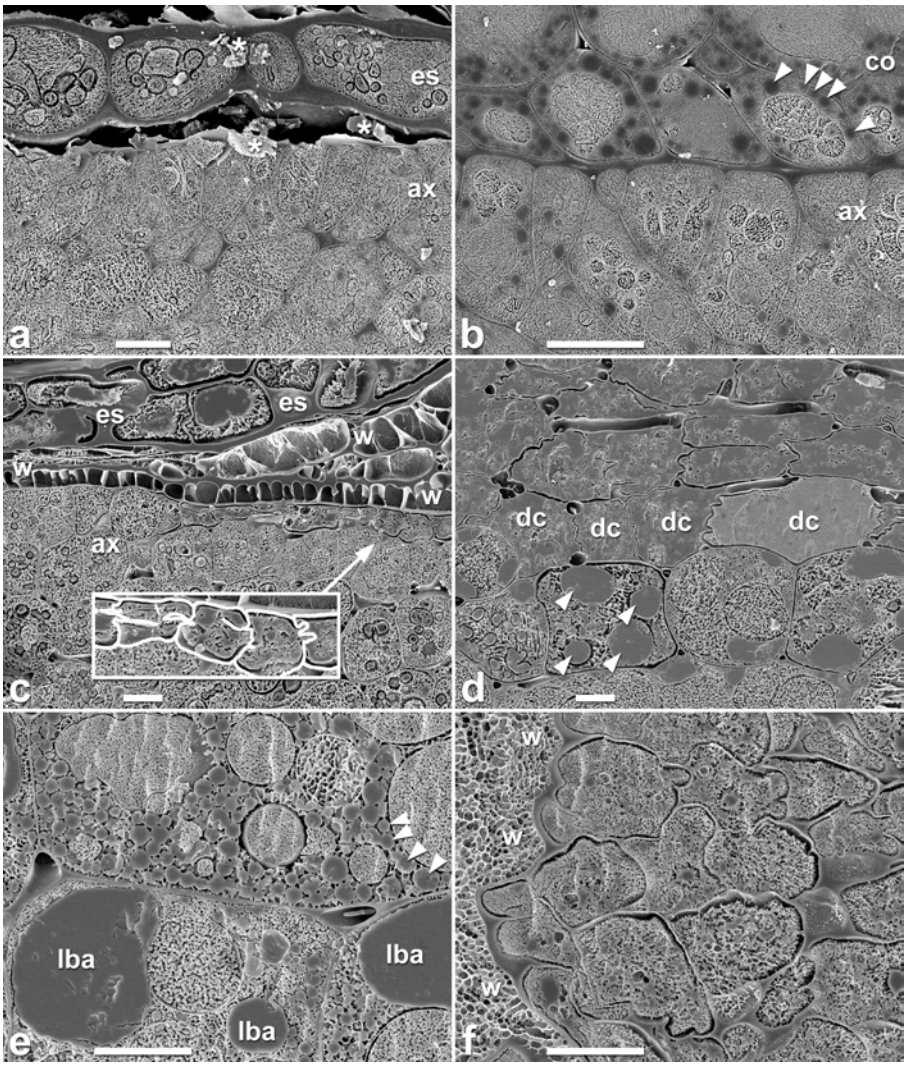

Figure 9. Cryo-SEM micrographs of a cryo-planed neem seed [moisture content (MC) = 4%] soaked in water at either 0°C or 30°C for 1 h followed by incubation on moist filter paper at 30°C for 22 h. (a) Non-aged, 30°C-imbibed seed, showing axis (ax) and endosperm (es) cells. Cells of both tissue types are turgid and compartmentalized, which is a sign of being alive. The debris (e.g. asterisks) originates from the cryo-planing procedure. (b) From the same seed as (a), showing the boundary between axis (ax) and cotyledon (co). All cells are alive. Cotyledon cells contain a high amount of lipid bodies (arrowheads). (c) Non-aged, 0°C-imbibed seed, showing axis (ax) and endosperm (es) with a water-containing space (w) in between. All endosperm cells are dead, which is apparent from the irregular and clumped cell contents. The axis cells are alive, except a part of the epidermis, which is magnified in

the inset (cell walls highlighted). The dead epidermal cells lack turgor and have heavily folded walls. (d) Same treatment as in (c), showing cotyledon cells at the boundary of damaged and intact cells, located at 10 layers from the periphery; dead cells (dc) lack turgor, have dense, amorphous contents and have curved walls. Typically, in the neighbouring live cells, lipid body aggregates (arrowheads) can be noticed. (e) Cotyledon cells of aged, 30°C-imbibed seeds. The cell at the top of the micrograph has numerous small lipid bodies (arrowheads), similar to the cells of non-aged seeds. The cells at the bottom have very large lipid body aggregates (lba). (f) Detail of the peripheral dead axis tissue of the aged 0°C-imbibed seed, also shown in Fig. 8. Cell walls and axis surface are folded, and no cellular compartments are discernible; w, water between endosperm (not shown) and axis. All scale bars represent 10 μm.

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

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

periphery were injured, but the area occupied by these cells did not exceed 5–10% of the total cryo-planed surface.
The 0°C-imbibition of the aged seeds (0% germination) caused considerable cellular damage. All endosperm cells appeared dead, while approximately 35% of the axis cells in the crosssection were injured. All injured cells were located at the periphery (Figs. 8b and 9f). Large peripheral areas of the cotyledons had cells with damaged ultrastructure (up to 15 cell layers; Fig. 8b). It was estimated that 15–20% of the total number of cotyledon cells in the cross-section were injured during cold imbibition. As in other treatments, injured cells were never noticed in the interior of the seed tissues, and they were always found in clusters.

Discussion

Dried neem seeds have been reported to be sensitive to imbibitional stress (Sacandé *et al.*, 1998, 2000a), and the data in Fig. 1 confirm this. When freshly collected seeds are dried to a low water content of about 5%, their germination percentage is still high if imbibition takes place at 30–40°C, but is reduced upon imbibition temperatures ≤ 20°C. If rehydration is to be considered an essential part of desiccation tolerance (Hoekstra *et al.*, 2001), then the problems with neem seeds, as reported in the literature, point to limited desiccation tolerance. Nevertheless, the integrity of cells in seeds that are dried to low MC is maintained as long as these seeds are in the dry state. Aged seeds displayed a considerably reduced germination percentage (Fig. 2). This reduction could partially be reversed by imbibition at the high temperature of 40°C, higher temperatures being ineffective in this respect, even when applied during periods shorter than the 1 h used in this work (unpublished results).
A number of reports link seed ageing with increased sensitivity to imbibitional stress (Priestley, 1986; Zeng *et al.*, 1998). Typically, this could also be concluded for neem seeds, on the basis of the data in Fig. 2 (7% MC, non-aged versus aged). While the nonaged 7% MC seeds were not sensitive to differences in imbibition temperature (15–40°C), the aged 7% MC seeds performed worse at 15–25°C. Furthermore, the effect of after-drying to 4% MC was greater in the aged seeds than in the non-aged seeds (particularly the 15–20°C range). To study a possible involvement of ageing in the sensitivity to imbibitional stress in more detail, we resorted to methods that can give an estimate of the proportion and location of dead cells.
Both ageing and imbibitional damage have been linked to the loss of plasma membrane barrier properties (Golovina *et al.*, 1997; Hoekstra *et al.*, 1999). If not a primary damage, reduced integrity at least will be evident 1 d after the onset of

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

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

appears to be in contrast to the pattern of ageing in neem seeds. Whether the increased sensitivity of neem seeds to imbibitional stress stems from changed physical properties of the plasma membrane, or from changed properties of the cell walls that allow for an increased water influx, has to be determined.

Ageing also had a specific effect on lipid bodies. Upon rehydration, the small lipid bodies still present in the dried cotyledons fused into giant droplets of up to 20 µm. Cells with these droplets, nevertheless, showed clearly discernible organelles and were turgid, suggesting that they were still alive. It is suggested that the giant lipid droplets may be involved in the delay of germination of the aged seeds. Lipid-body coalescence has been observed repeatedly in aged seeds (reviewed in Priestley, 1986). In this respect, the occurrence of giant lipid aggregates in the boundary layer between imbibitionally damaged and healthy cotyledon cells (non-aged; Fig. 9d) is indicative of a mild stress that is widespread in cotyledons of aged seeds, even after high temperatures of imbibition.

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

Acknowledgements

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Chapter 3

Germination capability of *Lannea microcarpa* and *Sclerocarya birrea* seeds as influenced by fruit maturity stage, desiccation, and dry storage

Oblé Neya, Moctar Sacandé and Folkert A Hoekstra

Abstract

Seeds from two African savannah tree species, both belonging to the Anacardiaceae family, were processed from fruits at three distinct maturity stages and then investigated for their survival of dehydration and dry storage. Although seed germination capability was low and variable throughout, an effect of fruit maturity stage on germination upon drying could be established for *L. microcarpa*, but not for *S. birrea*. The fact that seeds germinated when dried below 0.1 H_2O.g DM^{-1}, albeit at low percentages, is proof that the seeds of both species are desiccation tolerant and suggests that dormancy is involved. During drying, *S. birrea* seed germination improved substantially in all lots, suggesting that the dormancy is of the physiological type. Because germination was low for all *L. microcarpa* seed lots irrespective of fruit maturity status, it is likely that dormancy in this species is of a physical nature.

There was no difference in life-spans at low water contents between *L. microcarpa* seeds prepared from either green, green-red or purple-black fruits. The seeds could maintain their initial viability for up to 12 months at 15°C or -20°C, but storage at 25°C and, to a lesser extent, at 4°C, was considerably less effective. Percentage germination of *S. birrea* seeds improved in the course of dry storage to approx 80% after 15 months at a range of temperatures from –20 to 25°C. We conclude that both seed types behave as orthodox seeds and suggest that the variable germination percentages of fresh and dried seeds is the result of varying levels of dormancy.

Introduction

Germination and storability of savannah tree seeds have received little attention in spite of the need for *ex-situ* conservation of forest resources. Savannah tree species, in particular, are considered endangered as a result of over-exploitation and repetitive severe drought spells in recent decades. Seeds of many tropical tree species are intolerant of desiccation (Kamra, 1990; Hong *et al.*, 1996; IPGRI/DFSC, 1998, 1999; Ouédraogo *et al.*, 1999; Flynn *et al.*, 2004), which has a direct implication for their long-term storability. The better a seed survives dehydration; the longer it can be stored at a broad range of conditions without losing viability (Roberts, 1973; Dickie *et al.*, 1990; Ellis *et al.*, 1992; Mwasha *et al.*, 1996). Several factors are held responsible for desiccation intolerance, such as seed developmental stage, *i.e.* immaturity of embryos, or the lack of accumulation of protective compounds, e.g. sugars and late embryogenesis abundant (LEA) proteins (Ellis and Hong, 1994; Hoekstra *et al.*, 1994; Vertucci and Farrant, 1995; Dure, 1997; Gorecki *et al.*, 1997; Hay *et al.*, 1997; Pammenter and Berjak, 1999; Corbineau *et al.*, 2000; Hong *et al.*, 2000; Sliwinska, 2003).

Lannea microcarpa and *Sclerocarya birrea*, both belonging to the Anacardiaceae family, are important multipurpose tree species from the African savannah. They are used to prevent soil erosion, improve soil microclimate and provide shade, boundaries, fuel wood and timber. In addition, they contain highly effective medicines and pesticides, and the pulp of their fruits is used to produce several types of beverages.

L. microcarpa inflorescences are grapes, in which it is usual to find fruits of different maturity stages as in neem (*Azadirachta indica*) inflorescences (Neya *et al.*, 2004a). The fruit is a drupe, and the colour goes from green to purple-black during ripening. Previous studies indicated that *L. microcarpa* seeds are sensitive to desiccation (Hong *et al.*, 1996) and exhibit non-orthodox seed storage behaviour (Daws *et al.*, 2004a). The seeds also have been claimed to display orthodox behaviour (Flynn *et al.*, 2004; Pritchard *et al.*, 2004a), however, without proper reference to fruit maturity stage.

S. birrea flowers are reddish or greenish, with the male and female flowers occurring on separate trees (Coates-Palgrave, 1977; Pooley, 1993). The fruit often drops from the tree when it is still green and then ripens to pale yellow on the ground (Pooley, 1993; Nerd and Mizrahi, 2000). Reports on desiccation tolerance and storage behaviour of *S. birrea* seeds are rather contradictory. According to Ouédraogo and Verwey (1987) and Kamra (1990), *S. birrea* seeds exhibit recalcitrant storage behaviour, losing viability after only one month of storage. However, Were and Munjuga (1999) have reported the seeds to display intermediate storage behaviour. Other studies point to orthodox storage behaviour because the seeds have been found to tolerate desiccation to 10%

moisture content or even less, with retention of their initial viability for up to four years of storage (Msanga, 1998; Ouédraogo *et al*., 1999). Support for this contention comes from Gaméné *et al*. (2004), who found that well-dried seeds can survive 18 months of storage at -18°C. Several reasons, among them seed developmental stage, might have led to these conflicting reports.

Studies on neem seeds have demonstrated the importance of fruit maturity stage at harvest for seed desiccation tolerance and the associated storability (Sacandé, 1995; Sacandé *et al*., 1998; Neya, 1999; Neya *et al*., 2003 and Neya *et al*., 2004a). Because none of the previous studies on *L. microcarpa* and *S. birrea* have clearly identified the type of fruits from which the seeds had been processed, we investigated several seed lots extracted from fruits collected at different stages of maturity. Here we report on the impact of fruit maturity stage on desiccation tolerance and the associated long-term storability of seeds of *L. microcarpa* and *S. birrea*.

Material and Methods

Material

Seeds of *Lannea microcarpa* Engl. & K. Krause and *Sclerocarya birrea* (A. Rich.) Hochst. were collected from natural stands in Bissiga and Dakakiri (Burkina Faso), respectively. They were collected at three different developmental stages on the basis of fruits visual colour at harvest. Three lots were made up from green, green-red and purple-black fruits of *L. microcarpa* and, in the case of *S. birrea*, from green, green-yellow and pale yellow fruits. Once back (1-2 days) at the Centre National de Semences Forestières (CNSF) in Ouagadougou, different seed lots were prepared. For both species, fruits were soaked in water and depulped immediately. After that, the seeds were polished with sand to remove mesocarp tissue and washed with water. Seeds surrounded by an intact endocarp were then selected and used for the different experiments.

Moisture content and germination

Three replicates of five seeds were used to determine the moisture content (MC) of seeds throughout the study. The term seed refers to both the seed components (testa, cotyledons and embryonic axis) and the endocarp. The seeds were weighed before and after drying at 103°C for 17h (ISTA, 1999). MC was expressed proportionally to seed dry mass (DM) as g H_2O.g DM^{-1}.

Four replicates of 25 seeds or 50 seeds in duplicate were used to determine percent of germinated seeds for *L. microcarpa*, while 25 seeds in duplicate were used for *S. birrea*. The seeds were sown in moist sand contained in plastic boxes (50 or 25 seeds/box) and incubated at ambient temperature in the laboratory, where temperature fluctuated between 25 and 30°C. Germinated seeds were scored at least twice a week until all viable seeds had germinated. Seeds were scored as germinated when the radicle had emerged to at least 2 cm. For S. *birrea*, one protruded radicle per kernel was taken into account for germination evaluation.

Desiccation

After preliminary tests (moisture content and germination), seeds were dried at ambient conditions in the laboratory at CNSF (Ouagadougou), with a temperature ranging between 25 and 30°C and a relative humidity (RH) between 40 and 45%, without any additional desiccant. During drying, samples were taken regularly from the different seed lots for moisture content assessment and germination assay. The frequency of sampling was every 3 and 4 days for *S. birrea* and *L. microcarpa*, respectively.

Storage

For storage trials, seeds from the three fruit maturity stages of *L. microcarpa* fruits were utilized, whereas only seeds from the pale yellow fruits of *S. birrea* were used. After drying as described above, seeds were subdivided in samples packed in polyethylene bags and closed following practice at CNSF, *i.e.* folding the open end and using staples to fix the folds. The packed samples were stored at four different temperatures (25, 15, 4 and -20°C), and the viability was assessed at intervals of 3 months from the date of storage to determine survival. The content of each bag was used only once. Besides germination, moisture content was monitored during the storage period.

Data analysis

The software MINITAB was used to analyze the data. To adapt the data to the software each seed was considered as a replication. The method balanced ANOVA (analysis of variance) was used to access differences among means at a degree of confidence of 95%.

Results

Effects of fruit maturity stage and dehydration on *L. microcarpa* seed germination

During the first 4 days of drying a great reduction in MC was noticed for all seeds lots followed by a slight further reduction from 4 to 8 days (Fig. 1A). With initial MCs in the range of 0.37-0.45 H_2O.g DM^{-1} for seeds excised from all three maturity stages, the MC in all seed lots decreased to 0.11-0.15 H_2O.g DM^{-1} after 4 days of drying and reached a MC of approx. 0.085 H_2O.g DM^{-1} between 8 and 12 days.

The response to MC reduction in terms of germination was seed lot dependent. When MCs of the different seed lots were reduced to about 0.11-0.15 H_2O.g DM^{-1}, no deviation from the initial germination capability of freshly depulped seeds was noticed for seeds processed from green and green-red fruits, while seeds excised from purple-black fruits had germinated considerably less (Fig. 1B). Further drying to 0.085 H_2O.g DM^{-1} reduced the percentage of germinated seeds from green-red and purple-black fruits, whereas an improvement of germination was observed for seeds originating from green fruits. At 0.085 H_2O.g DM^{-1} the seeds germinated for 64, 24 and 20% in the case of seeds prepared from green, green-red and purple-black fruits, respectively (Fig.1).

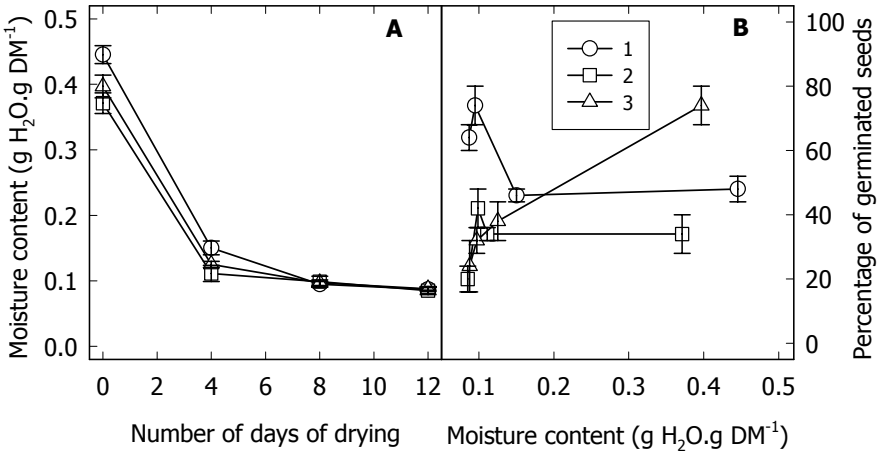

Figure 1. Effect of drying on moisture content (A) and percentage germination (B) of *L. microcarpa* seeds excised from fruits at three stages of advancing maturity designated 1 (green), 2 (green-red) and 3 (purple-black). (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 50 seeds in duplicate. Vertical bars represent standard errors.

Statistical analysis showed a significant effect (P<0.01) of MC reduction on germination of seeds prepared from green and purple-black fruits, but there was no significant effect in the case of seeds from green-red fruits.

Effects of fruit maturity stage and dehydration on *S. birrea* seed germination

With initial values in the range of 0.37-0.47g H_2O.g DM^{-1} for seeds excised from all three fruit maturity stages, MCs decreased to values in the range of 0.15-0.20 g H_2O.g DM^{-1} after 3 days of drying. After this 3-days period, in which the seeds lost more than half of their initial MC (Fig. 2A), a lesser further decrease in seed water content was observed. The seeds reached a MC of approx. 0.10 g H_2O.g DM^{-1} between 6 and 9 days of drying (Fig. 2A).

All the seed lots responded similarly to water reduction as assessed by germination assays. With decreasing MC, percentages germination increased (Fig. 2B). Initial germination was in the range of 38-54% for all seed batches. After 9 days of drying to a MC of 0.10 H_2O.g DM^{-1}, germination percentages became much higher (Fig. 2B: range 72-90%). Soaking these dried seeds in water for 4 hours at temperatures ranging from 5 to 40°C prior to sowing at 30°C did not affect germinability. Therefore *S. birrea* seeds are insensitive to imbibitional stress.

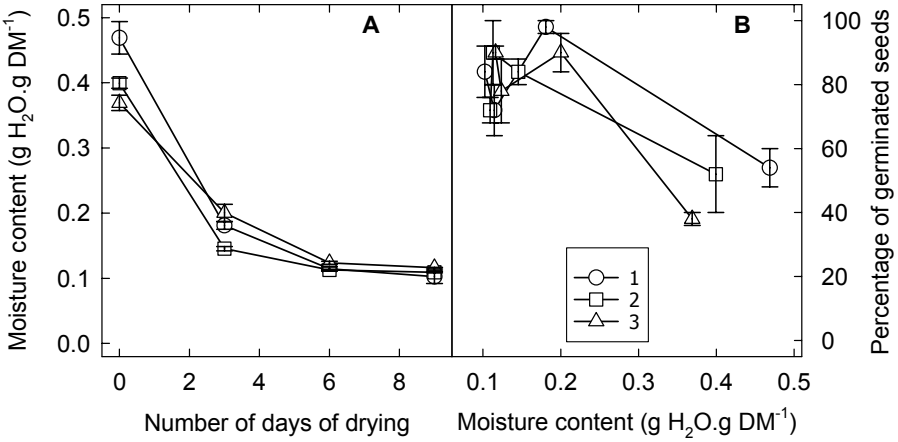

Figure 2. Effect of drying on moisture content (A) and percentage germination (B) of *S. birrea* seeds excised from fruits at three stages of advancing maturity designated 1 (green), 2 (green-yellow) and 3 (yellow pale). (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 2 replicates of 25 seeds. Vertical bars represent standard errors.

Statistical analysis showed that desiccation had a significant (P<0.05) effect on seed germination, while no difference between seed lots could be established.

Effect of fruit developmental stage and storage temperature on germination of *L. microcarpa* seeds

The MCs prior to the storage trials were 0.063, 0.067 and 0.63g H_2O.g DM^{-1} for seeds processed from green, green-red and purple-black fruits, respectively. During storage some variations in these initial MCs were recorded (Figs. 3A-5A), but all measured MC values were < 0.10g H_2O.g DM^{-1}.

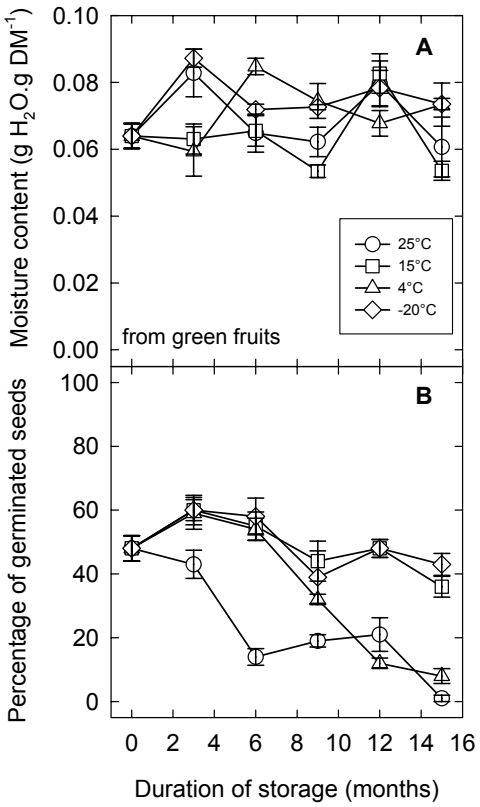

Figure 3. Moisture content (A) and germination percentage (B) of *L. microcarpa* seeds, prepared from green fruits, during storage at 4 temperatures (25°C, [circles], 15°C [squares], 4°C [triangles] and -20°C [diamonds]). (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 4 replicates of 25 seeds. Vertical bars represent standard errors.

Germination tests at regular intervals of 3 months from the date of effective storage at four different temperatures gave the percentages germination as shown Figs. 3B-5B. Regardless of seed lot, L. microcarpa seeds could maintain their initial viability more or less for 12 months if they were stored at 15 or –20°C, whereas a rapid decrease in germination ability was noticed for all seed lots stored at 25°C. In this respect storage at 4°C gave intermediate results. Statistical analysis showed a significant ($P<0.05$) effect of storage temperature and storage duration on seed viability, but the effect of seed lot was not significant ($P>0.1$).

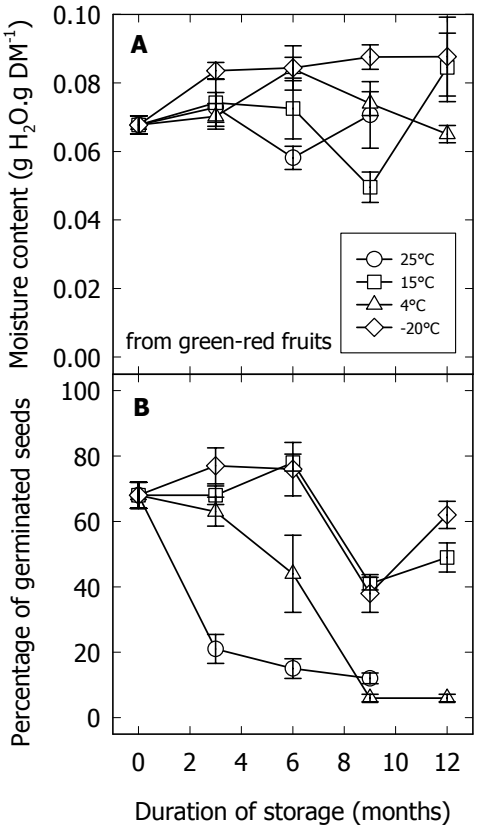

Figure 4. Moisture content (A) and germination percentage (B) of *L. microcarpa* seeds, prepared from green-red fruits, during storage at 4 temperatures (25°C, [circles], 15°C [squares], 4°C [triangles] and -20°C [diamonds]). (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 4 replicates of 25 seeds. Vertical bars represent standard errors.

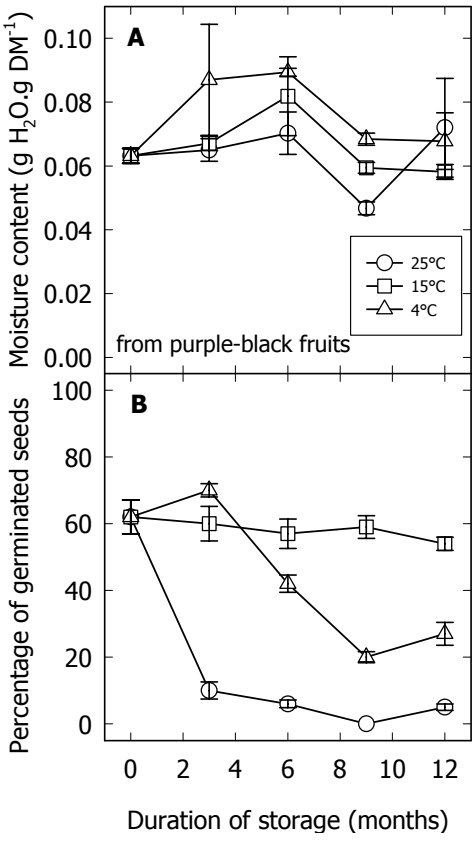

Figure 5. Moisture content (A) and germination percentage (B) of *L. microcarpa* seeds, prepared from purple-black fruits, during storage at 3 temperatures (25°C, [circles], 15°C [squares] and 4°C [triangles]). (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 4 replicates of 25 seeds. Vertical bars represent standard errors.

However, all measured values were in the range of 0.10-0.13g.g^{-1} after 15 months of storage at the different temperatures. During storage, *S. birrea* seeds exhibited rather unusual germination behaviour, regardless of storage temperature. After a decline of germination percentages during the first 6 months of storage, a significant increase in germination capability to 68-84% was recorded between 6 and 9 months (Fig. 6B), and this germination percentage was maintained up to 15 months of storage. Statistical analysis showed a significant (P<0.01) effect of storage duration, while no significant effect of storage temperature was found (P>0.1).

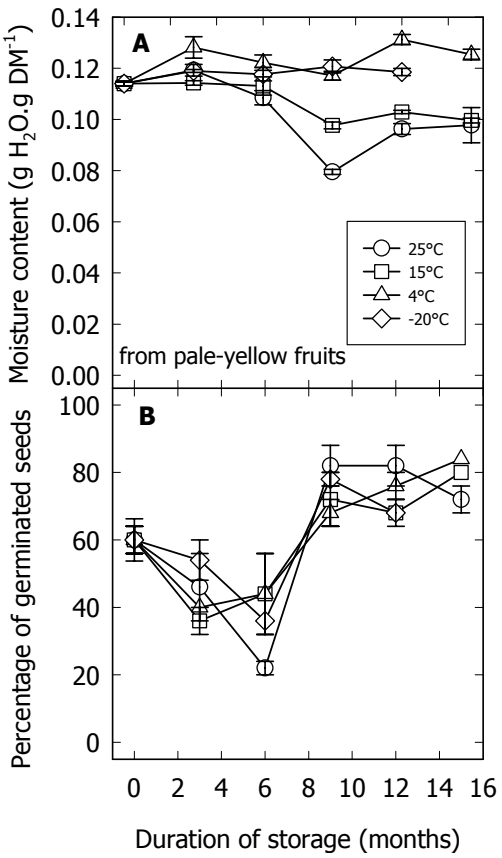

Figure 6. Moisture content (A) and germination percentage (B) of *S. birrea* seeds, prepared from pale yellow fruits, during storage at 4 temperatures (25°C, [circles], 15°C [squares], 4°C [triangles] and -20°C [diamonds]). (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 2 replicates of 25 seeds. Vertical bars represent standard errors.

Discussion

Are the seeds desiccation sensitive?

Typically, recalcitrant seeds reach lethal water contents during drying in the range of 20-30% MC (Pritchard, 2004). This range coincides with the water contents of our freshly depulped seeds. Since further drying did not lead to an entire loss of germination capability, the seeds from both species cannot be considered as displaying recalcitrant behaviour.

As a whole, germination percentages of *L. microcarpa* seeds were low including those of fresh seeds. Reducing seed MCs to approx. 0.085 g H_2O.g DM^{-1} did not lead to complete loss of viability of any seed lot, although some reduction in germination occurred in all seed lots at MCs < 0.1g H_2O. g DM^{-1} (Fig. 1). These findings corroborate the results of Daws *et al*. (2004a) and Pritchard *et al.* (2004a), who concluded that *L. microcarpa* has desiccation-tolerant seed. An improvement of germination ability of seeds processed from green fruits was noticed with progressive drying (Fig. 1B), which could be attributed to a gain in maturity during drying. Similar observation have been made with "immature" *Prunus africana* seeds (Were *et al*., 2004) and seeds of *Aesculus hippocastanum* collected at different stages of maturity (Daws *et al*., 2004b).

However, the overall low germination capability including that of fully ripened seeds processed from purple-black fruits could be a sign of dormancy as suggested once by Pritchard *et al*. (2004a). In L. microcarpa seeds, such dormancy will be likely physical as the low germination occurred in seeds collected at all developmental stages, without improvement during dry storage.

S. birrea seeds behaved contrary to what occurs when recalcitrant seeds are dehydrated. Drying had a positive effect on germination capability, and ultimately 76-90% germination was obtained for seeds from all fruit developmental stages at 0.10g H_2O.g DM^{-1} (Fig. 2B). This corroborates the results of Pritchard *et al*. (2004a), who observed that seeds of S. birrea germinated at higher percentages following dehydration to low moisture contents. The increase in germination could result from a gain in maturity of the seeds during drying as observed in prematurely harvested neem seeds (Neya, 1999). This hypothesis of gain in maturity may be supported by the fact that S. birrea fruits often drop from the tree when still green and ripen to pale yellow on the ground (Pooley, 1993; Nerd and Mizrahi, 2000). However, there was no significant difference between the initial germination capabilities of the different seed lots in spite of the distinct maturity stages of fruits.

The fact that drying improved germination ability in all seed lots, suggests that the dormancy involved in this species germination is of a physiological type. Poor seed performance has been shown in fresh seeds of a number of other tropical tree species

e.g. *Warburgia satularis* (ugandensis) (Kioko *et al*., 2004), *Sterculia quiqueloba* (Chanyenga, 2004), *Phoenix reclinata* (Berjak *et al*., 2004). A germination inhibitor has been suggested to be involved, ensuring that seeds that retain relatively high embryo water contents do not germinate prematurely (Berjak *et al*., 2004).

Effect of fruit maturity stage and temperature on storage longevity

The statistical analysis showed a significant effect of storage temperature and storage duration on *L. microcarpa* seed germination, but the effect of fruit maturity stage was not significant. Storage at 15°C and -20°C gave the best maintenance of germination capability. The seeds stored at 25°C performed much worse, with those stored at 4°C being intermediate (Figs. 3B-5B). Our results diverge from those of Daws *et al*. (2004a), who found that it is possible to store *L. microcarpa* seeds with similar MC (6%) for up to 14 months at 25°C, but that seeds are sensitive to storage at -20°C. A possible explanation for these contradictory results is that the extent of constraint to germination (probably a physical dormancy) experienced by the seeds varies within seed accessions and/or among seed lots. Moreover, the difference in germination of seeds stored at different temperatures may be attributed to differential influences of these temperatures on the depth of the dormancy.

It is known that many aspects of seed collection, handling and transport from field to laboratory and preparation, all can contribute to variation in seed germinability (Berjak and Pammenter, 2004, Whitaker *et al*., 2004). Heterogeneity within and among the seed accessions used in different studies is easily conceivable; this heterogeneity could be expressed in seed size, seed vigour and seed-coat properties, with subsequent effects on germination and/or dormancy (Matilla *et al*., 2005).

L. microcarpa seeds appeared to withstand subzero storage (-20°C), since it was possible to store seeds having about 0.06g H_2O.g DM^{-1} for up to 12 months, without significant loss of their initial viability (Figs. 3B-4B). Therefore; we conclude that *L. microcarpa* seeds exhibit orthodox storage behaviour. In practice, seeds from all three fruit maturity stages can be collected for conservation purposes.

Storage experiments with *S. birrea* seeds showed two patterns. During the first 6 months of storage a decrease in germination from 60% to 40% or less (Fig. 6B) occurred at all storage temperatures, while there was little change in MC of the different seed batches (Fig. 6A). Subsequently, germination percentages increased to about 80% independently of storage temperature. Comparable results have been reported by Shone (1979), who reported that the immaturely shed *S. birrea* seeds reached 100% germination only after 7 to 8 months of dry storage. Similarly, an increase in total germination has been reported after 3 months of storage under

ambient temperature (Were and Munjuga, 1999), while Von Teichman *et al*. (1986) concluded that germination rate increased following storage at 21°C for one year. Also, Gaméné *et al.* (2004) reported an increase in some *S. birrea* seed batches from Burkina Faso following 6 months storage under ambient conditions.

Hard-seededness and the associated physical dormancy of seeds as a result of an impermeability of the seed coat for water have been reported for members of the Anacardiaceae family (Baskin and Baskin, 1998; Li *et al*. 1999a). Drying and dry storage of seeds is expected to render the coat more impermeable and deepen dormancy. The observed improvement of *S. birrea* seed germination during drying and/or storage thus makes a possible physical dormancy unlikely. In accordance with this view, Pritchard *et al.* (2004a) noticed that removal of the potential constraint to germination - the cap above the embryo - did not improve germination of fresh seeds, unless the seeds were dried to low moisture contents. There is some evidence that the seeds can after-ripen in the dry state (Von Teichman *et al*., 1986), and our data in figure 6 are consistent with that. This favours the idea that seed dormancy in *S. birrea* is of a physiological origin.

As germination percentages were high after 15 months of dry storage without differences between the most divergent storage temperature regimes (25°C and -20°C) we confirm that the seeds of *S. birrea* display orthodox storage behavior (Msanga, 1998; Ouédraogo *et al*., 1999; Gaméné *et al*., 2004).

Acknowledgement

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Chapter 4

Seed coat-imposed constraints induce variability in *Lannea microcarpa* seed germination

Oblé Neya, Moctar Sacandé and Folkert A Hoekstra

Abstract

The few studies on the biology of *Lannea microcarpa* seeds, a multipurpose tropical species of the Anacardiaceae family, have reported contradictory germination and/or storage behaviour. To understand this discrepancy, seeds harvested in three successive years from natural stands in Burkina Faso were investigated for factors influencing their germination and storability. Germination tests on seeds excised from fruits at three different stages of maturity, either directly after depulping or after additional drying and/or subsequent storage, revealed considerable variation among and within seed lots. However, seeds from all these seed lots displayed high germination percentages (≈80%) when they were mechanically scarified prior to incubation. The data indicate that the generally low germination capability of the seeds is caused by seed coat-imposed constraints to germination. Differences in the extent of such constraints are likely the cause of the variability in germination responses observed among and within seed accessions. The seed coat-imposed constraint of germination is not due to an impermeability of the endocarp to water as reported for several other species of the Anacardiaceae, because water uptake also occurred in dried, non-scarified seeds. The seeds were not sensitive to low temperatures of imbibition, also not after scarification. Extended periods of dry storage at 15°C did not increase the low sensitivity to imbibitional stress.

The results reported in this study substantiate that the seeds have orthodox storage behaviour as they withstand dehydration to water contents as low as 0.05g H_2O per gram dry matter and can be stored at a broad range of temperatures (-20 to 15°C) for more than 1 year and retain high germination capability (≈80%).

Introduction

The role of natural resources in the fulfilment of energetic, alimentary and medicinal needs is crucial for populations in the tropics. In addition, the importance of biodiversity conservation compels sustainable management of natural forests, reforestation and the development of rational agroforestry and agro-silvi-pasture systems (Pritchard *et al.*, 2004b). Propagation of tropical trees species is generally via seeds in nurseries. Therefore, the unavailability and lack of sufficient knowledge about the biology of Sahelian tree seeds is a major problem for the sustainable management and implementation of successful reforestation programs using local species.

L. microcarpa is an important multipurpose tropical species; it is an excellent shade tree, which can reach 16m high by 2m girth and is used to improve microclimate and protect soils against erosion. The edible fruit is a drupe, the colour of which changes from green to purple-black with maturation. They are used for cooking and production of sweet or fermented beverages (juice, wine). The fruits represent a significant food supply for rural populations, particularly during years of cereal shortage. Despite the multiple uses of this tree for mankind and its undeniable importance for the environment, little attention has been paid to research on the seeds. The few earlier studies on *L. microcarpa* seed biology have shown that the seeds are difficult to store without loss of viability (Hong *et al.*, 1996), thus exhibiting non-orthodox storage behaviour (Daws *et al.*, 2004a). In contrast, in an earlier study we found that the seeds display orthodox storage behaviour, albeit that they are generally characterized by low and variable germination (Chapter 3). This low and variable germination may have several reasons, among them seed dormancy and sensitivity to relatively low soaking temperatures as has been established for neem tree seeds (Sacandé *et al.*, 1998; Neya *et al.*, 2004b).

Seed dormancy is the temporary failure of a mature viable seed to germinate under environmental conditions that would normally favour germination (Hilhorst, 1995; Li and Foley, 1997). Many flowering plants exhibit some level of primary seed dormancy that can be of one or both of the two following types: coat-imposed or embryo dormancy (Bewley and Black, 1982; Li and Foley, 1997). Seeds of the majority of dry savannah tree species are thought to be dormant, and physical dormancy is the most common type of dormancy (Baskin and Baskin, 1998; Li *et al.*, 1999a, 1999b; Baskin *et al.,* 2004; Lacerda *et al.*, 2004). Physical dormancy is mainly the result of seed coat impermeability regarding water uptake and oxygen diffusion into the embryo (Morris *et al.*, 2000; Baes *et al.*, 2002; Olvera-Carrillo *et al.,* 2003; Briggs *et al.*, 2005). Such a barrier should be removed in some way before seeds can germinate. Under natural conditions, the effects of high temperature, abrasion with soil, fire and ingestion by

large herbivores act on the seed coat to release dormancy (Bewley and Black, 1994; Baskin and Baskin, 1997; 1998; 2000; Baskin *et al.*, 2000; Morris, 2000; Manzano *et al.*, 2005). A variety of pre-sowing treatments such as hot water, sulphuric acid and mechanical scarification has successfully been used to overcome seed coat-imposed dormancy (Teketay, 1996; Teketay and Tigabu, 1996; Tigabu and Odéon, 2001; Ren and Tao, 2004; Bhatia *et al.*, 2005; Phartyal *et al.*, 2005). However, the optimal level of the various scarification treatments varies among species and needs to be determined empirically for any given species. Seed coat-induced dormancy has been found in several species of the Anacardiaceae family (Li et *al.*, 1999a; 1999b), to which *L. microcarpa* belongs. For *L. microcarpa* seeds, dormancy has once been suggested as a possible reason for the low germination percentages (Pritchard *et al.*, 2004a). The variation in the depth of such dormancy between and among seed lots might be responsible for the contradictory seed biological reports (Chapter 3).
This study was aimed at investigating the factors influencing *L. microcarpa* seed germination. We examined factors underlying the overall low and variable germination. This paper reports on the efficiency of mechanical scarification as a pre-sowing treatment to break coat-imposed constraints of seed germination. The implication of such constraints for the variability of germination between and among seed lots is discussed.

Material and Methods

Plant material

Seeds of *Lannea microcarpa* Engl. & K. Krause from Burkina Faso were harvested from natural stands in Bissiga (July 2002 and June 2003) and in Ipélcé (June 2004). Seeds in all collection years were obtained from fruits having the following maturity stages: green, green-red or purple-black. Seeds were then processed at the Centre National de Semences Forestières (CNSF) in Ouagadougou on the same day of collection. Fruits were soaked in water and depulped immediately. The seeds were rubbed with sand to remove the mesocarp tissue and washed with water. They were then dried at ambient conditions in the lab for 12 days (collection 2002) or 3-5 days (collection 2003 and 2004) and seeds surrounded by an intact endocarp were then selected and sent to Wageningen (Netherlands), in cotton bags. These pyrenes (embryo plus endocarp) were used for all experiments in this study, unless stated otherwise.

Moisture content and germination

Three replicates of five seeds were used to assess the moisture content (MC) of seeds throughout the study. The seeds were weighed before and after drying at 103°C for 17h (ISTA, 1999). The MC was expressed proportionally to seed dry mass (DM) as g H_2O.g DM^{-1}.

For germination assays, four replicates of 25 seeds or 50 seeds in duplicate were sown in moist sand or on moist filter paper contained in plastic boxes (11 cm x 17 cm x 5 cm) and incubated at a constant temperature of 30°C in an incubator, unless stated otherwise. Germinated seeds were scored at least twice a week until all viable seeds had germinated. A seed was considered as germinated when the radicle had emerged to at least 2 cm (sand) or 2 mm (filter paper).

Dehydration

All seeds were at least partially dehydrated upon arrival at Wageningen University. Seed lots were then subdivided in samples that were placed at different relative humidities (RHs) at a constant temperature of 20±2°C. The different RHs were obtained in closed drums with circulating air above saturated salt solutions ($MgCl_2$ for 33%, K_2CO_3 for 43% and NH_4NO_3 for 62%). At equilibrium, seeds having different MCs were thus obtained. Percentage of germinated seeds was used to assess the effect of dehydration on the different seed lots.

Mechanical scarification

To test the hypothesis that the low and variable germination capability of seeds was due to constraints imposed by the seed coat, mechanical scarification was applied as a pre-sowing treatment. Twenty five seeds in duplicate were utilized for each single assay. This treatment consisted of making a hole in the coat of seeds at the axis-side using a nail-clip, prior to incubation. Considerable attention was paid to avoid damage to the embryo during the treatment, and seeds with visibly damaged embryos were discarded. After this pretreatment, seeds were sown for germination as described above.

Water uptake and imbibition stress

Water uptake of 50 scarified and 50 non-scarified seeds was individually measured at several time intervals during the first 48 hours of incubation at 30°C. Seeds were individually weighed before placing them on moist filter paper in germination boxes. At

the end of each interval, each seed was removed quickly from the germination medium, surface-dried with tissue paper, weighed and immediately returned to the germination box. Percentage water uptake was calculated, using the following formula:
Percent water uptake = $(W_t - W_0)/W_0 \times 100$,
where W_t = seed weight at time t and W_0 = seed weight at the beginning of the experiment.

To test a possible sensitivity of the seeds to low temperatures of soaking, seed batches were allowed to imbibe for one hour in tap water (100 ml) at 0, 10, 20, 30, or 40°C. Subsequently, the seeds were removed from the water and sown on moist filter paper in plastic boxes at a constant temperature of 30°C.

Relationship between water activity and moisture content

Water activity (Aw) or equilibrium relative humidity (%ERH) measures the vapour pressure generated by the moisture present in a hygroscopic product (i.e. seed).
Aw = p / ps and %ERH = 100 x Aw,
where p is the partial pressure of water vapour at the surface of the product and ps is the saturation pressure or the partial pressure of water vapour above pure water at the product temperature. Water activity (Aw) of a seed then reflects the active part of moisture content or the part which, under normal circumstances, can be exchanged between the seed and its environment.
To establish the relationship between MC and Aw, seeds excised from green and purple-black fruits (2003 collection) were used. After equilibrium storage of seeds at three different relative humidities ($MgCl_2$ for 33%, $Ca(NO_3)_2$ for 55% and NaCl for 75% RH) and at constant temperature of 20±2°C, the Aw values of four replicates of 5 seeds (per seed lot) were measured using a water activity meter (HygroLab3, Rotronic, Germany), followed immediately by the assessment of the respective MCs (oven method as described above).

Storage trials

Seeds processed from green-red and purple-black fruits collected in June 2004 were used. After equilibrium at 33, 43 and 62% RH, seeds were subdivided in samples and packed in aluminium bags. The packed samples were stored at three different temperatures (15, 4 and -20°C), and the germination capability was assessed after 12 months of storage. Germination tests of scarified and non-scarified seeds were used to distinguish between seed coat-imposed constraints and problems associated with loss of viability in the differently stored seed batches.

Results

Effect of temperature on germination

The effect of temperature of incubation on the germination of intact seeds was studied to optimize conditions for germination. To this end seeds processed from purple-black fruits collected in June 2003 were used upon drying to a MC of 0.13g H_2O.g DM^{-1}. Figure 1 shows that the seeds had germinated in the range 15 - 40°C, but not at 10°C. Germination rate ($1/T_{50}$) was the highest for the seeds incubated at 35 and 40°C (0.2 and 0.25 d^{-1}, respectively), but the final germination percentages at these temperatures were comparatively low (28 and 18% respectively), with a high proportion of abnormal seedlings, at 40°C particularly (data not shown).

Seeds incubated at temperatures in the range 15 - 30°C germinated between 38 and 48%. In this range, the germination rate increased from 0.05 to 0.08 d^{-1} with increasing temperature. We therefore selected 30°C as the best temperature for all other germination tests.

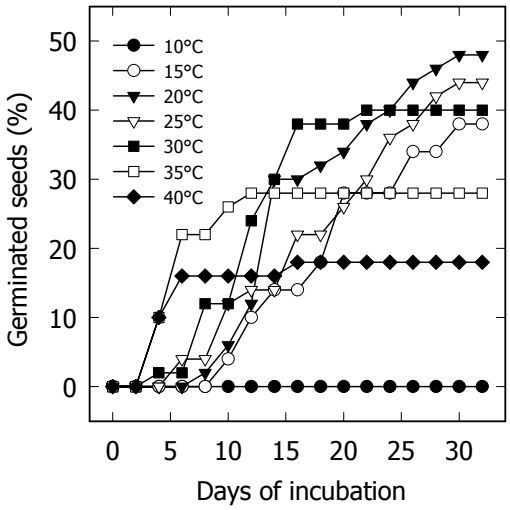

Figure 1. Effect of constant incubation temperature in the range 10-40°C on the germination of intact *L. microcarpa* seeds (0.13g H_2O.g DM^{-1}) processed from purple-black fruits. Data are the means of duplicate experiments of 25 seeds each.

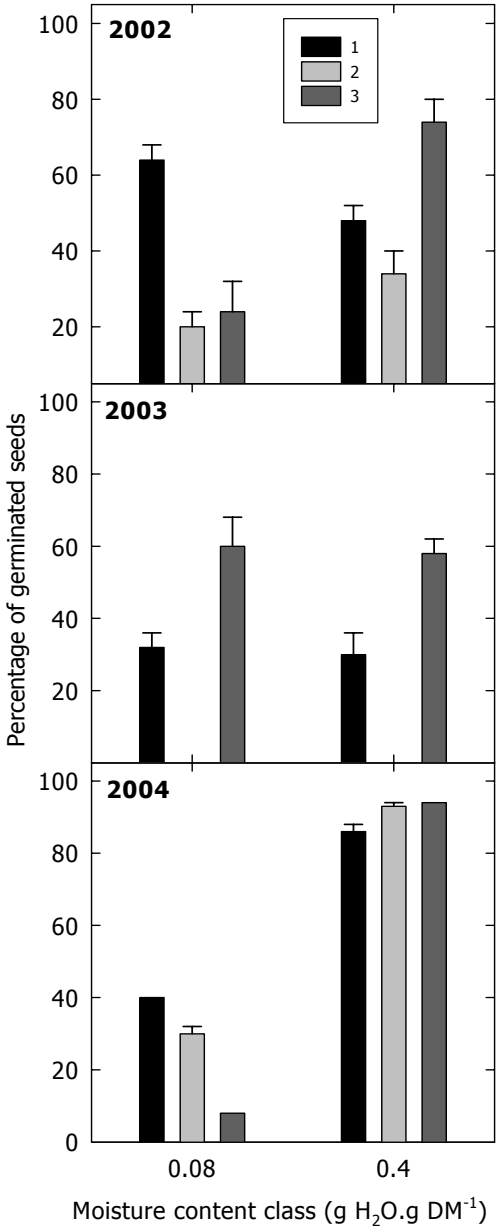

Figure 2. Variability of germination capability of intact seeds of *L. microcarpa* in three successive years. Germination was assessed directly after depulping (approx. 0.4 g H_2O.g DM^{-1} and upon drying to approx 0.08 g H_2O.g DM^{-1}. Seeds were from seed lots processed from fruits at three stages of advancing maturity, designated 1 (green), 2 (green-red) and 3 (purple-black). There were no seeds from green-red fruits in 2003. Data are the means of four replicates of 25 seeds each ±*SE*.

Variation in the germination of intact seeds

Germination of intact seeds was followed over 3 successive years of collection from natural stands in Burkina Faso. Large variation in germination of both fresh and dried seeds was observed from year to year and among seed batches derived from three stages of fruit maturity (Fig. 2). No link between germination capability and fruit maturity stage could be established. Seeds collected in 2002 and 2003 had a generally low germination and seeds from 2003 were not particularly sensitive to dehydration.

In contrast, the initially high germination percentage of seeds from the 2004 accessions was considerably reduced upon drying to approx. 0.08 g H_2O.g DM^{-1}, which would suggest some sensitivity to drying. However, since some seeds of all accessions and fruit maturity stages apparently survived dehydration to low water content, *L. microcarpa* seeds are unlikely to have a general sensitivity to desiccation. Rather, another, variable factor depressed germination without affecting viability.

Effect of scarification

As physical dormancy was suspected here, seeds were mechanically scarified directly before incubation in water at 30°C. Figure 3 shows the germination performance of the

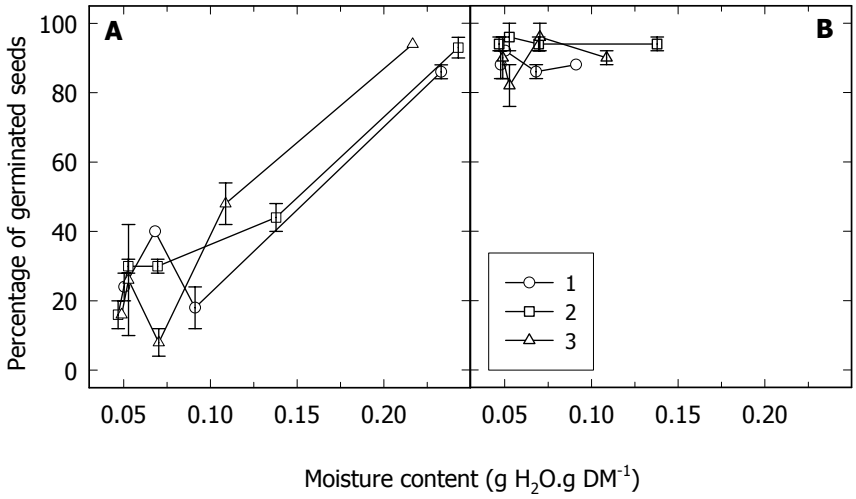

Figure 3. Effect of scarification on the germination of *L. microcarpa* seeds excised from fruits at three stages of advancing maturity, designated 1 (green), 2 (green-red) and 3 (purple-black) during drying. (A) Intact seeds; (B) seeds scarified prior to incubation at 30°C. Each point is the mean of 4 replicates of 25 seeds ± *SE*. SE bars are indicated when they exceed the symbol size.

seeds of all the 2004 lots in the course of drying to 0.05g H_2O.g DM^{-1}. Whereas drying reduced germination considerably in the non-scarified controls (Fig. 3A), this did not occur with the scarified seeds (Fig. 3B). No significant differences between germination percentages of scarified seeds having MCs between 0.14 and 0.05g H_2O.g DM^{-1} were found. In addition, the effect of dry storage on germination was investigated with or without scarification prior to imbibition. To this end, seeds dried to ca. 0.05 g H_2O.g DM^{-1}, processed from fruits at three stages of advancing maturity and stored at 15°C for 0, 2, 14 and 25 months, were used. Germination of the non-scarified seeds

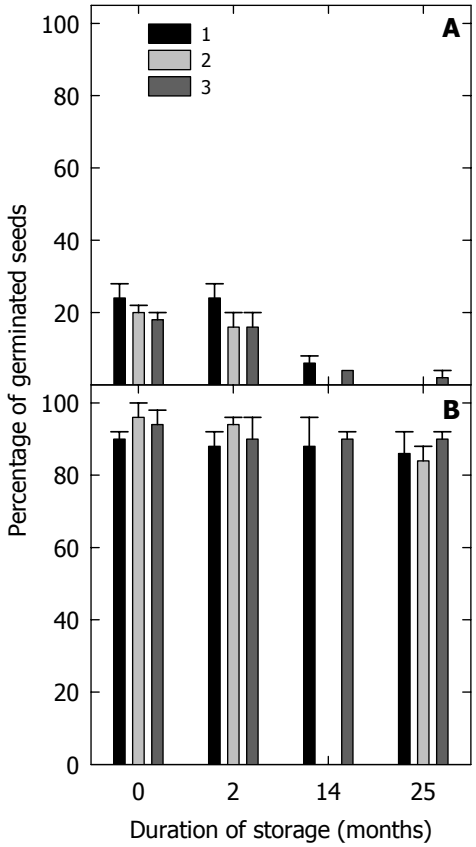

Figure 4. Effect of scarification on germination percentages ±*SE* of intact (A) and scarified (B) *L. microcarpa* seeds from different fruit maturity stages in the course of dry storage. Seed were excised from fruits at three stages of advancing maturity designated 1 (green), 2 (green-red) and 3 (purple-black). They were dried to ≈ 0.05g H_2O.g DM^{-1} and stored at 15°C for 0, 2, 14 or 25 months. Scarification was done immediately before incubation at 30°C. There were no 14-months-stored seeds from green-red fruits.The data represent means of duplicate experiments of 25 seeds each.

decreased to the range of 16-26% after 2 months, 6-8% after 14 months and 0-2% after 25 months of storage (Fig. 4). However, scarified seeds from the same seed lots germinated in the range of 90-96, 90-92 and 86-92% upon storage for 2, 14, and 25 months, respectively. These results show that there was a huge difference between percentages germination of intact and scarified seeds from the same batch. Apparently, the low germination in the different seed lots was caused by seed coat constraints and not by insufficient desiccation tolerance, ageing, or induction of physiological dormancy. The question whether or not the seed coat-imposed constraint is due to impermeability of the coat to water needed to be addressed.

Seed coat permeability

Seed coat effect was tested in an imbibition experiment using 50 scarified and 50 non-scarified seeds. During the first hour of imbibition, the average weight increase was slightly higher in intact seeds (12%) than in the scarified ones (10%) (Fig. 5). Subsequently, the rate of water absorption was reversed. After 48 h of incubation the average weight increase was 37% for intact seeds and 45% for the scarified ones. More than 50% of scarified seeds had developed visible radicles at that stage, while the intact seeds had not.

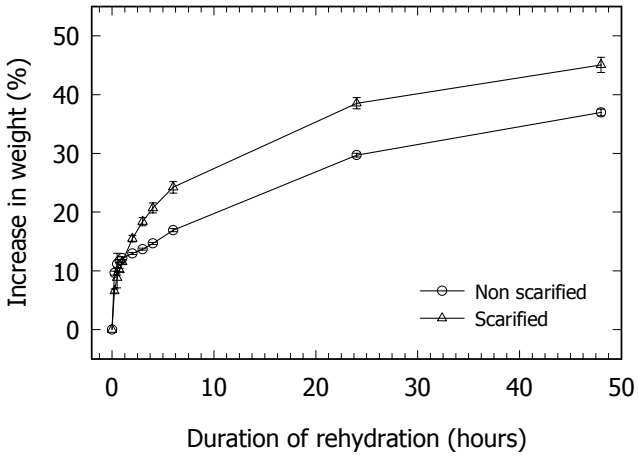

Figure 5. Effect of scarification on the water uptake of *L. microcarpa* seeds processed from purple-black fruits and dried to ≈ 0.05g H_2O.g DM^{-1} during the first 48 hours of rehydration at 30°C. Each point is the mean of 50 individual seeds. SE bars are indicated when they exceed the symbol size.

Sensitivity to imbibitional stress

To compare the sensitivity to temperature of the imbibition medium, scarified and non-scarified seeds from purple-black fruits collected in 2004 were used in two replicates of 25 seeds. Imbibition for one hour at a temperature range 0 - 40°C did not significantly affect germination of both scarified and non-scarified seeds (Fig. 6).

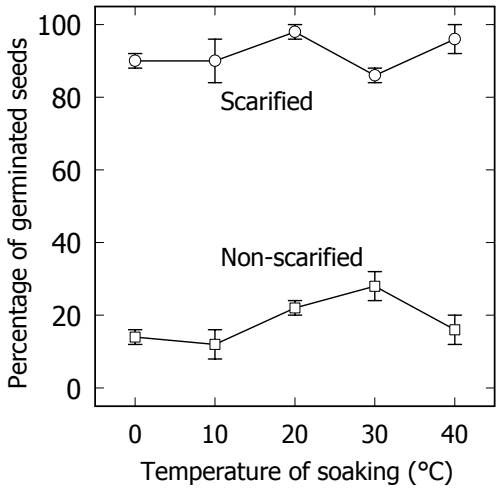

Figure 6. Effect of soaking temperature on the germination of non-scarified and scarified *L. microcarpa* seeds excised from purple-black fruits and dried to ≈ 0.05g H_2O.g DM^{-1}. Scarification was done immediately before soaking for 1 hour in water of different temperatures, and subsequently the seeds were further incubated at 30°C. Data points are means ±SE of duplicate experiments of 25 seeds each. SE values are indicated when they exceed the symbol size. The seeds were a few weeks old.

Similar to the 2004 collection, seeds stored at 15°C for 25 months (collection 2002) or 14 months (collection 2003) did not show any sensitivity to imbibition temperature in the range of 0 to 40°C, as far as their germination was concerned (Fig. 7). The differences observed between germination capabilities were seed lot dependent rather than the result of imbibitional damage. Therefore, no difference in sensitivity to imbibitional stress was found between seeds of different storage history.

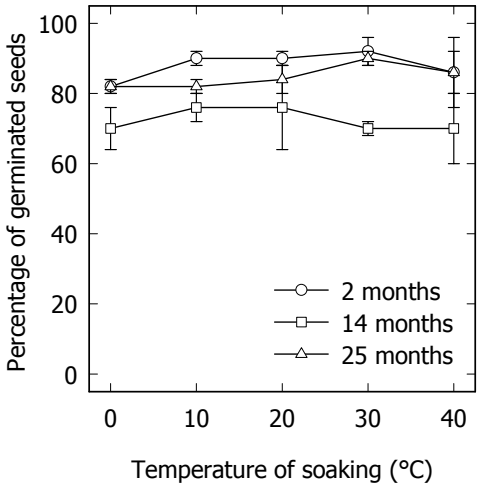

Figure 7. Effect of soaking temperature on the germination of stored *L. microcarpa* seeds that were scarified immediately before soaking. Seeds were processed from purple-black fruits, dried to ≈ 0.05g H_2O.g DM^{-1} and stored at 15°C for 2, 14 or 25 months. Scarification was done immediately before soaking for 1 hour in water of different temperatures, and subsequently the seeds were further incubated at 30°C. Data points are means ±*SE* of duplicate experiments of 25 seeds each. SE bars are indicated when they exceed the symbol size.

Relationship between moisture content and water activity

Plot of moisture content against water activity of *L. microcarpa* seeds showed that there was a positive linear correlation (R=0.958) between MC and water activity. Seeds that were dried to 0.05g $H_2O.g^{-1}$ DM had an Aw of approx. 0.35, while that of seeds dried to 0.10g $H_2O.g^{-1}$ DM was 0.75 (Fig. 8). Considering the physiological meaning of water activity this parameter is obviously a more accurate measurement of water status in stored seeds.

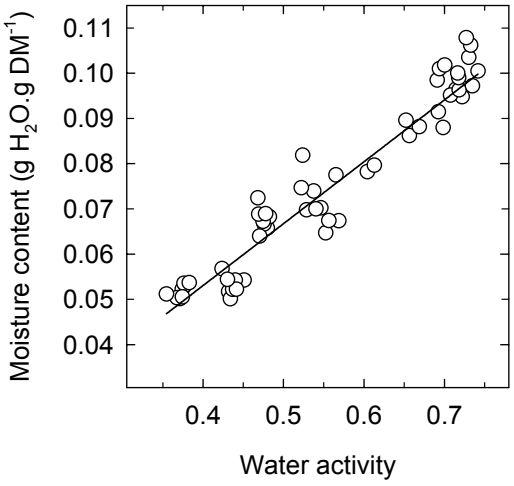

Figure 8. Plot of moisture content against water activity of *L. microcarpa* seeds. Each point was obtained from a sample of 5 seeds. There was a high and linear correlation (R=0.958) between MC and water activity.

Effect of low storage temperatures

In the light of the sensitivity of seeds from tropical regions to storage at low temperatures, even when seeds are dried, a one-year storage experiment was conducted with seeds excised from green-red and purple-black fruits which were dried to 0.05 or to 0.07 g H_2O.g DM^{-1} before storage. If the seeds were scarified prior to germination tests, their initially high germination capability was reasonably well maintained after 12 months of closed storage at 15, 4 and -20°C, particularly in the case of seeds excised from purple-black fruits (Table 1).

A certain reduction in the germination of seeds excised from green-red fruits, which were stored at 4°C, was observed, irrespective of their initial MC. Because of the general reduction in germination of intact seeds after 12 months of storage regardless of seed batch and storage temperature (Table 1, data in brackets), it can be concluded that the constraints to germination imposed by the seed coat might increase with duration of storage.

Table 1. Effect of three different storage temperatures on the one-year survival of *L. microcarpa* seeds from different seed lots at two moisture contents. Germination percentages of seeds that were scarified immediately before incubation at 30°C are presented, with the germination percentages of intact, non-scarified seeds in brackets. Seeds were excised from green-red or purple-black fruits and dried to 0.05 or to 0.07 g H_2O.g DM^{-1} before storage. The data represent means of duplicate experiments of 25 seeds each.

		12 Months storage			
	Initial MC (g H_2O.g DM^{-1})	Control	15°C	4°C	-20°C
From green-red fruits	0.05	71 (11)	74 (10)	51 (0)	74 (1)
	0.07	70 (6)	60 (8)	56 (2)	80 (2)
From purple-black fruits	0.05	95 (23)	-	83 (3)	83 (8)
	0.07	94 (30)	74 (8)	76 (2)	88 (10)

Discussion

Temperature sensitivity of germination

Seeds of many tropical species are chilling sensitive. The minimum temperature of *L. microcarpa* seeds, below which no germination is expected, appeared to lie below 15°C, but the optimum temperature for germination was in the range of 20-30°C (Fig.1). This corroborates the results of Pritchard *et al*. (2004a) and is in line with the results of other studies on thermal requirements for tropical tree seed germination (Tompsett and Kemp, 1996; Sacandé *et al*., 2001; Gaméné and Eriksen, 2004; Pritchard, 2004). On the other hand, germination was to a certain extent thermo-inhibited when seeds were incubated at ≥ 35°C (Fig. 1), and a high proportion of abnormal seedlings, was found at such temperatures. However, the germination of seeds was generally low, even at optimum temperatures. Therefore this low germination cannot be attributed to sub-optimal incubation.

Variability of germination

Germination of both fresh and dried seeds varied considerably from one accession to another (Fig. 2). Some seed batches showed high germination capability at harvest

(Fig. 2, 2004), while other seed accessions had a relatively low initial germination, regardless of the maturity stages of fruits, from which they were excised (Fig. 2, 2002 and 2003). On the other hand, the germination of the different seed batches after drying to 0.08 g H_2O.g DM^{-1} was also variable among seed lots. Drying the 2003 collection seeds to 0.08 g H_2O.g DM^{-1} did not affect their initial germination capability, indicating that *L. microcarpa* seeds are desiccation tolerant as previously stated (Daws et *al*., 2004a, Pritchard *et al*., 2004a, Chapter 3). However, seeds from the 2004 collection and to a certain extent those from the 2002 collection, showed a considerable reduction in germination competence with reduction of their MC from approx. 0.4 to 0.08 g H_2O.g DM^{-1}, suggesting that the seeds may be sensitive to desiccation, as has been claimed earlier (Hong et *al*., 1996). These observations raised suspicion that the seeds experience other constraints of germination.

Seed coat-imposed inhibition of germination

When mechanically scarified prior to germination tests, all seed batches under investigation germinated at high percentages (>80%), as shown in Figs. 3 and 4, regardless of their MC and age. Further than a mere suggestion of dormancy (Pritchard *et al*., 2004a), these results demonstrated that the seed coat imposes major constraints to germination of *L. microcarpa* seeds. We therefore interpret the variation in germination directly after depulping and after drying between seeds of the different seed lots as resulting from differences among and within seed batches in the depth of seed coat-related constraint of germination. Seed-dormancy has an environmental and a genetic basis (Li and Foley, 1997; Foley and Fennimore, 1998; Koornneef et *al*., 2002). Therefore, the intensity of dormancy in a given species exhibits a high degree of variation at several levels: among populations, within populations and even between seeds collected in different years from the same population (Andersson and Milberg, 1998; Meyer and Pendleton, 2000). Seed-dormancy variation in natural populations has been found for many tropical tree species, such as *Senna multijuga* and *Plathymenia reticulata* (Lacerda *et al.,* 2004).

Seed coat-imposed dormancy may be caused by a water-impermeable endocarp and is classified as physical dormancy (Baskin and Baskin, 2004). This type of dormancy has been reported in several species of the Anacardiaceae family, to which *L. microcarpa* belongs (Baskin and Baskin, 1998; Li et *al*., 1999a; 1999b). However, there was only a slight difference (<10%) between the weight increase of intact and scarified seeds after the first 48 hours of rehydration at 30°C (Fig. 5). This indicated that the seed coat is water permeable in accordance with data of Pritchard *et al*., 2004a, who argued that the seeds imbibed well during incubation. We thus conclude that, in contrast to seeds

of other Anacardiaceae species, seed coat-imposed constraints of germination in *L. microcarpa*, is not caused by a water-impermeable endocarp.
Aside from the water impermeable model, a number of other mechanisms have been proposed, by which the seed coat can impose dormancy on a seed (Bewley and Black, 1994). These include: (1) mechanical restriction of germination of the embryo: the mechanical barrier model; (2) prevention of the exit of germination inhibitors from the embryos: the inhibitor exit model; (3) presence in the seed coat of chemical inhibitors of germination: the seed coat inhibitors model and (4) restriction of oxygen uptake: the oxygen diffusion model.
Although with the data of the current study we are unable to discuss all the above-mentioned models, the seed coat inhibitors model is unlikely in *L. microcarpa* seeds, as the presence of the seed coat around scarified seeds did not reduce germination. Also, soaking intact seeds in water for 48h prior to incubation (which would have led to the leakage of any water soluble inhibitor from the seed coat) did not improve germination (data not shown). Considering the thickness of the coat, the mechanical barrier model may be a more plausible mechanism, by which the seed coat imposes dormancy. Yet further investigations are needed to verify this hypothesis experimentally and discard the inhibitors exit and oxygen diffusion models.

Imbibitional stress

Soaking dried scarified or non-scarified seeds for one hour in the temperature range 0 - 40°C prior to incubation at 30°C did not affect the competence to germinate. The germination percentages of scarified seeds soaked at 0°C and 40°C were 90 and 96%, respectively (Fig. 6). Also, the extension of the soaking duration at the two extreme temperatures (0 and 40°C) to 4 hours prior to germination did not lead to considerable changes in percentage of germinated seeds (data not shown). The hole made in scarified seeds obviously favoured a direct contact between water and the embryo, but without effect of low soaking temperatures. These results indicate that *L. microcarpa* seeds are not sensitive to imbibitional stress. In contrast, dried neem seeds have been reported to be highly sensitive to imbibitional stress (Sacandé et *al.*, 1998), and this sensitivity has been found to increase with ageing (Neya et *al.*, 2004b; Chapter 2). In *L. microcarpa* seeds, no increase in the sensitivity to imbibitional stress with ageing was noticed. Seeds stored at 15°C for 2, 14 and 25 months did not show any difference in their sensitivity to imbibition temperatures (Fig. 7). Differences in germination percentage were related to the inherent quality of the seed lots and not to an increased sensitivity to imbibitional stress associated with ageing.

Correlation between moisture content and water activity

As expected, a positive and linear correlation was found between water activity and moisture content of *L. microcarpa* seeds (Fig. 8). The water activity of a seed represents the exact amount of available water for exchange with its environment. Therefore it is a more accurate measurement of water status in a biological material such as seeds. Considering the importance of water status for seed viability during storage (McDonald, 1999, Merritt *et al.*, 2003), water activity measurement has a better physiological significance than moisture content. Furthermore, water activity measurement is fast and non destructive, so it may constitute a better alternative for the classic MC assessment of seeds.

Effect of dry storage on seed coat-imposed constraints to germination

Intact *L. microcarpa* seeds showed a reduced germination after 12 months of storage, regardless of the storage temperature applied (Table 1). This suggested that the constraints imposed on germination by the seed coat are enhanced upon dry storage. On the other hand, when scarified prior to incubation, seeds stored dry at 15°C for 25 months (Fig. 4), or at -20°C for 12 months (Table 1), germinated at high percentage. The low and variable germination percentage of intact seeds cannot be attributed to low vigor because seeds from all fruit maturity stages, irrespective of their storage history, germinated at high percentage when scarification was applied prior to incubation. These results confirm that the reported short storage longevity is due to constraints of germination imposed by the seed coat, as previously proposed (Chapter 3). However, the problem is not the result of endocarp impermeability to water as reported for several species of the Anacardiaceae family, which means that the exact mechanism still needs to be clarified. The results reported in this study further substantiate that *L. microcarpa* seeds are orthodox as they withstand desiccation to low water contents (≈ 0.05g H_2O.g DM^{-1}) and can be stored at a broad range of temperatures (-20 to 15°C) for at least one year and retain high viability (≈ 80%).

Acknowledgements

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Chapter 5

Mechanism of seed coat-imposed restraint of germination in *Lannea microcarpa* seeds

Oblé Neya, Elena A. Golovina, and Folkert A Hoekstra

Abstract

Lannea microcarpa, a multipurpose tree species from the dry African savannah, sheds seeds that display coat-imposed constraints of germination. The underlying mechanism was investigated using seeds processed from fully matured fruits collected from natural stands in Burkina Faso. Mechanical scarification of the coat, when applied at the axis-side, completely overcame germination constraints. Scarification at the side opposite to the axis led to delayed radicle emergence backwards through the orifice in ~40% of the seeds. The seed coat appeared permeable to water and respiratory gases. Water uptake and respiration increased with radicle emergence. Non-scarified seeds lost germination ability and cellular viability after approx 15d of moist incubation at 30°C, indicating that coat-imposed inhibition of germination does not prolong viability of seeds in the hydrated state. These seeds displayed a moderate rate of respiration with RQ values < 1. These results indicate that radicle emergence is inhibited mechanically and not by germination inhibitors or physiological dormancy.

Directly after imbibition an ethylene peak evolved from both intact and scarified seeds via *de novo* produced ACC. Intact, incubated seeds could be rescued by delayed scarification long after the ethylene production subsided, which abolishes possible involvement of ethylene in the dormancy or cellular decay. The variable and reduced germination upon drying or dry storage could not be ascribed to changes in seed coat composition as far as can be judged from FT-IR spectra, but rather to structural changes in the coat components. We suggest that the fast decay upon moist incubation at 30°C resembles the effects of an accelerated ageing treatment and might point to inadequate repair and/or antioxidant status in *L. microcarpa* seeds. The ecological consequence of such a fast decay is discussed.

Introduction

Seed dormancy is the temporary failure of a mature viable seed to germinate under environmental conditions that would normally favour germination (Hilhorst; 1995; Li and Foley, 1997). Plants are endowed with several mechanisms of dormancy to optimize the time of seed germination (Rees, 1997; Jones, 1999; Funes and Venier, 2006) in order to secure the successful establishment of their seedlings in natural populations (Bewley and Black, 1994; Li and Foley, 1997; Foley, 2001). Five classes of dormancy have been found to occur in seeds at maturity: physical, morphological, physiological, morphophysiological and combinational dormancy (Baskin and Baskin, 1998; 2004). These classes of dormancy have been defined on the basis of (1) permeability or impermeability of the seed (or fruit) coat to water, (2) whether the embryo is fully developed or underdeveloped at seed maturity, (3) whether the embryo is physiologically dormant or non-dormant, and (4) a combination of 2 or more of the above-mentioned circumstances (Nikolaeva, 1977; Baskin and Baskin, 1998; 2004). Seed dormancy caused by one or more water-impermeable layers of palisade cells in the seed or fruit coat (*i.e.* physical dormancy: Li *et al.*, 1999a, 1999b; Baskin *et al.*, 2000; Baskin and Baskin, 2004) is common in species that inhabit arid and semi-arid regions (Baskin and Baskin, 1998).

Coat-enhanced dormancy can be eliminated by artificial treatments such as mechanical or acid scarification, natural causes such as fire, high temperature or fluctuation in temperature, or biological actions such as gut passage or micro-organisms (Baskin and Baskin, 1998; 2000; Jones, 1999; van Assche *et al.*, 2003; Manzano *et al.*, 2005).

Seed biology of *Lannea microcarpa*, a multipurpose tropical tree species of the Anacardiaceae family, has been described in various ways: the seeds were classified as recalcitrant (Hong *et al.*, 1996), non-orthodox (Daws *et al.*, 2004a), and orthodox (Flynn *et al.*, 2004; Pritchard *et al.*, 2004a). Recently, (Chapters 3 and 4) we demonstrated that the generally low germination capability and the variability in germination response, particularly after drying and/or dry storage of the seeds is due essentially to seed coat-imposed constraints of radicle emergence. The variation in the extent of such constraints between and among seed lots has been proposed to have lead to the different descriptions of *L. microcarpa* seed biology (Chapters 3 and 4).

The seed coat of *L. microcarpa* is water-permeable (Chapter 4) and the seed imbibes well during incubation (Pritchard *et al.*, 2004a). Therefore, the dormancy is not of the physical type *sensu stricto* (Baskin and Baskin, 2004). However, apart from water-impermeability or hardseededness, a number of other mechanisms have been proposed, by which the seed coat can impose dormancy (Bewley and Black, 1994; Adkins *et al.*, 2002). These mechanisms include: a) mechanical restriction of embryo

expansion or growth; b) prevention of the exit of germination inhibitors from the embryos; c) presence in the seed coat of chemical inhibitors of germination, and d) restriction of oxygen uptake. Apart from the water-impermeability model, also the coat inhibitor model of seed coat-imposed dormancy is unlikely to apply to *L. microcarpa* seeds. Indeed, the results of our previous work (Chapter 4) have shown that neither the presence of seed coats affected germination of mechanically scarified seeds nor soaking of intact seeds in water for 48h prior to incubation lead to improvement of germination. In the latter case leaching of any water-soluble inhibitor from the coat would have improved germination ability. In the present study, germination patterns of non-scarified and scarified seeds were used to test the hypothesis of mechanical restriction of embryo expansion. In addition, the respiratory activity of seeds receiving these treatments was helpful to examine the hypotheses of restriction of oxygen uptake and presence of germination inhibitors.

If, as we suspected, a physical constraint imposed by the seed coat is involved in the inability of radicles to emerge, it makes sense to investigate the composition of the seed coat. Therefore, the composition of the seed coat and possible changes therein after drying and dry storage was analyzed by Fourier transform infrared microspectroscopy (FT-IR). Also, the plant hormone ethylene has been shown to be involved in the endogenous regulation of seed dormancy and germination (Kepczynsky and Kepczynska, 1997; Edelstein *et al.*, 2001; Ribeiro and Barros, 2004; 2006). Thus, this aspect has been investigated in the case of intact and scarified *L. microcarpa* seeds.

In this paper we report about the underlying mechanism of the seed coat-imposed restraint of germination and discuss the ecophysiological consequences of the observed germination behaviour of *L. microcarpa* seed.

Material and Methods

Plant material

Seeds of *Lannea microcarpa* Engl. & K. Krause were harvested from a natural stand in Ipélcé (Burkina Faso). Seeds were collected from fully mature (purple-black) fruits and were, at the same day, soaked in water and depulped at the Centre National de Semences Forestières (CNSF) in Ouagadougou. The seeds were rubbed with sand to remove the mesocarp tissue and washed with water. Subsequently, they were dried at

ambient conditions in the laboratory for 2-3h, and seeds surrounded by an intact endocarp were then selected and sent the next day to Wageningen (Netherlands), in cotton bags, arriving two days after harvest.

Moisture content and germination

Three replicates of five seeds were used to assess seed moisture content (MC) throughout the study. Seeds were weighed before and after drying at 103°C for 17h (ISTA, 1999), and their MCs were calculated on the basis of dried mass (DM) as g H_2O.g DM^{-1}. The MC during incubation was determined by temporary removal of the seeds from the germination medium and surface-drying with tissue paper before the fresh mass was weighed. From the increase in mass the actual MC was calculated.

For each single germination assay 25 seeds in duplicate (unless stated otherwise) were used. The seeds were sown on moist filter paper contained in transparent plastic boxes (11 cm x 17 cm x 5 cm) for germination and incubated at a constant temperature of 30°C in an incubator. Germinated seeds were scored at least twice a week until all viable seeds had germinated. Seeds were scored as germinated when the radicle had emerged to at least 2 mm. Soft and rotten seeds were considered as non-viable and removed from the boxes.

Mechanical scarification

Scarification consisted of making a small hole in the coat of seeds, at the axis side or opposite to that side, using a nail-clip or a razor blade. Great attention was paid to avoid damage to the embryo during the treatment, and seeds with visibly damaged embryos were discarded. Seeds were scarified prior to sowing or later during incubation. In the latter case, seeds were first incubated intact (non-scarified) at 30°C for 3, 6, 9, 12 or 15 days before being scarified at the axis-side and returned to the germination medium for further incubation at 30°C.

Electron Paramagnetic Resonance (EPR) spectroscopy

EPR spectra were recorded at room temperature with an X-band EPR spectrometer (Bruker, Rheinstetten, Germany, Model Elexsys 500). The water-soluble nitroxide radical, perdeuterated TEMPONE (PDT; kindly provided by Prof. I. Grigoriev, Institute of Organic Chemistry of the Russian Academy of Sciences, Novosibirsk, Russia), was used as the spin probe for testing the integrity of the plasma membranes of the cells in excised axes, according to the method described by Golovina and Tikhonov (1994).

The seeds were first decoated and rehydrated for 2 h or incubated intact at 30°C on moist filter paper for 15 d. After that, axes were isolated and incubated in 1 mM PDT + 120 mM potassium ferricyanide for at least 20 min. Because of spin label reduction after some time (if incubation periods were longer), the solution was renewed if the EPR signals became low. EPR spectra of PDT in the isolated axes were recorded at 0.25G modulation amplitude and 18 dB (3.17 mW) to increase the signal/noise without the necessity for scan accumulation. The spectra were recorded on individual axes, and the ratio of amplitudes of Water/Lipid (W/L) was calculated for each spectrum.

Respiration and ethylene measurements

Respiration of seeds was investigated using headspace analysis. The different seeds were allowed to respire in air-tight 7 mL-vials for about 5 h at a constant temperature of 30°C. In the case of seeds that were incubated in water for certain periods prior to respiration measurement, pieces of wet filter paper were placed at the bottom of the vials to avoid drying of the samples. After the appropriate accumulation times, 40-µL samples were injected into a F&M Dual model 700-00 gas chromatograph (GC) equipped with a catharometer detector as described by Leprince et and Hoekstra (1998), which allowed the O_2 uptake and CO_2 release to be measured simultaneously. The sensitivity of the GC detection was enhanced by an additional amplifier, connected to an electronic integrator (Spectra Physics, SP 4100). The carrier gas was helium. Calibration of the respiratory gases was performed with pure CO_2 and ambient air assuming a gas composition of the air at atmospheric pressure of 20.95 vol % O_2, 0.93 vol % Ar, and 78.09 vol % N_2. Accuracy of the quantification was enhanced by using the fact that the sum of the injected gases (CO_2, O_2, Argon and N_2) equalled 100%.
Concurrently to respiration analysis, samples of the headspace gas of intact and axis-side scarified seeds were taken from corresponding vials for ethylene production measurements. One-mL samples were taken and analyzed using a Shimadzu GC8A GC equipped with an alumina column and a flame ionization detector. Temperatures were 180 and 200°C for the oven and the detector, respectively. Ethylene peaks were registered by an integrator coupled to the GC and quantified by comparison with peaks of authentic ethylene standards.

2-Aminoethoxyvinylglycine (AVG) treatments

Five AVG concentrations (0, 0.1, 1, 5 and 10 mM) were used to investigate the effect of this molecule on ethylene biosynthesis via inhibition of ACC synthase and to get

details on a possible regulatory role of ethylene on *L. microcarpa* seed germination. Five individual seeds, scarified at axis-side, were used for each solution of AVG. Seeds were incubated at 30°C in Petri dishes containing filter paper soaked in one of the 5 AVG solutions. At daily intervals, the different seed batches were removed from the Petri dishes and individually put into air-tight 7-mL vials, where they were let to accumulate ethylene for about 2h. Subsequently, headspace gas samples were taken for ethylene analysis as described above.
To investigate the possible regulatory role of ethylene in seed germination, 25 seeds in duplicate were used per concentration of AVG. Seeds were sown in transparent plastic boxes containing filter papers moistened with one of the 5 above- mentioned AVG solutions and incubated at a constant temperature of 30°C. Germination of the different seed batches was assessed as described above.

Fourier transform infrared microspectroscopy (FT-IR)

In situ Fourier transform infrared microspectroscopy (FTIR) was used to analyze the main components of the seed coat and possible changes in these components after drying and dry storage of the seeds. Transmission FT-IR spectra were recorded at room temperature on a Perkin-Elmer 1725 IR-spectrometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK), equipped with a narrow band, liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin-Elmer microscope. The optical bench was purged with dry CO_2-free air (Balston, Maidstone, UK). The acquisition parameters were 4 cm^{-1} resolution, 32 co-added interferograms, 2 cm^{-1} moving mirror speed, and 3,600 to 800 cm^{-1} wavenumber range. Spectral analysis and display were carried out using Spectrum version 2.00 (Perkin Elmer).
Material scratched by scalpel from the outer or inner side of the seed coat was placed between two diamond windows that were mounted into a temperature-controlled holder. The sample was pressurized until sufficient transparency was obtained. To determine the components of the seed coat, recorded spectra were compared with spectra of pure lignin (Organosolv, from Sigma) and cellulose (fibrous, from Sigma) – constituents of seed coats –, and of the natural reference materials cotton (almost entirely cellulose) and kapok (a mixture of cellulose, lignin and hemicellulose) fibers.

Results

Germination pattern and viability

Figure 1A shows that on incubation, the intact air-dried seeds absorbed water, but less than the scarified seeds. The water uptake of the intact seeds levelled off after 3 d of incubation, whereas that of the scarified seeds continued to increase, likely as the result of cell expansion associated with seedling emergence. Intact seeds did not germinate during the two weeks that the incubation for testing germination lasted (Fig.1B).

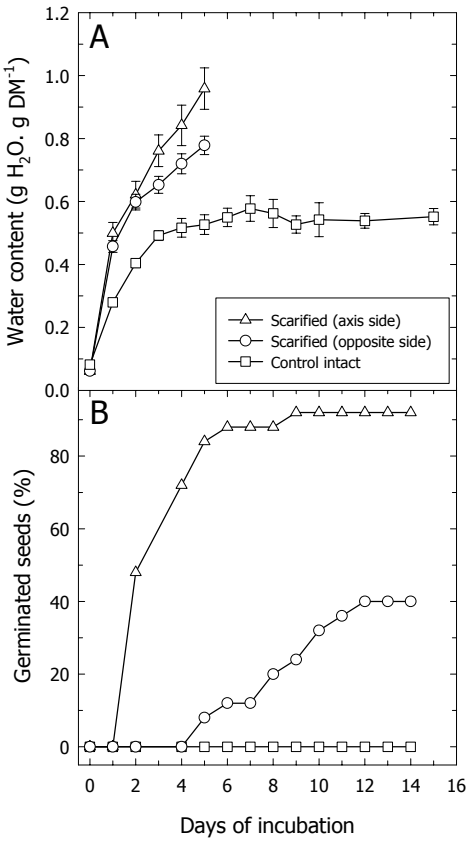

Figure 1. Effect of scarification on water uptake (A) and germination (B) of *L. microcarpa* seeds incubated on moist filter paper. Air-dried seeds of a few weeks old were either scarified at the axis-side or at the side opposite to the axis, or left untreated. Scarification was carried out directly prior to incubation at 30°C. Each point is the average water content of 10 individual seeds ± *SE* (A) or the average germination percentage of 2 batches of 25 seeds each (B).

In contrast, radicles of the seeds that were scarified at their axis-side emerged already on the second day of incubation in the germination test, and a high germination percentage was reached after 5d. For practical reasons the effect of soaking in H_2SO_4 (concentration: 98%) was tested as an alternative of mechanical scarification. Maximum improvement of germination to 48% was obtained after 30 min of soaking, after which the harmful effect of the acid overruled the beneficial effect of the treatment.

The cellular viability of those seeds that failed to germinate was analysed by spin label technique. Briefly, the axes were brought in an aqueous solution of spin label (PDT) and broadening agent (ferricyanide ions). By measuring the relative heights of the peaks in the EPR spectra, which are caused by the spin label in aqueous cytoplasm and lipid bodies, an estimate can be obtained about the extent to which cells still have intact plasma membranes. In leaky cells the aqueous cytoplasmic signal will be wiped out because of the broadening effect of ferricyanide ions that have entered the cells through damaged membranes, whereas the lipid signal remains untouched. Figure 2 shows an example of a spectrum of PDT in cells of an axis imbibed for 2h in water, having 100% viable cells. The spectrum contained three superimposed components:

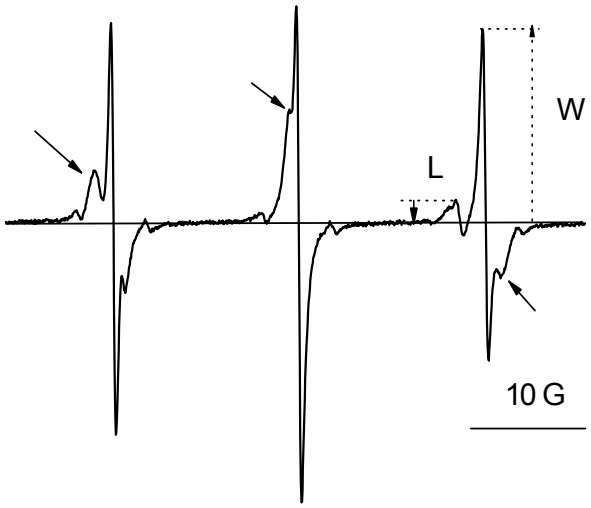

Figure 2. Representative EPR spectrum of PDT in cells of an axis excised from a *L. microcarpa* decoated seed that was allowed to imbibe for 2h (the horizontal line represents the base line). The spectrum contains 3 superimposed components. Determination of the heights of the lines representing aqueous cytoplasm (W) and lipid bodies (L) is as indicated in the figure. The origin of the 3rd component (indicated by arrows) is not clear and left out from further considerations. The ratio W/L can be used as an indicator of cellular integrity.

one from aqueous cytoplasm (W) and another one from lipid bodies (L), as described previously (Golovina and Tikhonov, 1994; Golovina *et al*. 1997; Neya *et al*., 2004b). The origin of the 3rd component (indicated by arrows) is not clear and will not be considered further, but for the sake of avoiding overlap with the 3rd component the upper part of the high field (right-hand side) water line was used to estimate the aqueous component of the spectra. Thus, axes with low W/L ratios are indicative of cells that are essentially non-viable, whereas those with high values contain viable cells.

Figure 3 shows the distribution of W/L values of individual axes isolated from decoated, 2h rehydrated seeds and from intact seeds after 15d of moist incubation at 30°C. Axes

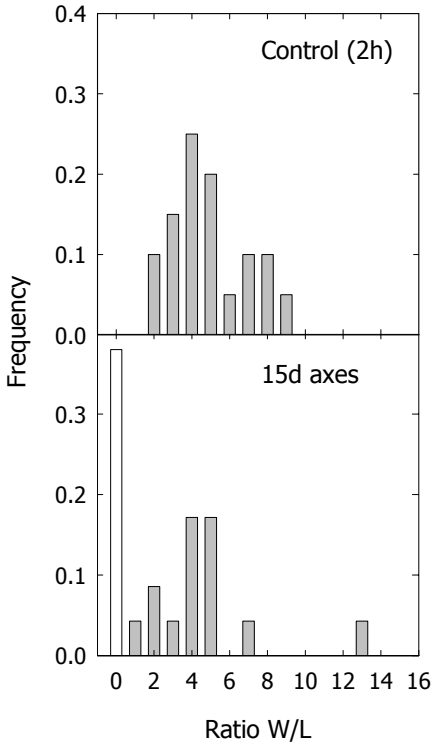

Figure 3. Distribution of the W/L ratios calculated from the EPR spectra of PDT in individual axes from *L. microcarpa* seeds. Axes were isolated from decoated seeds that were rehydrated at 30°C for 2 h (n=20) or from intact seeds after 15 days of incubation at 30°C (n=50). The 38% of 15-d moist-incubated axes, which lost firmness and did not stain in tetrazolium chloride are assumed to have a W/L value of 0 (no real EPR measurement; white column).

from the 2h incubated seeds had a distribution of W/L values ranging from 2-9, whereas those from the 15-d incubated seeds had a distribution that was slightly shifted to lower W/L values. Thirty eight percent of the latter lost firmness and did not stain in tetrazolium chloride. For completeness this fraction was assumed to have a W/L value of 0. The remaining firm axes were clearly on the way to viability loss. One seed had germinated. This analysis shows that the seed coat-imposed inhibition of germination does not prolong cellular viability of seed axes in the hydrated state.
Analysis of the gas-exchange rates might shed some light on the possible lack of oxygen and the rate of metabolism during prolonged incubation.

Respiratory activity during drying of fresh seeds and rehydration of air-dried seeds

If the permeability of the seed coat for water or respiratory gases would change during drying of the seeds or dry storage, it could be expected that respiration characteristics change. Figure 4A shows the O_2 uptake and CO_2 evolution during drying of freshly depulped seeds from approximately 0.4 g H_2O.g DM^{-1} to the air-dried state. Respiratory activity decreased with the loss of water to reach immeasurably low values around 0.1 g H_2O.g DM^{-1}. The respiratory quotient (RQ) was around 0.7, a value that fits values found for other oily seeds. On drying there was no indication of an unbalanced respiration caused by a possibly impeded O_2 uptake. On rehydration of the air-dried seeds, during the first two d, respiration increased to values similar to those of the freshly depulped seeds at comparable water contents. Also, RQ values returned to approximately the same values. This indicates that the seeds were able to recruit sufficient O_2 to avoid fermentative metabolism, at least during the first two d of rehydration.

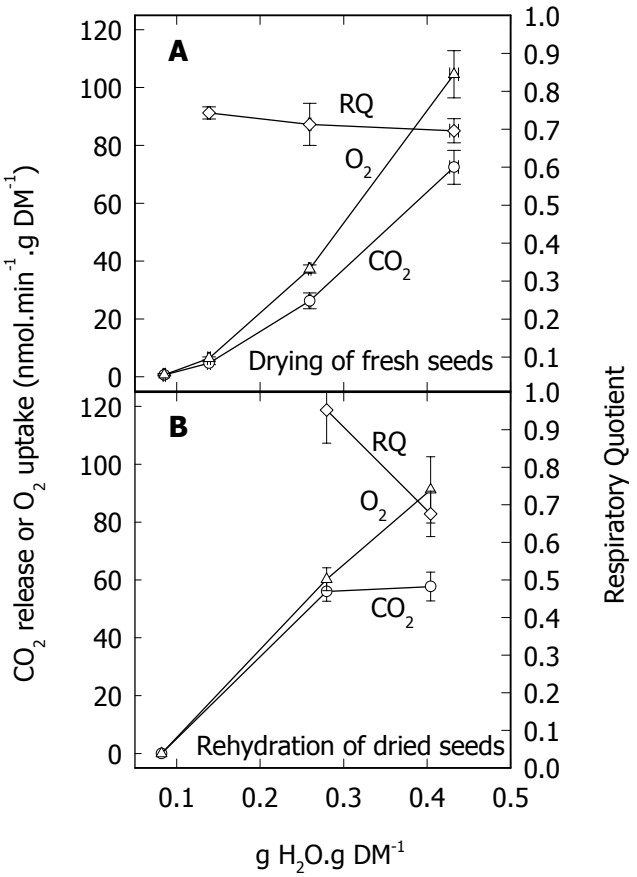

Figure 4. Effects of drying of freshly depulped (A) and rehydration of intact, air-dried (B) seeds of *L. microcarpa* on respiratory gas exchange and RQ. Respiration of the seeds was analyzed by GC at 30°C in 7-mL flasks. Conditions for the depulped seeds (A) were 8d total drying time, 5 seeds per flask (n=4) ±*SE* and for the rehydrating seeds (B) were 2d rehydration time, 1 seed per flask (n=6) ±*SE.*

Effect of scarification on seed respiratory metabolism

As scarified seeds were able to germinate and intact, non-scarified seeds were not, it can be expected that respiration starts to diverge as soon as cell expansion occurs in preparation for emergence of the radicle. Figure 5A shows that even one d after the start of imbibition the gas exchange rate from the seeds scarified at the axis side was already higher than that from the intact seeds and continued to increase during radicle

emergence. The respiratory activity of the non-germinating intact seeds remained constant during days 1 through 6 and then slightly increased. The latter increase might be linked with the decay of some of these seeds.

More insight in the restraining role of the seed coat was obtained by scarification at the side opposite to the axis. Thus, oxygen and water have free access to the embryo and possible endogenous germination inhibitors can leak out. Figure 1A shows that the water uptake was higher than in the case of intact seeds, but difficulties with germination remained. Only approximately 40% of the seeds displayed radicle protrusion after some delay (Fig. 1B). This germination was abnormal in the sense that the radicle appeared through the hole made at the opposite side. Because gas exchange measurements were performed on individual seeds, categories of non-germinating and germinating seeds could be tested for respiratory activity. From Fig. 5B it is clear that the upsurge of respiration coincides with the emergence of the radicle. On the basis of these data we conclude that mechanical constraints established by the seed coat impede germination of *L. microcarpa* seeds.

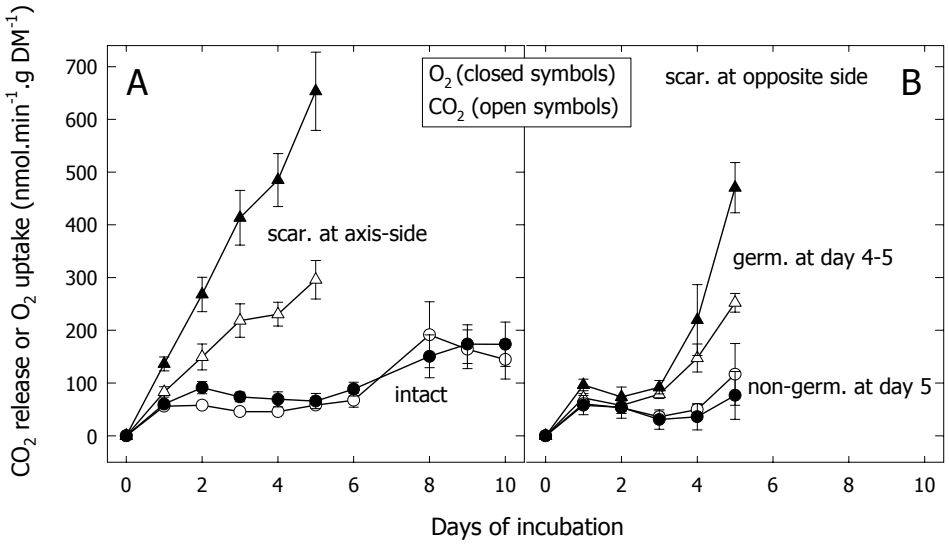

Figure 5. Effect of scarification on respiratory gas exchange from rehydrated *L. microcarpa* seeds at 30°C. (A) Seeds were intact and did not germinate or were scarified at the axis side, which allowed for emergence of the radicle from day 2 on; points are the average (±*SE*) of measurements of 6-10 individual seeds. (B) Seeds were scarified at the opposite side of the axis and categorized according to whether they displayed radicle emergence at day 4-5 (n=6) or lacked signs of outgrowth at day 5 (n=4).

Loss of germination ability of intact seeds during prolonged incubation

Upon rehydration, air-dried, intact seeds did not germinate within the next 15 d of moist incubation at 30°C (shown earlier in Fig. 1). In addition, the test for cellular viability in Fig. 3 showed that part of the embryos underwent considerable decay and that the remaining firm embryos consisted of cells having reduced cellular integrity. By scarification at intervals during incubation, information was obtained about the germination capacity of the seeds. Figure 6 shows that germinability gradually

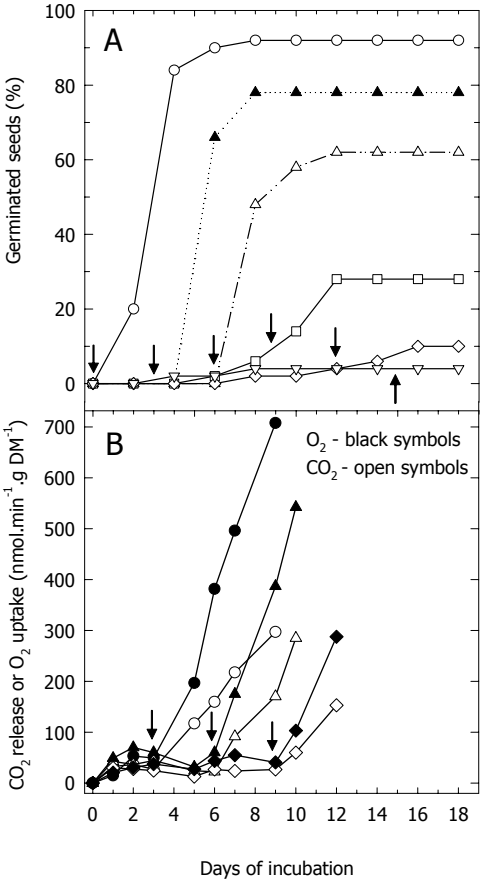

Figure 6. Effect of delayed scarification on the ability of intact seeds of *L. microcarpa*, incubated on moist filter paper at 30°C, to resume radicle emergence (A) and metabolic activity (B). The seeds in (A) were scarified at their axis side at t= 0, 3, 6, 9, 12, and 15 days after the start of incubation, and each point represent the average of 2x 25 seeds. The seeds in (B) were scarified at their axis side at t= 3, 6, and 9 days, and each point is the average value of 5 individual seeds.

decreased until day 15 when scarification did not anymore lead to radicle protrusion. Scarification at day 3, 6 and 9 led to an upsurge in respiration, but with decreasing intensity.

Possible role of ethylene in the regulation of seed germination

The possibility of ethylene being involved in the lack of germination and decay of incubated intact seed was investigated. Figure 7 shows the evolution of ethylene from rehydrating intact and scarified seeds. After a peak in the production on day one in both samples, the production fell to low values, particularly for the intact seeds after day 3. The intact controls tended to also have a high production at day 2, which might be explained by slower ethylene release from seeds having an intact coat. The data nevertheless show that the seed coat is permeable to ethylene. The production by scarified seeds remained approximately 0.5 $nL.h^{-1}.g\ DM^{-1}$ at day 4 and 5, which could be associated with radicle growth occurring at the same time.

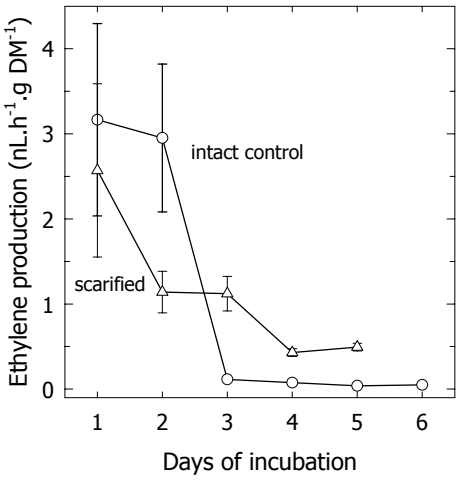

Figure 7. Ethylene biosynthesis by intact and scarified (axis-side) *L. microcarpa* seeds upon incubation at 30°C. Each point is the average value of the ethylene production of 10 individual seeds ±*SE.* The ethylene was allowed to accumulate for approx. 9h in the headspace of 7-mL flasks.

The effect of the ACC-synthase inhibitor amino-ethoxyvinylglycine (AVG) on ethylene evolution was investigated to test whether the ethylene evolution was the result of the conversion of pre-existing amino-cyclopropane carboxylic acid (ACC)

into ethylene or *de novo* synthesised ACC. To this end scarified seeds were incubated in solutions of a range of AVG concentrations.

Figure 8A shows that incubation in AVG solutions >1 mM during the first 4h of incubation almost completely inhibited ethylene production. From these data it emerges that *de novo* synthesis of ACC is involved in the ethylene evolution from the seeds upon rehydration and not the conversion of pre-existing ACC. Furthermore, AVG concentrations > 1 mM had a negative effect on the percentage germinated seeds (Fig. 8B). The much higher value of ethylene production of the control in Fig. 8A as compared to that of Fig. 7 stems from the shorter accumulation time in the former, which allows the early peak in ethylene production to be more accurately measured.

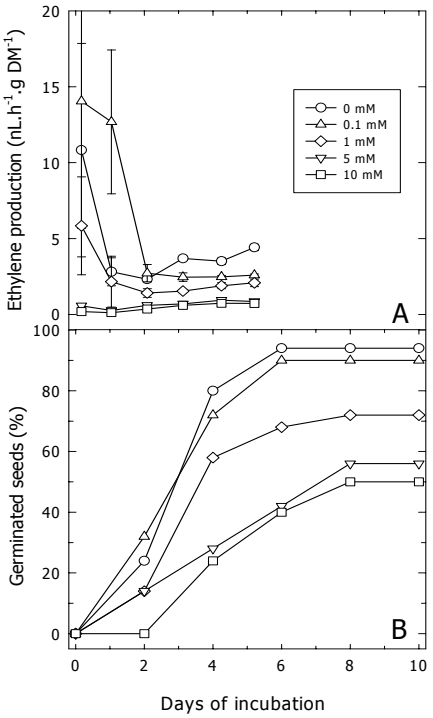

Figure 8. Effect of amino-ethoxyvinylglycine (AVG) on ethylene biosynthesis (A) and percentage germination (B) of *L. microcarpa* seeds upon incubation at 30°C. All seeds were scarified at their axis side prior to rehydration in the AVG solutions. Each point in (A) is the average ethylene production of 4 individual seeds ±*SE.* Ethylene was allowed to accumulate for 1.5-2.5h. Each point in (B) is the average percentage germination of 2x25 seeds.

Seed coat composition

Germination has been shown to decrease during drying of the seeds and subsequent dry storage in a variable manner (Chapter 4). Since physical constraint imposed by the seed coat appears the main factor in the inability of the radicles to emerge, the composition and possible changes therein after drying and dry storage was analyzed by FT-IR. Figure 9A shows the IR-spectrum of material scraped from the inner side of the seed coat. Using FT-IR, the main biological components can be determined on the basis of specific IR-absorption brought about by their unique molecular features. Thus, the lack of major contributions at 2853 cm^{-1} (symmetric CH_2 stretching vibration; spectral region not shown) and 1650/1540 cm^{-1} (C=O stretch/N–H bending) is evidence of the absence of lipids and proteins, respectively. However, the spectrum revealed a number of IR-absorption peaks that can be linked to structural carbohydrates and lignin (Yu *et al.*, 2005). Peaks were found at 1050, 1159, 1243, 1332, 1373, 1424, 1461, 1505, 1595, and 1737 cm^{-1}.

The carbonyl C=O stretching absorbance at 1737 cm^{-1} refers to the ester bonds of the hemicelluloses, lignins, and cellulose , and the absorbance at 1243 cm^{-1} points to the presence of hemicelluloses (Yu *et al.*, 2004, 2005). These peaks are absent in the spectra of authentic cellulose or cotton fibers (spectra not shown). The peaks at 1595, 1505 and 1461 cm^{-1} are associated with aromatic ring vibrations in lignin molecules (Dorado *et al.*, 2001; Sun *et al.*, 2004), whereas the main peak at 1050 cm^{-1}, associated with C–O stretching, and another at 1159 cm^{-1} can be assigned mainly to cellulose (Ilharco *et al.*, 1997; Sun *et al.*, 2004). The other peaks may be the result of both lignin and cellulose, as these compounds have overlapping peaks in this wavenumber region (Dorado *et al.*, 2001).

For comparison, the spectrum of kapok fibers is shown in Fig. 9A, which resembles the spectrum of the inner seed coat of *L. microcarpa* to a large extent. Kapok has a composition of approximately, 43% cellulose, 13-15% lignin and 32% hemicellulose (Prosea, 2003). From this similarity, it is assumed that the seed coat of *L. microcarpa* has approximately the same composition. Figure 9B shows the spectra of material from the outside of the seed coat, isolated from fresh seeds (0.43 g H_2O.g DM^{-1}) and seeds after 10 months of dry storage (0.04 g H_2O.g DM^{-1}). There was no large difference in peak position and area under the peaks, as evidenced by an almost flat difference spectrum.

FT-IR analysis of 2 or 3 specimens from 3 seeds each scraped from the outside of coats indicated that there was no significant difference in peak position between fresh and 10-months-old seeds (data not shown). The same held true for material scraped from the inside of seed coats (data not shown). From this it can be concluded that the

increased toughness of the seed coat with drying and dry storage is not reflected by a shift in the composition of structural carbohydrates.

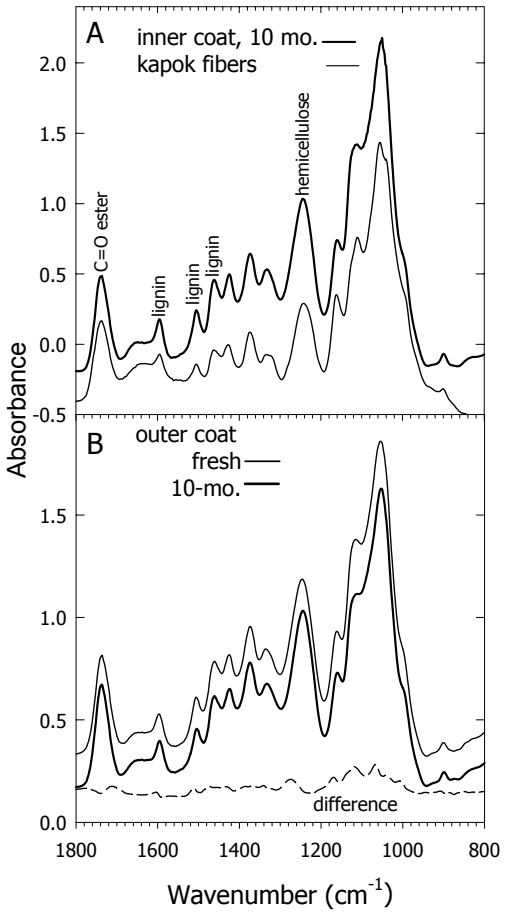

Figure 9. Fourier transform IR spectra of material scraped from coats of *L. microcarpa* seeds. (A) Material from the inner side of the coat of a 10-months old air-dried seed (0% germination), with dry kapok fibers shown for comparison. The position of peaks associated with the carbonyl C=O stretching vibration (1737 cm^{-1}), the lignin-specific aromatic ring vibrations (1461, 1505, 1595 cm^{-1}) and hemicellulose (1243 cm^{-1}) are indicated. (B) Comparison of material from the outer coat of fresh (16% germination) and 10-months old seeds, with the difference spectrum shown at the bottom.

Figure 10 shows that not all coats from different seed species have the same composition. Thus, the IR-spectrum of the *Khaya senegalensis* seed coat differed considerably from that of the *L. microcarpa* seed coat in that hemicelluloses were low and lignins were almost absent. Growing radicles of *K. senegalensis* always easily break through the seed coat (Neya, personal observation). However, neem (*Azidarachta indica*) seed, whose radicle emergence benefits from seed coat removal (Neya, unpublished data), exhibited an IR spectrum of the coat suggesting a composition almost identical to that of the *L. microcarpa* seed coat.

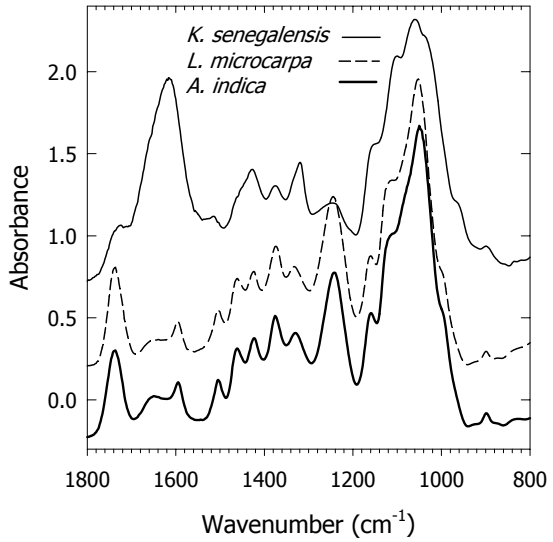

Figure 10. Fourier transform IR spectra of material scraped from the outer coats of seeds of *Azadirachta indica* and *Khaya senegalensis*, with that of *L. microcarpa* as a reference.

Discussion

Seed coat impedes radicle protrusion

Dormancy has been suggested to occur in *L. microcarpa* seeds (Pritchard *et al.*, 2004a). We have found that this dormancy is seed coat-imposed (Chapter 4). Yet, it was not known how the seed coat inhibits germination. The data in Fig. 1A showed that both intact and scarified, air-dried seeds promptly absorbed water upon rehydration, which excludes coat impermeability as a possible mechanism of dormancy. In many seeds that readily imbibe water but require scarification or smoke for germination, the embryo has the capacity to germinate, the constraint being one or more tissues that surround the embryo (Bewley and Black, 1994; Adkins *et al.*, 2002). Germination capability of *L. microcarpa* seed was not only related to whether or not seeds were mechanically scarified prior to incubation, but also to the scarification site. Indeed, more insight in the restraining role of the seed coat was obtained by the result of scarification at the side opposite to the axis. Seeds scarified at the axis-side germinated faster and at a more than twice higher percentage than seeds scarified at the opposite side (Fig. 1B). Moreover, germination of the latter seeds was abnormal in the sense that the radicles protruded backwards through the hole made at the opposite side of the axis. This indicates that the restrained radicle protrusion may be essentially due to mechanical restriction imposed by the seed coat.

Germination capacity of intact seeds incubated at 30°C was tested by scarifying them at different periods after the onset of incubation (Fig. 6A). Germination fell to almost zero when the seeds were scarified at day 15. The issue of cellular integrity of these seeds was addressed using spin label technique. Apart from 38% soft, obviously rotten seeds after these 15 d of incubation at 30°C, the remaining seeds were still firm, but the cellular integrity of their axes was reduced in comparison with that of the axes of 2-h imbibed seeds (Fig. 3). This can be interpreted to mean that the cells from the 15-d incubated seeds were clearly on the way to viability loss. These results allow us to conclude that the seed coat-imposed constraint of germination does not prolong viability of *L. microcarpa* seeds in the hydrated state. How this can be interpreted in an ecological context is discussed later. Whether an obstruction of the exchange of respiratory gases is involved in the early death of the incubated seeds is discussed next.

Respiratory gases exchange and ethylene evolution

Seed coats have been reported to impede respiratory gas exchange, for example in pea seeds early during rehydration, prior to the penetration of the seed coat by the radicle (Wager, 1974; Rolletschek *et al*., 2002). This leads to a lack of oxygen for normal respiratory metabolism. In an attempt to compensate for the lack of ATP via oxidative phosphorylation, glycolysis is increased leading to fermentation (Pasteur Effect). The end products of such fermentation may be damaging to the seed, although not in the case of pea. Diagnostic for the lack of sufficient oxygen in a seed is a high CO_2/O_2 ratio (RQ), reaching values considerably above 1. From the low RQ values (<1) in Fig. 4 it can be deduced that there is sufficient oxygen available during rehydration of intact seeds. Since the germination ability of fresh seeds is usually better than those of seeds after drying and/or dry storage (Chapter 4), it is expected that if problems with the permeability would arise, this should occur in the rehydrating seeds. Moreover, the rates of gas exchange were almost the same at similar water contents in both, dehydrating fresh seeds and rehydrating seeds (Fig. 4). Scarified seeds attained much higher rates of respiration than the non-scarified seeds (Fig. 5A) and the seeds scarified at the opposite side to the axis (Fig. 5B). In the case of restriction of oxygen permeation through intact seed coats, we would expect the latter seeds to display a much higher rate of respiration, the more so as the water uptake was almost that of the axis-side scarified seeds (Fig. 1A). High respiratory activity appeared to be closely associated with radicle protrusion (Fig. 5A and B). This is comparable with the results of Derkx *et al*. (1993), who found that in seeds of *Sisymbrium officinale* dormancy breaking does not cause an upsurge in O_2 uptake, but germination does. The above results allow us to conclude that the seed coat does not impede respiratory gas exchange in *L. microcarpa* seed during both dehydration and rehydration.

A peak in ethylene production was noticed early after the onset of incubation in both intact and scarified seeds, after which the production fell to low values (Fig. 7). This demonstrates that the seed coat is permeable to apolar gases like ethylene. Also, the ethylene is not involved in the slow decay of the hydrated, intact seeds, because radicle emergence could be evoked by delayed scarification of the incubated, intact seeds (Fig. 6A), long after the ethylene had dissipated. The inhibition of the ethylene production by AVG (Fig. 8A) suggests that the ethylene is produced via *de novo* produced ACC and not by conversion of pre-existing ACC. Some involvement of ethylene in radicle emergence and growth might be deduced from the reduction of the germination percentage by AVG (Fig. 8B).

Ecological consequences of *L. microcarpa* seed germination behaviour

Dormancy is the strategy to postpone germination until a time that is more favourable for seedling establishment. In that respect, the loss of viability in the hydrated state (Figs. 3 and 6A) within 15 days at 30°C is clearly not the strategy followed in nature, but an artefact of forestry practice. Seedling establishment in Burkina Faso is often observed at the beginning of the rainy season, when the fruits mature (Arbonnier, 2002). To minimalize weight losses as a result of respiration, fresh seeds probably remain hydrated only for a short time and survive in the dry state, at least until the next rainy season. We found that the seeds can withstand an additional cycle of drying after rehydration without loss of viability, although scarification was still necessary to let them germinate. This is an indication that successive cycles of drying and rehydration may play a key role in the natural survival of *L. microcarpa* seeds. Successive drying cycles might also lead to a weakening of the seed coat, apart from weakening by gut passage, fluctuations in temperature and humidity, abrasion by soil, and the actions of micro-organisms. The CO_2 production of approx. 60 $nmol.min^{-1}.g\ DM^{-1}$ at 30°C by hydrated, intact seeds (Fig. 5) leads to a (calculated) weight loss of approx 0.3% of the DM per day. This metabolic rate is not so high in comparison with that of hydrated deeply dormant *Sisymbrium officinale* seeds. These seeds from the temperate climate zone have a CO_2 production of approx 38 $nmol.min^{-1}.g\ DM^{-1}$ (normalized to 30°C), when fully hydrated (cf Derkx *et al.*, 1993). Survival of *S. officinale* may be > 10y under natural conditions in the soil. To bridge such a period and not to lose too much weight by metabolic activity, the average temperature must be considerably lower than 30°C and the MC considerably lower than at full hydration. With comparable metabolic rates in the non-germinating condition, *L. microcarpa* seeds lose germinability after 15 d at 30°C, which clearly shows that these seeds lack the ability to avoid lethal damage. This fast decay of *L. microcarpa* seeds resembles the effects of an accelerated ageing treatment and might point to inadequate repair and/or antioxidant status (Benamar *et al.*, 2003; Goel *et al.*, 2003; Calucci *et al.*, 2004).

The importance of seed coat composition in the control of germination

The seed coat is the outer cover of every mature seed. The seed coat is therefore the main intermediary between the internal structures of a seed and the external environment (Dubbern de Souza and Marcos-Filho, 2001). It is obvious that the properties and functionality of a given seed coat are influenced by composition. In contrast to the wealth of research done on the anatomy of seed coats, little has been

done on seed coat compositions and the impact on germination. FT-IR spectroscopy is generally used for the analysis of wood and cell wall components. We assume that FT-IR analysis of seed coat reveals most of its components. From our current FT-IR spectra we cannot ascribe the reduction in *L. microcarpa* seed germination upon drying or dry storage to changes in seed coat composition (Fig. 9), although we cannot completely rule out the involvement of other components lacking IR-absorbing molecular groups. It is a known phenomenon that tensile strength of cellulose fibers may irreversibly change during water loss (Derbyshire *et al.*, 1996; Turkulin *et al.*, 2004). Because such changes in tensile strength cannot easily be observed by FT-IR, we suggest that structural changes in seed coat components with drying could be involved in the reduced germinability. Considering the differences and resemblances between species in terms of their seed coat composition (Fig. 10) and the key role played by the seed coat in germination, investigation of the main components of the coat may be of great importance when comparing the seed physiology of different species.

Acknowledgements
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Chapter 6

Ageing related changes in membranes composition, structure and integrity of *Khaya senegalensis* seeds

Oblé Neya, Elena A. Golovina, Jaap Nijsse and Folkert A. Hoekstra

Abstract

Khaya senegalensis is a multipurpose tropical tree species with documented difficult seed storage behaviour. This study was undertaken to reveal the causes of this behaviour. Freshly harvested seeds had low water content. Further drying to 0.04g H_2O g DM^{-1} did not affect the initially high germinability (90-100%), indicating that the seeds display orthodox storage behaviour. While dry, intact seeds were completely insensitive to low imbibition temperatures, decoated seeds were moderately sensitive to rehydration ≤ 10°C

Storage at 4°C was more detrimental to seeds than storage at -20°C or 15°C. Viability loss at 4°C storage coincided with the loss of approx. 25% of the membrane polar lipids (PLs). PLs steadily decreased and free fatty acids accumulated with natural ageing at 15°C. The critical loss of PLs was between 15% and 25%. PL losses were approx 80% in 20-years old seeds that were already non-germinable for, probably, 15 years. High resolution SEM images after freeze fracturing of these dry old seeds nevertheless showed intact membranes with regularly distributed intra membrane particles. This indicates that a membrane phase separation, thought to occur during viability loss, is unlikely to happen in the dry state. However, upon rehydration, membrane structure was lost.

As phospholipases can act in dry membranes and be more active during a phase transition, the phase behaviour of membranes in dry axes was investigated. Using ESR spin label technique it was found that membranes in dried and rehydrated axes were rigid at −20°C, in the liquid crystalline phase at 15°C, and in mid-melting condition at 7°C. This might explain the faster decay of seeds at low above zero (°C) temperatures assuming that phospholipases are involved in the deesterification of the PLs. Neutral lipids had an average T_m of −10.4°C, which rules out their involvement in the fast viability loss at 4°C. We suggest that the low above zero (°C) anomaly in the storage behaviour of tropical seeds is caused by phospholipases being active at relatively high mid melting temperatures of the membranes.

Introduction

Khaya senegalensis is a multipurpose tropical tree species, the wood of which was probably the first African mahogany to be exported from West Africa (from Gambia particularly) on a commercial scale in the early part of the nineteenth century. It has been (and still is) heavily exploited for this trade as also for its other numerous local uses with the result that it has become scarce in places, e.g. Burkina Faso, Ivory Coast (West Africa in general). The seed is not dormant and normally germinates freely, but regeneration is frequently poor due to depredation by insects. Nursery propagation via seeds and transplanting of seedlings is the usual practice.

In contrast to the various reports on the phytopathological and phyto-pharmaceutical properties of its by-products (Govindachari and Krishna-Kumari, 1998; Khalid *et al.*, 1998; Kayser and Abreu, 2001; El-Aswad *et al.*, 2003; Bamaiyi and Bolanta, 2006), few investigations have been conducted on *K. senegalensis* seed biology and conservation. Hong and Ellis (1998) have described the seed to display intermediate storage behaviour. The seeds can be dried to moisture contents (MC) as low as 5%, but are sensitive to cold temperatures, which prevents long term storage. Recently, Gaméné and Eriksen (2004) reported orthodox storage behaviour for these seeds. However, in practice some difficulties resulting in sporadic and fast losses of viability have occurred even upon dry storage at low temperatures (≤5°C) (CNSF, unpublished data).

Seed longevity during storage is strongly dependent on water content and temperature. Although under dry storage conditions longevity is enhanced, eventually seeds deteriorate and lose the capability to germinate. For tropical recalcitrant or intermediate seeds, low above-zero temperature storage is often not a good option. The difficulty in storing seeds at these temperatures is due to their chilling sensitivity, which is thought to be caused by the relatively high gel-to-liquid crystalline phase transition temperature (T_m) of their membranes (Crowe *et al.*, 1989b, Sacandé *et al.*, 2001). In dry neem seed, also the extreme sensitivity to low rehydration temperatures has been argued to depend on the intrinsically high T_m of the plasma membranes (Sacandé *et al.*, 2001).

Seed deterioration involves many biochemical and biophysical changes, including the loss of enzymatic activities and membrane integrity (Stewart and Bewley, 1980; Priestley and Leopold, 1983; Ferguson *et al.*, 1990). Lipid peroxidation and the associated free radical oxidative stresses are widely considered to be major contributors to seed deterioration (Priestley, 1986; Wilson and McDonald, 1986; Hendry, 1993). There are many types of peroxidative reactions, in which lipids serve as substrates. Among the unsaturated acyl chains, the polyunsaturated ones are preferentially degraded, leading to the accumulation of toxic products (Chan, 1987). On

the other hand, a commonly observed phenomenon is the breakage of the ester linkage between the acyl chain and the glycerol backbone (McKersie *et al.*, 1988), which leads to the accumulation of free fatty acids and lysolipids (Van Bilsen *et al.* 1993, 1994). This deesterification can be caused by free radical activity (Niehaus, 1978; McKersie *et al.*, 1988) or the activity of phospholipases (Salama and Pearce, 1993). The observation that phospholipases also can be active at low water contents (approx 10%; Oliver *et al.*, 1997) makes it possible that these enzymes play a role in the deesterification of acyl chains in dried seeds. Both neutral and polar lipids are subject to these reactions, and if these reactions occur, the chemistry of the lipid components of the seeds is likely to change (Vertucci, 1992). Although polar lipids constitute a major component of membranes, it is not clear whether membrane damage is a primary or secondary effect of seed deterioration. Nevertheless, viability loss is often attributed to the loss of membrane integrity (Roberts, 1972 Maguire, 1977; Bewley and Black, 1994; Golovina *et al.*, 1997). Therefore, the analysis of membrane composition, structure, and integrity during ageing of seeds constitutes a widely used tool to study changes associated with viability loss (Zacheo *et al.*, 1998; 2000; Murthy *et al.*, 2003). Reports on the biochemical and biophysical changes in membranes of tropical tree seeds are almost nonexistent, in contrast to those of seeds of crop species (Sung and Chiu, 1995; Zacheo *et al.*, 1998; 2000; Tammela *et al.*, 2000; Goel and Sheoran, 2003; Murthy *et al.*, 2003).

The current study was conducted to investigate the fate of *Khaya senegalensis* seeds during short-term storage at different temperatures and long-term storage at 15°C. In this paper we report on the changes in membrane physical structure associated with ageing and viability loss. In addition, the quantitative and compositional changes in polar lipids and free fatty acids were studied. Finally, we discuss the possible role of phospholipases in membrane degradation during storage at low water contents and low, above zero °C temperatures.

Material and methods

Seed material and germination

Seeds of *Khaya senegalensis* (Desr.) A. Juss. were collected from several stands in Burkina Faso. They were processed from mature, fresh fruits (still closed) or mature, dried fruits (open and ready for natural dispersal). For "natural" ageing studies, seeds

prepared from mature, dried fruits of six different accessions collected in 2004, 2003, 2002, 2001, 1990 and 1985 were stored at 15°C for 1, 2, 3, 4, 15 and 20 years, respectively.
Four replicates of 25 seeds were used for each single germination assay. Intact seeds were sown on moist filter paper contained in plastic boxes (25 seeds per box) and incubated at a constant temperature of 30°C. Germinated seeds were scored at least twice a week until all viable seeds had germinated. A seed was considered as germinated when the radicle had protruded 2 cm without any infection.

Moisture content and water activity

Moisture contents (MC) of seeds (including the endocarp) were determined gravimetrically using the oven-drying method. The seeds were weighed before and after drying at 103°C for 17h (ISTA, 1999) and the MC was expressed proportionally to seed dry mass (DM) as g H_2O.g DM^{-1}.
Water activity (Aw) or equilibrium relative humidy of a seed reflects the active part of moisture content or the part which, under normal circumstances, can be exchanged between the seed and its environment (Chapter 4). The relationship between MC and Aw of seeds was established using seeds excised from both mature, fresh and mature, dried fruits. After equilibrium of seed MC under conditions of three different RHs obtained in closed drums with circulating air above saturated salt solutions ($MgCl_2$ for 33%, $Ca(NO_3)_2$ for 55% and NaCl for 75%), the Aw of 4 replicates of 5 seeds (per seed lot) was measured using a water activity meter (HygroLab3, Rotronic, Germany), followed immediately by the assessment of the respective MCs by the oven method as described above.

Storage trials

Seeds processed from mature, fresh and mature, dried fruits were used to investigate the effect of relative humidy (RH) on seed storability. Each seed lot was subdivided in samples that were placed at three different RHs at a constant temperature of 20±2°C. The three RHs were obtained in closed drums with circulating air above saturated salt solutions ($MgCl_2$ for 33%, $Ca(NO_3)_2$ for 55% and NaCl for 75%). The effect of storage environment on germination capability was assessed after 3, 12 and 24 weeks of exposure to the above RHs.
To investigate the effect of storage temperature, seeds processed from mature, dried fruits with an MC of approx. 0.04g H_2O.g DM^{-1} were packed in aluminium bags closed by folding the open end and using staples to fix the folds. These packed samples were

stored at 4 different temperatures (-20, 4, 15 and 20°C), and the germination capability of the different samples was monitored at several time intervals over 30 months of storage. After 30 months of storage, biochemical and biophysical changes in the different batches associated with the loss of viability were assessed.

Electrolyte leakage

Embryonic axes were isolated from dry seeds. Conductivity measurements were performed at room temperature (≈20°C) on 25 individual axes per seed batch, using a CM100 Conductivity Meter (Reid & Associates CC, Durban, South Africa). The conductivity data were expressed as μS.mg DM^{-1} of axis in 1.5 mL of deionized water.

Low Temperature Scanning Electron Microscopy (LTSEM)

To prepare samples for high-resolution microscopy of membrane surfaces, excised embryos were mounted onto specimen holders using a drop of carbon conducting cement (Leit-C; Neubauer Chemicalien, Münster, Germany) and immediately plunge-frozen in liquid nitrogen slush (–210°C< temperature< –196°C). The samples were subsequently fractured and freeze-etched using Cressington equipments (Cressington, Watford, UK) and immediately double-layer coated with 3 nm platinum at an angle of 45° and with 5–10 nm carbon at 90° (Walther *et al.*, 1995). These samples were cryo-transferred to a semi in-lens field emission SEM (JEOL 6340F Field Emission SEM, Tokyo, Japan) and analysed at –140°C and 10 kV.

To detect possible imbibitional damage, we used cryo-planing followed by low temperature scanning electron microscopy. After imbibition of dry seeds at either 0° or 30°C, embryos were placed in a drop of Tissue-Tek on top of small rivets (4-mm-long aluminium pins with a head) and immediately plunge-frozen in liquid propane. These rivets fitted into sample holders for fracturing, cryo-planing and SEM investigation of the specimens. The embryos were cryo-planed (reviewed by Nijsse and Van Aelst, 1999) to examine cell contents. For cryo-planing, the frozen samples were sectioned with a glass knife in a cryo-ultramicrotome (Reichert-Jung Ultracut E/FC4D, Vienna, Austria) to the desired plane through the material. The last sections were cut using a diamond knife at decreasing section thicknesses from 0.5 μm to 20 nm, and at decreasing sectioning speeds down to 0.2 mm s^{-1}. During planing, the sample temperature was –90°C and the knife temperature was –100°C. The samples were cryo-transferred to a cryo-SEM (JEOL 6300F Field Emission SEM, Tokyo, Japan) equipped with an Oxford 1500 HF cryo-system (Eynsham, UK). The planed samples

were freeze-etched for 3 min at –89°C to enhance contrast and remove water-vapour contamination, sputter-coated with platinum, and subsequently analysed at –190°C with an accelerating voltage of 2.5 kV.

Polar lipids and free fatty acid analysis

About 15 mg of embryo axes was used per seed batch. Lipid extraction was done in small mortars by grinding the axis in 3x 1mL chloroform: methanol $CHCl_3$: MeOH (2:1 v/v). Internal standards for polar lipids (PLs) and free fatty acids (FFAs) (diheptadecanoyl-phosphatidyl-choline and heptadecanoic acid, respectively) were added immediately to the axes before grinding. The $CHCl_3$/MeOH extract was sonicated for a few min in a round-bath sonicator (Lab. Supplies Co., Hicksville NY, USA, model G112SP1) to improve extraction and cleared by centrifugation for a few min at 3000 rpm. Subsequently, the supernatant was washed with 20 vol% of a 0.9% NaCl solution, and after centrifugation for a few min at 3000 rpm, the phase separated $CHCl_3$ layer was recovered and passed over a column of anhydrous Na_2SO_4. After vacuum-evaporation, the residue was taken up in 1 mL $CHCl_3$ and separated into a neutral and a polar lipid fraction by passage over a SEP-PAK silica cartridge (Waters Associates, Milford, Mass. USA, catalog no. 51900). The neutral lipid fraction (containing the free fatty acids) was eluted with 20 mL of anhydrous acetone, followed by elution of the polar lipids with 30 mL methanol.

The PL fraction was transmethylated with 0.2 M KOH in methanol for 15 min at 70°C under vigorous shaking. After cooling on ice, the fatty acid methylesters were phase-separated to hexane and passed over an anhydrous Na_2SO_4 column prior to GC-analysis. The neutral lipid fraction was loaded onto a SEP-PAK silica cartridge and eluted with 20 mL of acetone:ether (1:0.02 v/v), to reduce di and triglycerides, followed by the elution of the FFAs with 30 mL of methanol. The FFA fraction obtained was methylated using freshly prepared diazomethane in diethylether.

The fatty acid methylesthers 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 Chromjet integrator. Identification was done by comparing retention times with standards and GC Mass Spectral analysis as described by van Bilsen *et al.* (1993), while the quantification of the different components was achieved by comparing the total peak surface of methyl esters with that of methyl esters of the respective internal standards.

Fourier transform-IR spectroscopy (FT-IR)

In situ FTIR microspectroscopy was used to determine lipid melting in hydrated 10-day old seedling roots and dry axes. Transmission FT-IR 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. The acquisition parameters were 4 cm^{-1} resolution, 32 co-added interferograms, 2 cm^{-1} moving mirror speed, and 3600 to 1000 cm^{-1} wavenumber range. Spectral analysis and display were carried out using Spectrum version 2.00 (Perkin Elmer). A root tip was placed between two circular CaF_2 windows (2x13 mm) that were tightly mounted into a pre-cooled, temperature-controlled brass cell. In the case of dry axes, a thin slice was sandwiched between two diamond windows, and the sample was pressurized until sufficient transparency was obtained. 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 the cell. The sample temperature was recorded using two PT-100 elements that were located close to the sample windows. The temperature dependence of the FT-IR spectra was studied in the range -50°C to 60°C, starting with the lowest temperature. Spectra were recorded every min at temperature increments of 1.5°C min^{-1}. 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^{-1} was selected, and second derivative spectra were calculated after appropriate smoothing. The position of the symmetric CH_2 stretching vibration band of the hydrated membranes or neutral lipids around 2852 cm^{-1} was determined from these second derivative spectra. The mid point of melting (T_m) was derived from the maximum of the first derivative of the wavenumber-temperature plots.

ESR measurements

Phase behaviour of membranes in axes was studied using the membrane spin label 5-doxylstearic acid methyl ester (m-5-DS; from Aldrich Chemical Co, USA). Axes isolated from dry seeds were placed in a 1 mM solution of the label in hexane for at least 24h at room temperature. After 2 short washes in hexane, the axes were left for 5 days in air for the hexane to evaporate. Axes fully survived the hexane treatment. After the appropriate incubation, axes (approx. 10 mg) were placed in a 2-mm diameter capillary that was hermetically sealed. ESR spectra were recorded using an X-band ESR spectrometer (Bruker Analitik, Rheinstetten, Germany, model Elexsys 500). Spectra of m-5-DS in axes were recorded at a magnetic field scan sweep of 100 Gauss with the

field center at approximately 3480 Gauss. A microwave power of 5 mW, and 1.5 Gauss modulation amplitude were used for spectra recording, which prevented distortion of the line shape due to saturation and overmodulation. The temperature dependence of the ESR spectra was studied in the range −70°C to 60°C, starting with the lowest temperature. Two spectra were accumulated at temperature increments of 5°C. The time for sample equilibration at a given temperature was 2 min. Spectra manipulation was performed using Origin 7.0 (Microcal Software, Inc).

Results

Effect of relative humidy on seed viability

Seeds excised from fresh fruits had initial moisture contents (MC) that were twice higher than that of seeds processed from dried fruits. However, the MCs of both seed lots were ≤ 0.1g H_2O.g DM^{-1}, indicating that the seeds had undergone considerable

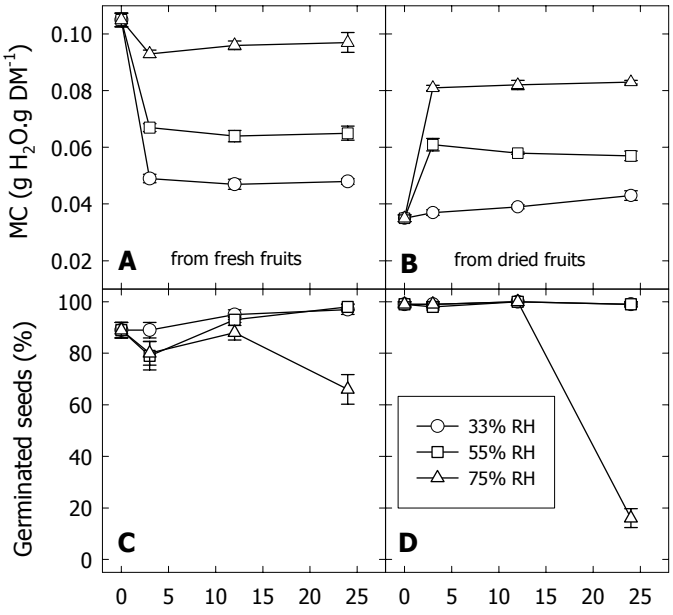

Figure 1. Effect of open storage at 20 ±2°C and three different relative humidities (33, 55 and 75%) on moisture content (A, B) and percentage germination (C, D) of intact *K. senegalensis* seeds processed from two fruit maturation stages (mature, fresh [A, C] or mature, dried fruits [B, D]). Germination data are the means of four replicates of 25 seeds each ± *SE*. Bars are indicated if they exceed symbol size.

drying upon maturation. Upon exposure to the different RHs (33, 55 and 75%) at 20°C, the two seed lots reached different equilibrium MC (Fig. 1, A and B); the equilibrium MC of seeds extracted from mature, fresh fruits was higher at all considered RHs than that of seeds processed from mature dried fruits. This difference in water status was confirmed by plotting the MCs of the two seed lots against their respective water activity values, as indicated by the two parallel regression lines (Fig. 2). In both cases, there was a positive linear correlation between MC and water activity.

Seeds from both types of fruits showed good initial germination (≥90%), and this initial high viability was maintained at least up to 24 weeks of exposure to RHs of ≤55% (Fig.1 C, D). On the other hand, exposure to high relative humidity (75%) resulted in a reduction of germination capability between 12 and 24 weeks, more so in seeds processed from dried fruits than in those from fresh fruits. As a whole, there was a negative correlation between seed survival and relative humidity, which is a normal and predictable trend in orthodox seeds upon storage.

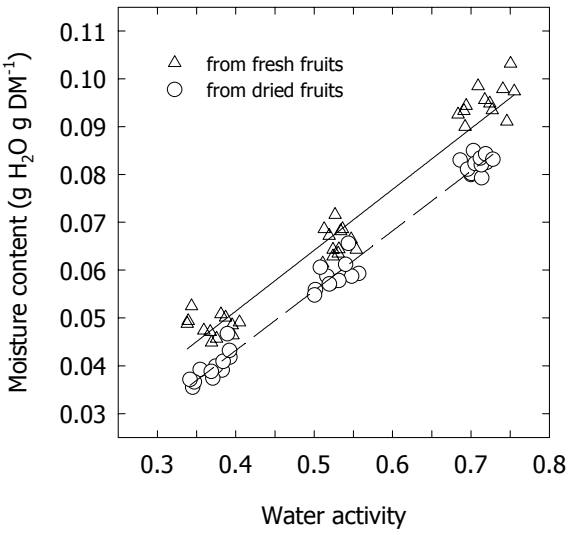

Figure 2. Plot of moisture content against water activity of *K. senegalensis* seeds prepared from mature fresh fruits or mature dried fruits. There was a positive linear correlation between MC and water activity in both seed lots (R=0.98 and R=0.94, for seeds from fresh and dried fruits, respectively). Each point was obtained from a sample of 5 seeds.

Imbibitional stress

In order to investigate possible sensitivity to low temperature imbibition, seed batches were soaked in tap water for 4h at temperatures ranging from 0-40°C, before further incubation on moist filter paper at 30°C. Figure 3 shows that seeds with an intact pericarp were entirely insensitive, even to soaking in ice water. However, seeds from which the pericarp was removed germinated less than the intact controls when soaking temperatures were ≤10°C. On the one hand, this indicates that the seed coat contributes to the prevention of imbibitional damage, but on the other hand, the relative insensitivity of the naked seed to low temperature imbibition suggests that ultrastructural features may play a role in preventing such damage.

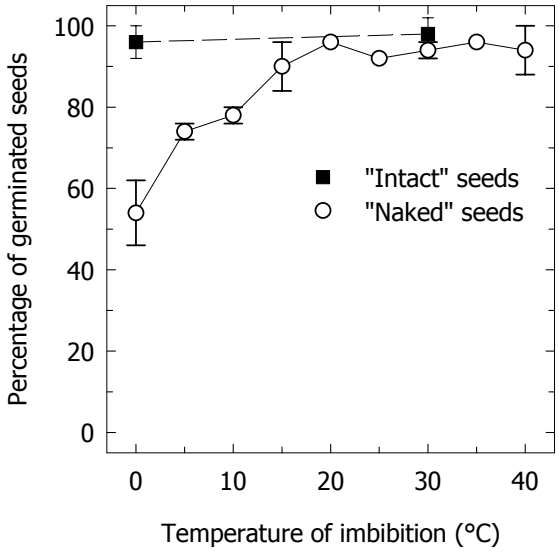

Figure 3. Effect of imbibition temperature on the germination of *K. senegalensis* intact and decoated seeds. Seeds were freshly collected and air-dried at room temperature to 0.05 g H_2O.g DM^{-1}. Soaking was for 4 hours in water of different temperatures, after which the seeds were further incubated at 30°C. Data points are means ±*SE* of duplicate experiments of 25 seeds each. Error bars are indicated when they exceed symbol size.

Figure 4 shows LTSEM images of the injury inflicted to the ultrastructure of axis cells by low temperature (0°C) imbibition. Healthy compartmentalized cells could be noticed after the 30°C-imbibition (Fig. 4A). As expected, damaged, collapsed, non-turgid axis cells were visible after the 0°C imbibition (Fig. 4C, D). These damaged cells were

observed mostly at the radicle tip (Fig. 4D). No sign of damage was observed in the remaining part of the axis and the cotyledons, where a dense, thick-walled epidermis was present (Fig. 4B). This dense cell layer might play a role in reducing imbibitional stress.

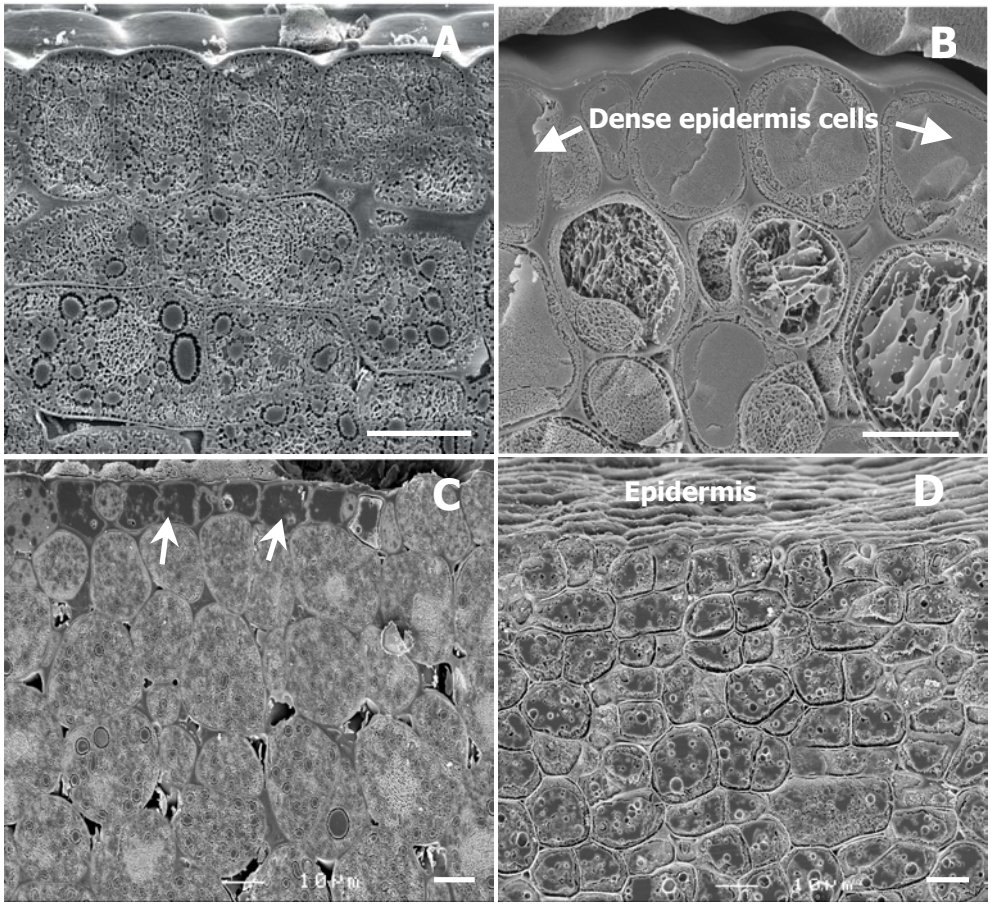

Figure 4. Cryo-SEM micrographs of cryo-planed *K. senegalensis* (decoated) seeds (MC = 0.05g H_2O.g DM^{-1}) subjected to imbibitional stress. The seeds were soaked in water at either 0°C or 30°C for 4h followed by incubation on moist filter paper at 30°C for 24 h. (A) 30°C-imbibed seed, showing healthy radicle tip cells, which are turgid and compartmentalized, without signs of damage. (B) 0°C-imbibed seed, showing a dense epidermis cell layer in a cotyledon, with no sign of damage. (C) 0°C-imbibed seed, with only some damaged epidermis cells (arrows) in the radicle tip area. (D) 0°C-imbibed seed, showing numerous non-turgid, damaged, radicle tip cells. All scale bars represent 10 µm.

Effect of storage temperature on seed survival

The effect of storage temperature is shown in Fig.5 using seeds that were processed from mature, dried fruits, with a MC of approx. 0.04g H_2O.g DM^{-1} (33% RH equilibrium). Seeds stored at -20, 15 and 20°C maintained almost 100% viability at least up to 30 months of storage, whilst seeds stored at 4°C lost their viability between 12 and 18 months. Although the increase in MC of the 4°C-stored seeds indicated that the storage bags were not hermetically closed, the relatively low level of MC (<0.08g H_2O.g DM^{-1}) at which seeds lost their viability at such a low temperature is unexpected.

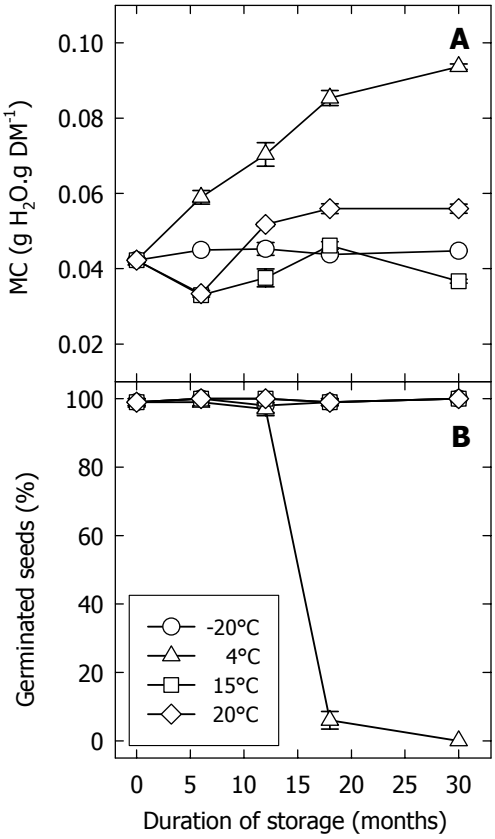

Figure 5. Effect of storage temperature on moisture content (A) and percentage germination (B) of *K. senegalensis* seeds prepared from mature dried fruits. (A): Each point is the mean of 3 replicates of 5 seeds. (B): Each point is the mean of 4 replicates of 25 seeds ± SE. Bars are indicated if they exceed symbol size.

The data suggest anomalies in the relationship between seed viability and temperature during storage. The current results show that in practice there is no beneficial effect of storing *K. senegalensis* seeds at 4°C in comparison with storing them at, for example, 15°C. This observation has considerable practical value for the short or medium-term storage of cold sensitive tropical tree seeds.

Effect of storage temperature on membrane permeability

Measuring conductivity of the leachate from plant tissue is a long-standing method for estimating membrane permeability. To analyze possible involvement of the loss of membrane barrier function in the decreased germination capacity of the seeds stored for 30-months at 4°C, conductivity measurements were carried out with embryonic axes isolated from the seeds used in Fig. 5.

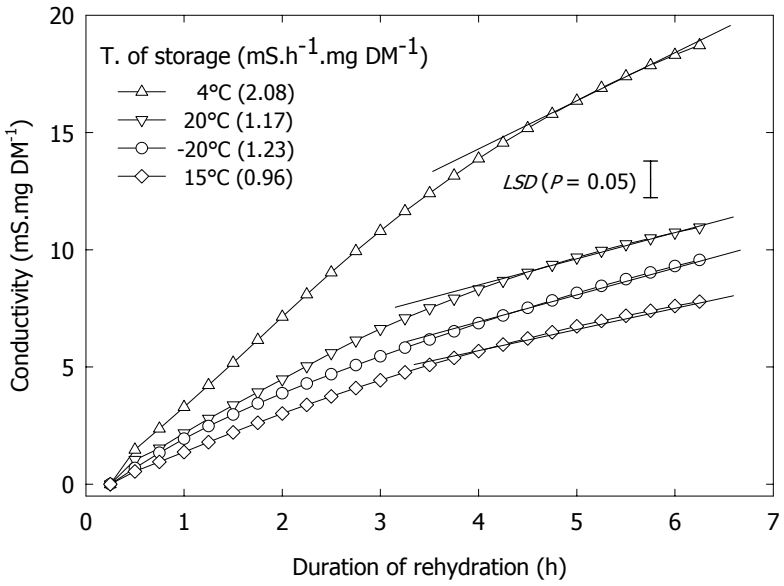

Figure 6. Effect of 30-months of storage of *K. senegalensis* seeds (from mature, dried fruits) at 4 different temperatures on the conductivity of the solution, in which isolated axes were rehydrated at 20°C Each point is the mean value of measurements of 25 individual axes. LSD (P=0.05) is indicated in the figure.

Twenty five individual axes were rehydrated in 1.5 mL water at 20°C, and the electrolyte leakage from each of them was followed during 6 h incubation (Fig. 6). After an initial fast leakage from axes of the 4°C-stored seeds, particularly, a steady leakage rate was obtained, which was twice higher for the axes of the 4°C-stored seeds than for those of the 15°C-stored seeds. The leakage rate of axes from the 20°C and -20°C-stored seeds were slightly higher than that from the 15°C-stored seeds. The greater leakage from the axes of the 4°C-stored seeds is a clear indication that membrane barrier function has decreased. In the next paragraph the question is addressed whether membrane polar lipid content and composition of the axes is affected during ageing of the seeds from Fig. 5.

Polar lipid and free fatty acid contents and composition during ageing

Effect of temperature: Embryonic axes isolated from seeds after 30-months of storage at the four above-mentioned temperatures were analyzed for polar lipid (PL) and free fatty acid (FFA) contents. Figure 7 shows that axes isolated from the −20°C-stored seeds had the highest PL content (2.7% of axes DM). In comparison, axes from

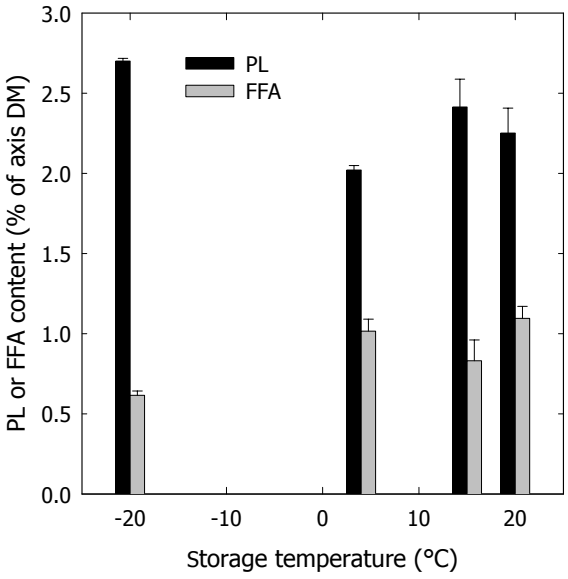

Figure 7. Polar lipids (PL) and free fatty acids (FFA) contents in axes of *K. senegalensis* seeds, processed from mature, dried fruits, stored for 30 months at 4 temperatures (-20°C, 4°C, 15°C and 20°C). Each bar is the mean of 2 separate extractions. Small vertical bars represent standard errors.

4°C-stored seeds showed a reduction of about 25% in their PL content. Values recorded for the 15°C- and 20°C-stored seeds were intermediate in that respect. The FFA content in axes of 20 and 4°C stored seeds were the highest; that of the −20°C stored seeds the lowest, with that of 15°C stored seeds being intermediate. Although the loss of viability of the 4°C-stored seeds could be linked to the greatest reduction in PL content, there was no direct connection between FFA content and viability loss. In an attempt to verify the link between PL degradation and loss of viability during ageing we expanded the research to long-term stored seeds.

Effect of long-term storage: Long term "natural" ageing was investigated in six initially high quality seeds accessions collected during the period 1985 - 2004, which were stored dry (MC, approx. 0.05g H_2O.g DM^{-1}) at 15°C for 1, 2, 3, 4, 15 and 20 years. Viability assessed by germination tests revealed that seeds stored for durations between 1 and 3 years maintained entirely their initial germinability, while seeds stored for 15 and 20 years had completely lost it. In that respect, four-year stored seeds gave an intermediate result, with 60% germination (Fig. 8, Ger).

In line with the germination capability, total PL content in the embryonic axes of the respective seed batches declined over the years of storage, from approx. 2.5% in one-year stored seeds to <0.5% in the 20-years-stored seeds (Fig. 8, PL). This suggests that membrane PLs undergo progressive degradation upon storage as seeds age. The decrease in total PL content coincided with a slight decrease in the average number of double bonds per PL molecule - the double bonds index (Fig. 8, DBI) and a considerable increase in FFA content (Fig. 8, FFA).

Gradual decay of membrane PLs with ageing was evident, even during the first few years of storage, during which no decline in germination occurred. Acyl chains with double bonds are more prone to decay, as suggested by the slight decrease in DBI. The question of type of fatty acids being involved is addressed next.

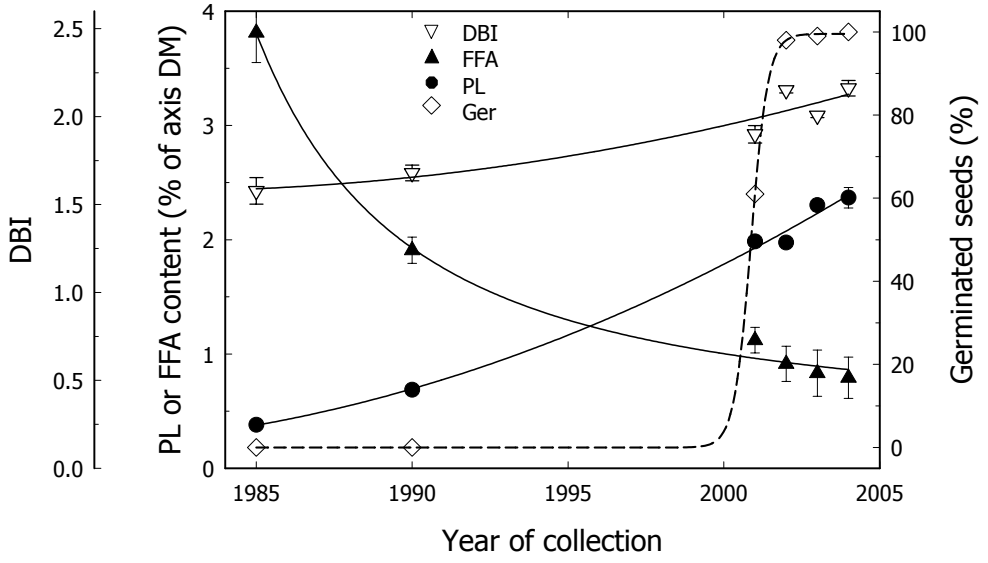

Figure 8. Effect of long-term dry storage at 15°C of *K. senegalensis* seeds (of different accessions, processed from mature, dried fruits) on percentage germination (Ger), polar lipid content (PL), free fatty acid content (FFA) and double bond index (DBI). The PL, FFA and DBI values are from isolated axes. The analyses were carried out in 2005. Ger data are means of 4 replicates of 25 seeds. PL, FFA and DBI data are means of two separate extractions. Vertical bars represent standard errors.

Figure 9A shows the relative proportion of five commonly found fatty acids esterified to the PLs with time of storage. With age, linoleic acid (18:2) - the most unsaturated of the three abundant acyl chains (18:1, 18:2, and 16:0) decreased proportionally more than the mono-unsaturated, oleic acid (18:1), whereas the contribution of palmitic acid (16:0) remained almost constant. Another observation was that the mol percent of 18:1 and 18:2 esterified fatty acids reciprocally varied from year to year in the 2001-2004 accession seeds, likely induced by differences in the temperature during maturation of the seeds. The years 2001 and 2003 were relatively warm during seed maturation (Temperature Survey Data: 1995-2004, Meteorology Centre, Ouagadougou).

In contrast to the mol composition of the PLs, that of the FFAs showed that the proportional contribution of the two main acyl chains (18:1 and 18:2) increased with increasing seed age, whilst the proportion of two (16:0 and 18:0) of the three other chains decreased (Fig. 9B). This trend was observed only in seeds batches <5 years

old; stabilisation of the proportional contribution of the different acyl chains was noticed in seeds stored for 15 and 20 years. The mole composition of free fatty acids in the oldest seeds was more like that of the PLs in the youngest seeds suggesting that initially present FFAs were proportionally diminished in favour of newly deesterified fatty acids.

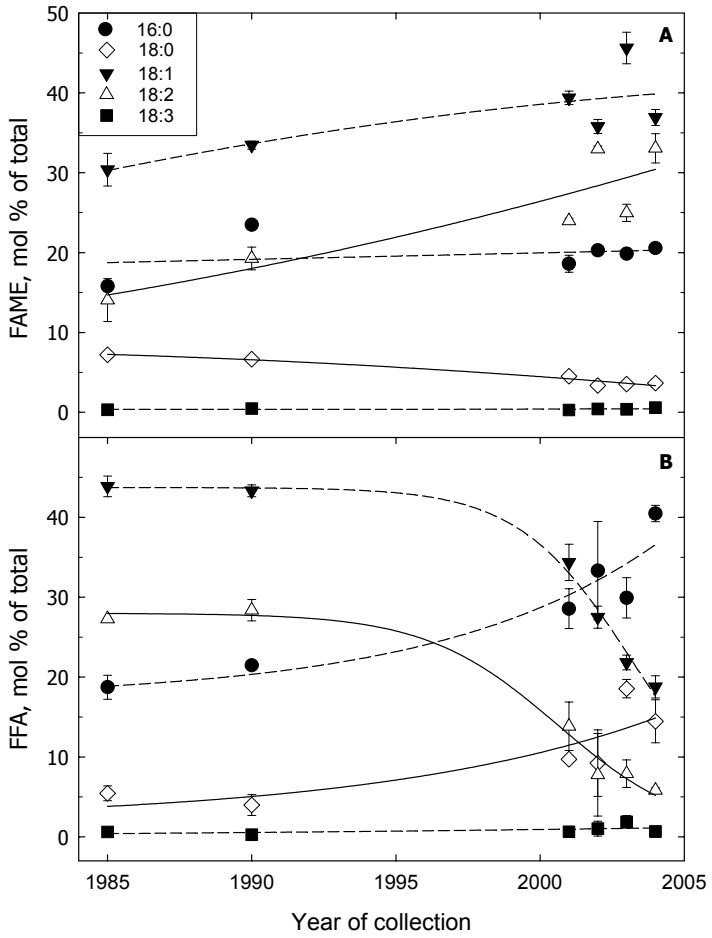

Figure 9. Effect of long-term dry storage on the mol percent of the esterified acyl chains of PLs (FAME in A) and of the FFA (B) in axes of six *K. senegalensis* seed lots differing in year of accession. All seed batches were processed from mature, dried fruits and stored dry at 15°C until analysed. The different experiments were carried out in 2005. Data ± SE are means of two separate extractions. Error bars are indicated when they exceed symbol size.

Since the axes contain considerable amounts of neutral lipids (NLs), FFAs also can be derived from deesterified NLs, besides from the PLs. Nevertheless, when PL contents were plotted against FFA contents, a negative linear correlation was observed (Fig. 10). This is an indication that a non-negligible part of the FFAs quantified in the different seed batches could be of PL origin. The impact of the loss of PLs in aged seed axes on membrane structure is dealt with in the next paragraph.

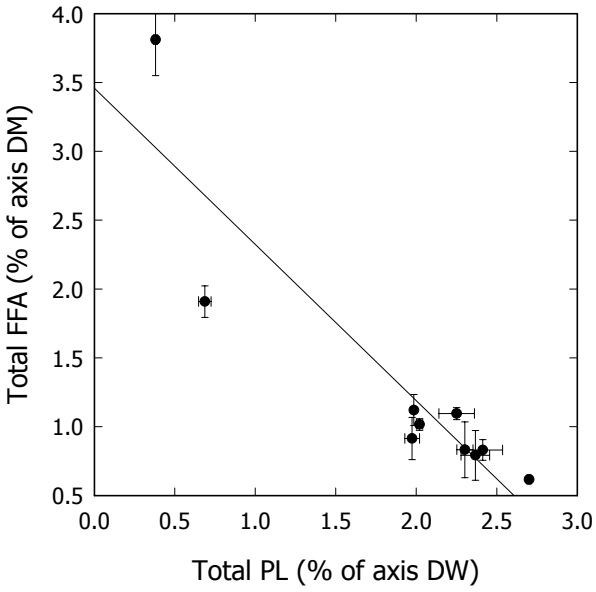

Figure 10. Plot of PL contents against FFA contents of axes of *K. senegalensis* seeds processed from mature dried fruits. The data were from 30-months stored seeds at 4 temperatures (-20°C, 4°C, 15°C and 20°C) and from seeds from different accessions stored dry during 1-20 years (seeds from Fig. 7). There was a negative correlation (R= 0.91) between PL and FFA content. Vertical bars represent standard errors.

Effect of ageing on membrane structure

Cryo-fracturing and high resolution SEM analysis were used to address the question whether or not deesterification of acyl chains from the polar lipid backbone during ageing leads to loss of membrane structure or to phase separation of membrane components. A representative cryofractured plasma membrane surface of a dry axis of a seed stored for 30 months at 4°C (0% germination, highest electrolyte leakage [Fig. 6]) is shown in Fig. 11A.

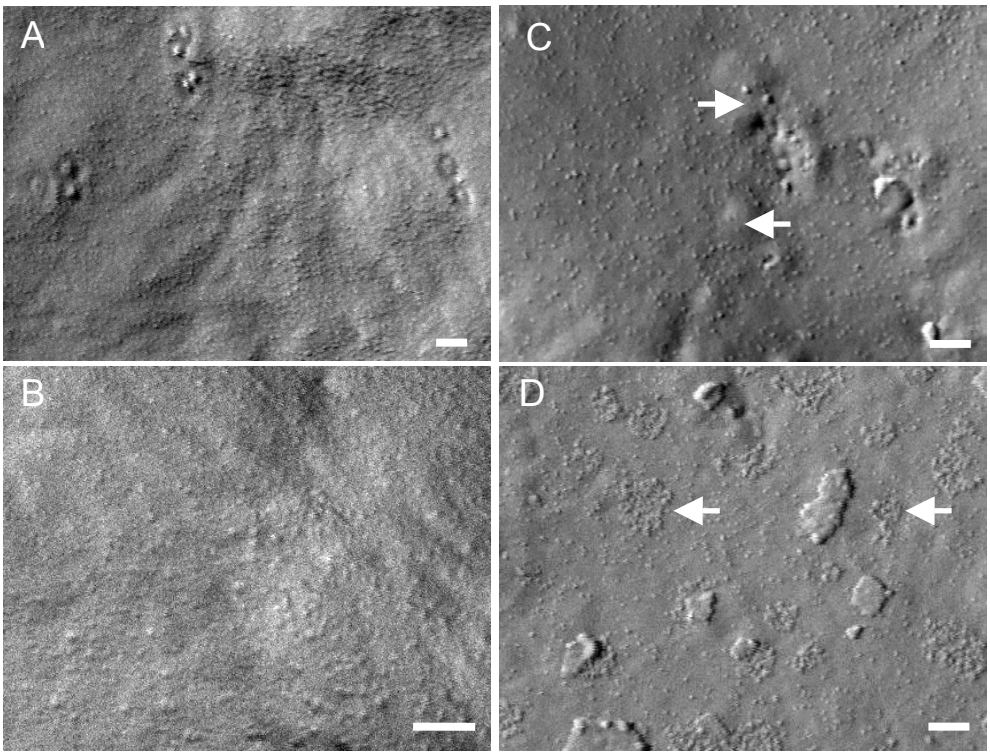

Figure 11. High-resolution cryo-fracture images of the plasma membranes of *K. senegalensis* axes after different treatments. (A) dry axis of a 30-months old seed stored at 4°C; the pits represent plasmodesmata. (B) rehydrated axis (40 min; non-damaged) of a 30-months old seed stored dry at 15°C. (C and D) rehydrated axes of 30-months old seeds stored at 4°C, showing different types of damage (indicated by arrows: upheavals in [C] and clustered IMPs in [D]). The numerous dots are intra-membrane particles (IMPs). Bar = 100 nm.

Large, non-disrupted membrane surfaces and a regular distribution of integral membrane proteins (intramembrane particles [IMPs]) can be noticed. Also plasmodesmata and imprints of cellulose fibers from the cell wall are visible. The same features can be noticed when inspecting plasma membranes of 30-months old seeds stored at 15°C, which retained viability (images not shown). On rehydration of the 4°C-stored seeds for 40 min at 25°C, still membrane surfaces can be noticed, but also numerous defects were apparent, ranging from small upheavals (Fig. 11C) to clustered IMPs (Fig. 11D), which are signs of membrane deterioration. As a control, a rehydrated 15°C-stored seed is shown in Fig. 11B, which displayed no features referring to damage.

The greatest losses of PLs had occurred in axes of the 20-year-old seeds. Figure 12 shows the impact of such losses on the intactness of the plasma membranes in dry axes. The cryo-fracture followed the membrane core and exposed seemingly intact membranes (Fig. 12A), also when inspected in greater detail (Fig. 12B). However, no membrane surface could be observed after rehydration (image not shown), suggesting that the membrane components completely phase-separated after their mobility was increased by adding water.

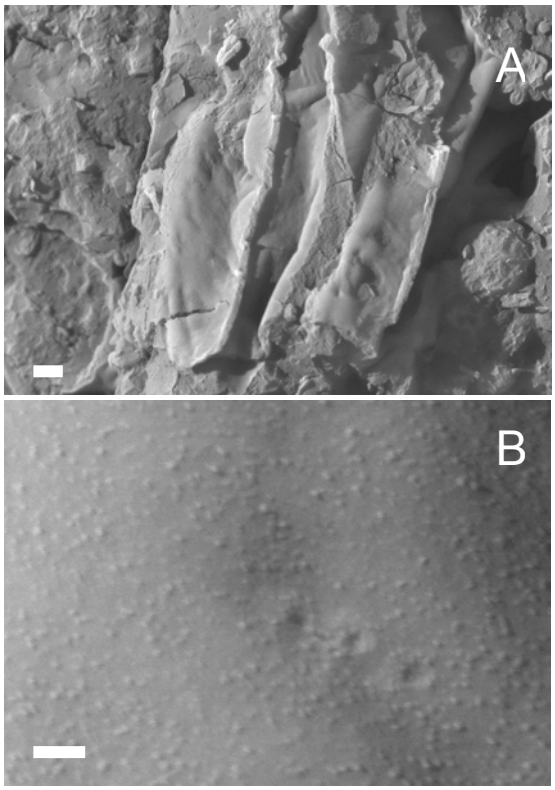

Figure 12. High-resolution cryo-fracture images of the plasma membranes of *K. senegalensis* axes of a dry, 20-y old seed (accession year 1985). (A) Overview of whole cells showing intact membranes. (B) Detail of the plasmamembrane and plasmodesmata (pits); the numerous dots are IMPs. Bar = 1 µm (A) and 100 nm (B).

Phase behaviour of membranes and neutral lipids

Deesterification of acyl chains can occur via free radical activity (Niehaus, 1978; McKersie *et al.*, 1988) or phospholipase activity (Salama and Pearce, 1993), or both. Free radical-induced deesterification leading to the decline of viability is unlikely to cause the anomaly of reduced longevity at low subzero (°C) temperatures as observed by us (Fig. 5; Chapter 3) and others (Hong and Ellis, 2002; Crane *et al.*, 2006). Phospholipases have been shown to be active at extremely low water contents (Oliver *et al.*, 1997). Phospholipase activity is, apart from temperature dependent, also influenced by the physical state of the bilayer. The activity is much greater at mid melting temperatures (Oliver *et al.*, 1995) than in either the gel or liquid crystalline phase. If, in the case of *K. senegalensis* seeds, the mid melting temperature of membrane PLs would lie around low above zero (°C) temperatures, the 4°C anomaly would be understandable. To this end, we analyzed the phase behaviour of membranes and neutral lipids *in situ*.

Differential scanning calorimetry (DSC) and FT-IR can give information on the melting behaviour of lipids *in situ*. DSC is not sensitive enough to detect the phase behaviour of PLs because of the minute amounts present in eucaryotic cells (few percent of DM). In addition, thermograms are confounded by the triacylglycerols that are generally present in large quantities in storage propagules, such as seeds. FT-IR analysis of PLs is valid only in cases when the amounts of triacylglycerols are very low.

To nevertheless get an impression of T_m in membranes of *K. senegalensis*, FT-IR analysis was performed on hydrated root tips of 10-day old seedlings, assuming that most of the oil had been consumed, thus allowing T_m of membranes to be determined. Figure 13 shows a wavenumber-temperature plot of the symmetric CH_2 stretching band around 2852 cm^{-1}, from which it can be derived that T_m was 11.3°C. Repeated analyses indicated that T_m was 11.5±2.8°C (SD). This value is comparable with the T_m of root tips of neem seedlings (9.6°C; Sacandé *et al.*, 2001) and of *Lannea microcarpa* (14.5±1.5°C; Neya, unpublished results). These elevated T_m values are typical for plants from the tropics (Crowe *et al.*, 1989b) and can be considered as a homeoviscous adaptation to the prevailing high temperatures.

However, our purpose was to reveal the T_m of membranes in axes of dry seeds. Wavenumber-temperature plots of slices of a dry axis indicated a T_m of −13.0°C (Fig. 13). This T_m is surely from triacylglycerols, because oil appeared as a result of the pressure applied. Repeated scans indicated an average T_m of −10.4 ±4.5°C. Another way to access membrane phase behaviour is *in vivo* spin labelling of membranes. We used the spin label, methylated 5-doxylstearate (m-5-DS), and ESR spectroscopy for

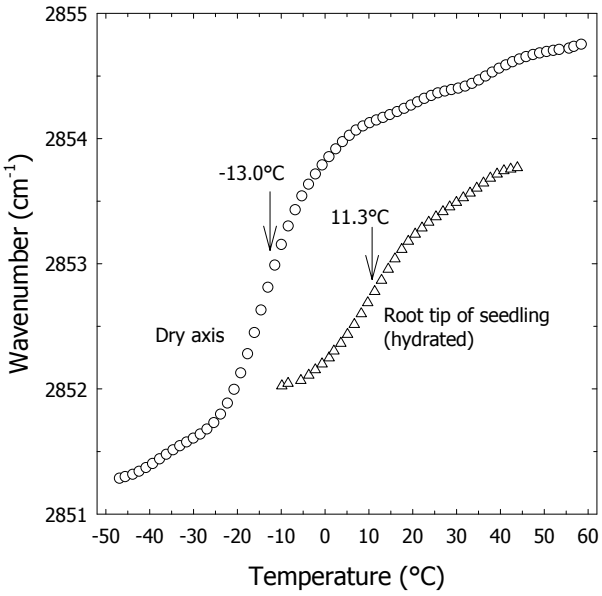

Figure 13. Wavenumber-temperature plot of the symmetric CH_2 stretching vibration band (FTIR) of a (hydrated) root tip of a 10-d old *K. senegalensis* seedling and of a dry axis, mainly oil. The mid value of the melting transition, which was determined on account of the maximum of the first derivative, is considered as T_m. These values were 11.3°C and −13.0°C for the hydrated root and the dry axis oil, respectively.

this purpose. Figure 14 shows ESR spectra of m-5-DS in dry axes around T_m. Below T_m, the spectra were anisotropic (top spectra), which is characteristic for an immobile state of the spin label in gel-phase membranes. The outermost splitting $2A_{max}$ characterizes this phase. The less the distance between the outermost extremes, the more is the motional freedom of the label (Marsh, 1981). Above T_m the spectra lose anisotropy (*i.e.* the outermost peaks move inwards), which is indicative of an increased freedom of motion of the spin label in the liquid-crystalline phase of membranes. The shift from anisotropic to isotropic occurred around 7°C, where it looks as if the spectrum contains two components – a mixture of the spectra at -3° and at 17°C (Fig. 14). We consider the shape of this spectrum with two coexisting phases as an indication of the phase transition.

Figure 15 shows the change in $2A_{max}$ with temperature for dry axes and axes that were rehydrated in saturated water vapour for 2.5 h at 25°C. The data were very similar, indicating that the presence of water did not influence the *in situ* T_m of axis membranes. Axes with higher water content essentially gave similar $2A_{max}$ values (data not shown). When the shape of spectra changes to isotropic the value of $2A_{max}$ cannot

be further calculated. Then, the mobility of the spin label is characterized by the rotational correlation time, calculated by the formula: $\tau c = 6.5 \times 10^{-10} W_0 (\sqrt{h_{-1}/h_0} - 1)$, where W_0 (see Fig. 14) is the peak-to-peak line-width of the central line, and H_{-1} and H_0 are the heights of the high-field (right-hand side) line and central line in isotropic ESR spectra, respectively (see insert of the spectrum at 42°C in Fig. 15). The rotational correlation time is proportional to the membrane viscosity in the liquid-crystalline phase. Values of the calculated τc above T_m decreased with the increase of temperature (Fig. 15). Membranes in hydrated axes had a lower τc (and viscosity) than membranes in dry axes, which corroborates the assumption of the spin label residing in membranes.

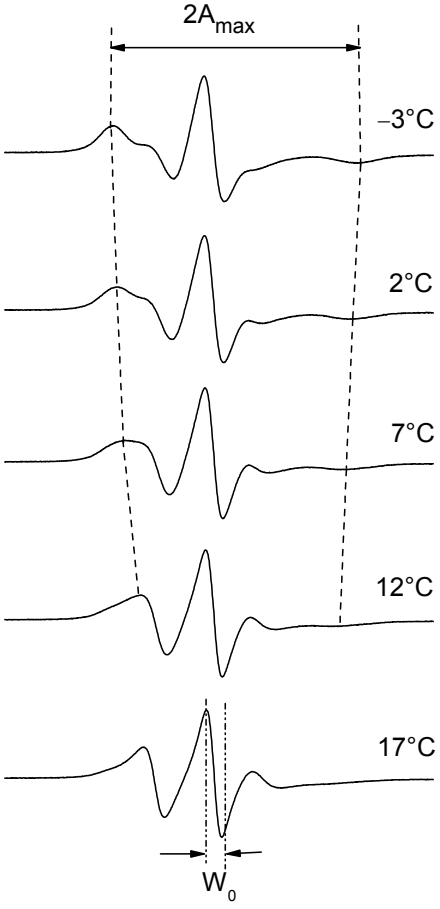

Figure 14. ESR spectra of methylated 5-DS in dry axes of *K. senegalensis* around T_m.

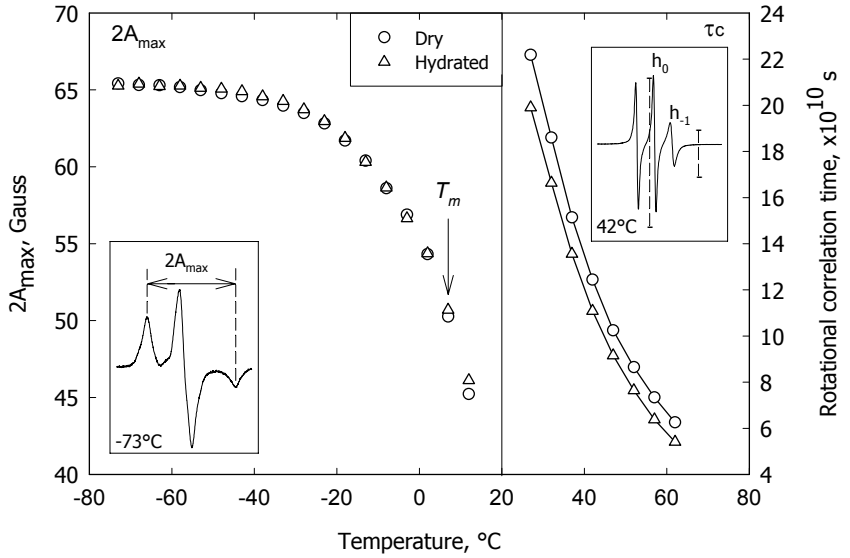

Figure 15. Temperature dependence of $2A_{max}$ (see insert at left-hand side of the figure; spectrum in dry axes at -73°C) and rotational correlation time τc for methylated 5DS in dry and rehydrated *K. senegalensis* axes. Hydration was achieved by 2.5 h in water vapour-saturated air at 25°C. The arrow indicates the mid of melting (T_m). The insert at the right-hand side of the figure shows a spectrum in dry axes at 42°C, with an indication of the line heights h_0 and h_{-1} used for the calculation of τc. The spectra around T_m are shown in Fig. 14.

Discussion

Survival of seeds under different conditions of storage and rehydration

Freshly processed seeds of *K. senegalensis* from the two types of fruits had low initial MC (<0.1g H_2O g DM^{-1}) and germinated well (90 - 100%). Further drying to 0.04 g H_2O g DM^{-1} did not affect germination capability, confirming that the seeds are desiccation tolerant (Hong and Ellis, 1998) and obviously orthodox (Gaméné and Eriksen 2004). Exposure to high RH (75%) condition at 20°C resulted in the loss of viability after 12 weeks, whereas seeds kept at RHs ≤53% maintained high initial viability for at least up to 24 weeks (Fig. 1). Thus, for effective long-term storage seeds should be kept at low RH.

Seeds with a drying history attained lower equilibrium MC on exposure to saturated salt solutions than did fresh seeds equilibrated to the same RHs. This was also reflected by

lower Aw values for the seeds with a drying history (Fig. 2). This behaviour has been described earlier (Fleischer and Werner, 1992), and is known as hysteresis of the sorption-desorption process of water. It might be interpreted as resulting from irreversible changes in water binding properties with drying.

Intact, dried seeds were not sensitive to rehydration in ice-water, whereas decoated seeds were damaged at rehydration temperatures ≤ 10°C (Fig. 3). However, these naked seeds were less sensitive to imbibitional stress than neem seeds. Although some injured cells were noticed, mainly at the periphery of the radicle tip (Fig. 4), more than 50% of seeds survived a 0°C imbibition treatment (Fig. 3). This relatively low sensitivity might be explained by the presence of a dense epidermis layer that could help slow down the rate of water penetration. Previously, the sensitivity of tropical seeds to imbibitional stress has been ascribed to the high gel-to-liquid crystalline transition temperatures (T_m) of their membranes. Despite similarly high T_m, *K. senegalensis* seeds were only moderately sensitive and scarified *L. microcarpa* seeds were insensitive (Chapter 4). This dismissed the hypothesis of a general sensitivity of all tropical seeds to cold rehydration.

We observed a discontinuity in the relationship between storage temperature and seed longevity (Fig. 5). While seeds stored at -20, 15 and 20°C were able to maintain their initially high viability over a period of 30 months of storage, those stored at 4°C lost completely germination capability between 12 and 18 months (Fig. 5B). It was noticed that the MC of seeds stored at 4°C increased during storage – the consequence of storage bags not being hermetically closed. However, it is remarkable that the viability loss occurred at such a low temperature and a MC of approx. 0.07 g H_2O.g DM^{-1} (Fig. 5). It is unlikely that the loss of viability observed in these seeds is the result of the increased MC. In that respect, the results of *L. microcarpa* seeds storability (Chapter 3) support a 4°C-storage as being in general less effective for tropical seeds in comparison with a -20 or 15°C-storage. Although in this species the overall low germination was determined by seed coat constraints, it has been consistently observed that seeds stored at 4°C performed less than those stored at -20 or 15°C, without any difference in MC. Similarly, the life span of arabica coffee seeds upon hermetic storage is longer at -20 and 15°C than at 0°C (Hong and Ellis, 2002). Also Crane *et al.* (2006) have found that viability of *Cuphea carthagenensis* seeds remains high at 25°C-storage, but is quickly lost at 5°C. On the basis of these results we conclude that *K. senegalensis* seeds have orthodox storage behaviour, but that a fast loss of viability may occur at 4°C even at water contents <0.1 g H_2O.g DM^{-1}.

Changes associated with viability loss and ageing

Viability loss is often attributed to the loss of barrier function of the plasma membrane (Roberts, 1972 Maguire, 1977; Bewley and Black, 1994; Golovina *et al.*, 1997). The leakage experiments of Fig. 6 confirm this. The 4°C-stored seeds that lost germination capacity showed a greater initial leakage and a twice higher steady state leakage than the still viable seeds stored at -20, 15 and 20°C (Figs. 5, 6). A similar relationship between elevated leakage and viability loss has been found for many other seed species (Sung and Chiu, 1995; Tammela *et al.*, 2000; Corbineau *et al.*, 2002).

The upsurge of electrolyte leakage from propagules during ageing often has been linked with chemical changes in the membrane polar lipids (Pearce and Abdel Samad, 1980; Pukacka, 1991; Corbineau *et al.*, 2002; Goel and Sheoran, 2003). Therefore, it was no surprise to find a loss of PLs of approx 25% in the axes of the 4°C-stored seeds as compared with the content in axes of the -20°C seeds (Fig. 7). Meanwhile, the loss of PLs was accompanied by an accumulation of FFAs suggesting that these phenomena are linked. One main event often described in the literature is the deesterification of acyl chains from the glycerol backbone, which has been attributed to free radical activity (Niehaus, 1978; McKersie *et al.*, 1988), but also to the activity of phospholipases (Salama and Pearce, 1993). The relationship between viability loss and the changes in PL and FFA contents was further investigated by analysing seeds from different accessions that were long-term stored at 15°C (Fig. 8). It appeared that losses of PLs and increase in FFAs occurred right from the onset of dry storage. Viability was maintained at high level during the first three years of storage, but started to fall off after 4 y. By then 15% of the PLs were lost. From this and the 4°C storage data it can be learned that the critical loss of PLs must lie around 15-25%. Whether this loss is from particular cells or from all the cells is unknown.

The loss of PLs coincided with an increase in FFAs. But because FFAs are deesterified also from the triacylglycerols, it is difficult to indicate a critical level of FFA accumulation. However, when the loss of PLs was plotted against the gain in FFAs a negative linear correlation was obtained (Fig. 10), suggesting that at least part of the FFAs is of PL origin. The number of double bonds per PL molecule (Double Bond Index), which could be calculated from the composition of the remaining acyl chains in the PLs displayed some decrease during the 20 years of storage. The greater relative loss of the unsaturated fatty acid, in particular linoleic acid, indicates a preferential loss of unsaturated acyl chains from the PLs (Fig. 9). This could suggest that lipid peroxidation also occurred, apart from deesterification. However, such a conclusion supposes that deesterification would take place at random, which is unknown. We have an indication that the mol composition of the FFAs resembles that originally present in the PLs (Fig. 9B). The small amount of other, preexisting, FFAs in the young seed axes

was overruled during ageing by the deesterified FFAs, to give the composition found after 20 years of storage.

Impact of polar lipid loss and free fatty acid accumulation on membrane ultrastructure

The bilayer core is typically the path of a freeze fracture because of its relatively low resistance – the reason why a fracture usually exposes large membrane surfaces. With a loss of almost 80% of the PLs in 20 years-old dry axes, it is difficult to believe that there is a membrane left. However, high resolution SEM images from these axes showed intact membrane surface, with a spaced random distribution of intra membrane particles (IMPs; Fig. 12). We interpret this to mean that the deesterified acyl chains must still be present at the site in the membrane interior where they originally resided. Apparently, the FFAs are prevented from leaving the membrane because the headgroups are immobilized by the glassy cytoplasm in the dry axis. The randomly distributed IMPs also indicate that, contrary to what was originally thought; membrane chemical changes do not lead to phase separation of the membrane components in the dry state, also the result of immobilization of the membrane components. Rehydration immediately led to phase separation and loss of extended membrane structure, resulting in our inability to find a membrane surface with cryo-SEM. Rehydration was less devastating in the 4°C-stored material that just lost germinability. Different patterns of damage could be observed in membranes, from small upheavals, to clustering of the IMPs – evidence of a phase separation (Fig. 11).

Possible causes of the 4°C anomaly in tropical seeds

In earlier work, deesterification of PLs during ageing has been attributed to the activity of free radicals and not to the activity of phospholipases (Van Bilsen *et al.*, 1993, 1994). This conclusion was based on the fact that the FFAs produced had undergone random deesterification, *i.e.*, they had the same composition as the fatty acids in the PLs, from which they were deesterified. Phospholipase A_2, for example would have led to a proportionally larger amount of unsaturated FFAs in comparison with the fatty acid composition in the PLs (Van Bilsen *et al.*, 1993). In addition, the authors have argued that it is unlikely that phospholipases can be active in dry seeds. Meanwhile, phospholipases have been reported to be active at extremely low water content (Oliver *et al.*, 1997), which brings them back as possible participants in the deesterification.

Reverting to the 4°C anomaly, we may consider the following. With free-radical mediated deesterification as a mechanism of PL breakdown one would not expect an activity that is particularly high at 4°C in comparison with that at 15°C. However with phospholipases being involved, higher activity may be possible under special conditions. It has been shown that the activity is much enhanced when membranes have two coexisting phases – gel phase and liquid crystalline – during a phase transition (Oliver *et al.*, 1995). Using ESR spin probe technique we found that membranes in dry axes clearly were at mid melting at approx. 7°C (Figs 14 and 15). Hydration did not change this value. For the low above zero (°C) anomaly, this means that it is very likely that during storage at 4°C, the membranes have two coexisting phases for extended periods of time, which would promote phospholipase activity. In contrast, the membranes are in gel phase at –20°C and mainly in liquid crystalline phase at 15°C. The low temperature and difficult access to the site of action would make activity unlikely at -20°C, whilst the single phase at 15°C probably allows for less activity than at 4°C.

One problem to solve is why the low above zero (°C) anomaly is typical for tropical seeds. Membranes of plants from the tropics are characterized by elevated T_m (Crowe *et al.*, 1989b; Sacandé *et al.* 2001; Fig. 13) as a homeoviscous adaptation to the high average temperature. Membranes from plants of the temperate climate zones have much lower T_m (Crowe *et al.*, 1989b). It can thus be expected that T_m values of axes membranes also are lower. Because of the low mid-melting temperature a possibly elevated phospholipase activity and the associated reduced life span might have gone unnoticed in temperate climate seeds.

Crane *et al.*, (2006) have suggested that the low above zero (°C) anomaly can be attributed to triacylglycerol phase changes, since the rate of deterioration of *C. carthagenensis* seeds stored at 5°C correlated with the rate of crystallization of triacylglycerols within the seeds. We find it difficult to envisage how changes in the lipid bodies can lead to direct leakage from the seeds on rehydration. Moreover, our determination of T_m of oils in *K. senegalensis* axes at -10°C (Fig. 13) suggests little involvement of oil in the 4°C anomaly that we observed in this species. Therefore, we suggest that the deesterification of acyl chains leading to the loss of viability of tropical seeds at low above zero (°C) temperatures occurs, at least partly, via phospholipase activity.

Acknowledgement

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Chapter 7

General discussion

Introduction

The multiple values of tropical tree species for humankind and the undeniable importance for the protection of the environment make them potentially suitable for use in reforestation programs and agroforestry systems. Unfortunately, difficult seed storage behaviour has thus far limited the utilization of these species in such programs. The current research project was therefore aimed at a better understanding of the seed biology, particularly focussed on the improvement of seed survival after drying and subsequent dry storage. A better understanding of the storage behaviour of tropical tree seeds is a prerequisite for their long-term preservation (Hong *et al.*, 1996). Additionally, the knowledge of the causes of the short life spans of seeds after drying and dry storage will facilitate the planting of endogenous tree species in Burkina Faso and other Sahelian countries.

The inability to withstand desiccation to low (≈5%) moisture contents and the sensitivity to low storage temperatures (subzero °C) are the major constraints in storing recalcitrant and intermediate seeds. The seeds of many tropical tree species are thought to display such intermediate or recalcitrant storage behaviour (Hong *et al.*, 1996, Flynn *et al.*, 2004). However, the reports on the biology of many tropical tree seeds, including those of *Lannea microcarpa*, *Sclerocarya birrea and Khaya senegalensis* are rather contradictory. This may suggest that the difficult storage behaviour of these seeds is caused by reasons other than desiccation intolerance and/or sensitivity to subzero (°C) storage temperatures. For example, for neem seeds, the difficult storage behaviour could largely be explained by the sensitivity to chilling /sub-zero (°C) temperatures and stresses associated with rehydration in water (imbibition) (Sacandé *et al.*, 1998; Sacandé, 2000), rather than intolerance to water loss as initially thought.

In the work presented in this thesis, seeds of neem (*Azadirachta indica*), *Lannea microcarpa*, *Sclerocarya birrea and Khaya senegalensis* from Burkina Faso were investigated for their sensitivity to dehydration and survival under different storage conditions. The results indicate that unlike in neem, where sensitivity to low imbibition

temperatures is the main factor limiting survival of the dried seed, various types of dormancy could be held responsible for the difficult storage behaviour of *Lannea microcarpa and Sclerocarya birrea* seeds. It was found that seeds of *Khaya senegalensis* have no particular sensitivity to desiccation or sub-zero (°C) storage temperatures, but appear to deteriorate faster when stored at 4°C than at -20, 15, or 20°C.

Desiccation tolerance and storability of the seeds

The criterion used to determine whether or not a seed is desiccation-sensitive is usually the capability of the rehydrated seed to germinate normally. Considering this criterion, the seeds of the species dealt with in this thesis had various responses to water reduction.

In neem (*Azadirachta indica*) a noticeable decrease in germination percentage was observed when the MC of the seeds was reduced from 27 to 5% (Neya *et al.*, 2004, Chapter 2). This reduction may be interpreted as intolerance of desiccation. However, when the seeds were rehydrated at elevated temperatures (≥25°C) for one hour prior to incubation at 30°C, seeds with MCs ranging from 27 to 5% all germinated at similarly high percentages (>85%). This seed is therefore desiccation tolerant, but during drying its germination requirements appeared to change. Changes in germination requirements after drying have been observed in other species such as cassava (Roberts *et al.*, 1984) and *Carica papaya* (Wood *et al.*, 2000). The difficult storage behaviour reported for neem seed may thus be attributed to sensitivity to imbibitional stress as previously reported (Sacandé *et al.*, 1998). This sensitivity increased considerably with seed ageing (Neya *et al.*, 2004; Chapter 2).

S. birrea seeds had low initial germination regardless of maturity stage of the fruits from which they were processed. However, during drying, germination ability improved substantially in all seed lots (Chapter 3). Improvement of germination with drying is completely in contrast with what would happen in a desiccation-sensitive seed. After an initial decrease, germinability increased to about 80% during storage, independently of storage temperature (Chapter 3). This high germination capability was at least maintained for 15 months of storage at all tested temperatures (-20, 4, 15 and 25°C). Seeds of *S. birrea* thus behave as orthodox seeds, but dormancy is involved in their germination behaviour.

Seeds of *L. microcarpa*, which were collected in three successive years, were extremely variable as to their germinability and storability. Seeds collected in 2002 and 2003 had a generally low germination, and the 2003 seeds were not particularly sensitive to

dehydration. In contrast, the seeds from the 2004 accession had an initially high germination that considerably decreased upon drying to approx. 0.08 g H_2O.g DM^{-1} (Chapter 4). This feature, of the latter accession particularly, would suggest sensitivity to desiccation, as has been claimed earlier (Hong *et al.*, 1996). However, when mechanically scarified prior to the germination test, all seed batches germinated at high percentages (>80%), regardless of their MC. Similarly high germination percentages were obtained when scarification was applied to seeds stored at 15°C for up to 25 months, or at -20°C for one year (Chapter 4). Therefore, the initially low germination observed in the 2002 and 2003 collections cannot be attributed to low vigour, but to differences in dormancy. Several factors, such as seed provenance, change in environmental conditions from year to year, and seed collection and processing may introduce variation in seed coat properties and, consequently, in the intensity of the germination constraint imposed. The difference in the intensity of such constraints between seed accessions and among seed lots is likely the cause of the apparent controversial reports about the seed biology. The results of the current study clearly demonstrate that drying does not lead to death of the seeds and that storage is possible at a wide range of temperatures (-20 to 15°C) for at least one year without reduction in viability. Therefore, we conclude that *L. microcarpa* seeds display orthodox storage behaviour and that dormancy is involved in the generally low and variable germination capability. The types of dormancy involved in *S. birrea* and *L. microcarpa* seeds are discussed the next section.

In the case of *K. senegalensis*, two seed batches were investigated in this study. The MC of both seed lots at harvest was low (<0.1g H_2O g DM^{-1}) and further reduction of the MC to 0.04g H_2O g DM^{-1} did not affect the initially high germinability (90-100%). The low MC at harvest and the capability to withstand further drying are an indication that this seed has potentially orthodox storage behaviour. During storage a relatively fast viability loss was observed for seeds stored at 4°C in comparison with those stored at -20, 15 and 20°C. This suggests the seeds to be more sensitive to low above-zero (°C) temperatures than to subzero (°C) and ambient temperatures (Chapter 6). A possible mechanism of such fast deterioration at low above-zero (°C) temperatures is discussed later. Our results contrast with those of Hong and Ellis (1998), who reported intermediate storage behaviour because of intolerance of subzero (°C) storage temperatures, but corroborate to a certain extent the results of Gaméné and Eriksen (2004), who reported that *K. senegalensis* seeds display orthodox storage behaviour.

Germination and dormancy in *S. birrea* and *L. microcarpa* seeds

The poor initial germination observed in *S. birrea* seeds may be attributed to embryo immaturity when the fruits are mature. However, no significant differences were observed between the germination capabilities of seeds processed from fruits of distinct maturity stages. The dormancy occurring in this species is therefore unlikely of the morphological type. During drying, germination ability improved substantially in all seed lots. Berjak *et al*., (2004) have observed a similarly poor initial germination in fresh *Trichilia dregeana* seeds and attributed this to the involvement of a germination inhibitor. In this way, seeds that retain relatively high embryo water content are prevented from germinating prematurely. Therefore, we conclude that dormancy in *S. birrea* seeds is physiologically determined, which also is supported by after-ripening – the phenomenon of increase in germination capability with dry storage (Chapter 3). Were and Munjuga (1999) and Gaméné *et al.* (2004) have reported similar increases in some *S. birrea* seed batches following 3 - 6 months of storage under ambient conditions. The exact causes of the low initial germination capability and variability in storage time before noticeable improvement of germinability are unknown. However, the irregularity in germination behaviour has obviously contributed to the discrepancies between reports on the species seed biology.

The extreme variability of *L. microcarpa* seeds as to their germinability and storability clearly indicated that seeds experienced germination constraints in the dried condition and to different extents when fresh (Chapter 4). The underlying mechanism of such constraints was investigated using seeds processed from fully matured fruits (Chapter 5). It was observed that mechanical scarification of the coat completely overcame germination constraints, when applied at the axis-side, while scarification at the side opposite to the axis led to delayed radicle emergence backwards through the orifice in ~40% of the seeds. A study of the metabolic activity upon rehydration at 30°C indicated that the seed coat is permeable to water and respiratory gases. However, increases in water uptake and respiration were closely related to radicle emergence. We thus concluded that radicle emergence is inhibited mechanically and not by germination inhibitors or physiological dormancy.

In contrast with deeply dormant seeds from the temperate climate zones, which survive for considerable period of time in the hydrated state, it was observed that non-scarified seeds lost germination ability and cellular viability after approx 15d of moist incubation at 30°C. Dormancy is the strategy of postponing germination until a more favourable time for seedling establishment. In that respect, the loss of viability in the hydrated state after 15 days at 30°C is clearly not the strategy followed in nature. This

suggests that the loss of viability of some seeds under laboratory conditions, which has been ascribed to desiccation intolerance, is simply an artefact of forestry practice.
Physical barriers to germination may lead or may have led to confusing reports on the storage behaviour of many other tropical tree seeds including *Ekebergia capensis*, *Gmelina arborea*, and *Azadirachta indica*. For the latter species, the endocarps were removed prior to sowing in a number of studies on its seeds (Bellefontaine and Audinet, 1993; Sacandé, 2000; Neya *et al.*, 2004, Chapter 2), but without mentioning explicitly that the seed coat might have constituted a physical barrier to germination.
In contrast to the wealth of research done on the anatomy of seed coats, little has been done on seed coat compositions and the impact on germination. From the FT-IR spectra in the current study, yet we cannot ascribe the reduction in *L. microcarpa* seed germination upon drying or dry storage to changes in seed coat composition. However, we suggest that structural changes in seed coat components with drying could be involved in the reduced germinability. Considering the differences and resemblances between species in terms of seed coat composition (Chapter 5) and the key role played by the seed coat in germination, investigation of the main components of the coat may be of great importance when comparing the seed physiology of different species.

Sensitivity to imbibitional stress

The exposure of initially dry specimens to the considerable forces upon rehydration can lead to loss of plasma membrane barrier properties and subsequent increase in cellular leakage (Hoekstra and Golovina, 1999). Known as imbibitional damage or injury, the rupture of membranes is thought to be caused by the fact that membranes in the gel phase have a higher rigidity as compared to those in the liquid crystalline phase (Hoekstra and Golovina, 1999; Hoekstra *et al.*, 1999). Low rehydration temperatures and low initial moisture content of organisms are the main factors that promote membrane injury upon imbibition.
Tropical tree seeds are thought to be particularly sensitive to low imbibition temperatures because of the relatively high gel-to-liquid crystalline phase transition temperature (T_m) of their membranes (Crowe *et al.*, 1989b; Sacandé *et al.*, 2001). However, not all tropical seeds appeared to be sensitive to low imbibition temperatures, despite a similar phase transition temperature of their membranes in the hydrated state. Dry neem seeds are very sensitive to low imbibition temperatures (Chapter 2), while *L. microcarpa* seeds, even when scarified prior to soaking, are not (Chapter 4). The same is true for *S. birrea* and, to a lesser extent, for *K. senegalensis* seeds, which were insensitive to imbibitional stress (Chapters 3 and 6).

In the case of the sensitive neem seeds, it was observed that soaking in water at low temperatures resulted in a considerable reduction of germinability. Furthermore, in slightly aged seeds (10 months of dry storage at 15°C) some damaged cells were observed even when rehydration took place at elevated temperatures (30°C). However, these damaged cells were only encountered at the periphery of the seed tissues (Chapter 2). In contrast, full ageing of orthodox seeds normally leads to patches of dead cells, also in the interior of tissues, as can be made visible by tetrazolium chloride staining (ISTA, 1993). The peripheral location of the damaged cells in the slightly aged neem seeds suggests involvement of rehydration stress rather than a general effect of ageing. We therefore conclude that slight ageing increases the sensitivity to imbibitional stress.
It might be thought that imbibitional damage, leading to loss of viability, involves the demise of all the cells in a seed. However, a combination of ESR data and cryo-SEM images of cross sections of imbibitionally damaged neem seeds indicated that germination is not supported when 30% or more of axis cells have died (Chapter 2). This means that there are still 70% or less of live cells in a non-germinating seed. From this observation, it may be argued that up to this lethal threshold, seeds are able to repair the damage caused by imbibitional stress or to survive with a number of lethally damaged cells. This is in agreement with the results of Nijsse *et al.*, (2004), who have shown that in multicellular organisms, such as seeds, one or more damaged cell layers might be overcome. Differences in tolerated number of damaged cells, in intrinsic repair capabilities, or in morphology of the outer cell layers (Chapter 6) may determine the level of sensitivity of a seed to imbibitional stress.

Ageing-associated loss of viability and membrane integrity

It was noticed that contrary to the expectations, cold storage of seeds sometimes did not promote survival in dry storage, conditions at 4°C being more detrimental than at -20°C or 15°C. This was prominent in *K. senegalensis* seeds and, to a lesser extent, in *L. microcarpa* seeds. The phenomenon also has been noticed in neem (Sacandé *et al.*, 1998), *Coffea arabica* (Hong and Ellis, 2002) and *Cuphea carthagenensis* (Crane *et al.* 2006) seeds.
Viability loss associated with ageing is often attributed to the loss of membrane barrier function (Bewley and Black, 1994; Golovina *et al.*, 1997). Our analysis of membrane polar lipids indicated that the content steadily decreased with ageing (Chapter 6). It was thus observed that the loss of germinability coincided with a loss of between 15% and 25% of the polar lipids. This percentage was based on data of *K. senegalensis* seeds stored dry at 15°C and at 4°C. The free fatty acid content rose with the decrease

in polar lipid content, but since the free fatty acid also can arise from degradation of neutral lipids a critical value for free fatty acids was difficult to establish. The loss of 80% of the polar lipids in dry, 20 year-old *K. senegalensis* seeds that were already non-germinable for a long period of time did not lead to changes in the structure of membranes as could be observed by LTSEM after freeze fracturing. The membranes still gave easy passage for the fissure during fracturing, indicating that the deesterified acyl chains were still at the position they originally had in the fresh seed. This was also the case for the 4°C-stored specimens that had just become non-viable. The IMPs (transmembrane proteins) were regularly distributed in the plane of the membrane, indicating the absence of a phase separation in the dry state. This is understandable considering the fact that the membrane components are immobilized in the glassy cytoplasmic matrix, thus impeding lateral diffusion in the membrane core. The data of the current study suggest that membrane phase separation, thought to occur during viability loss, is unlikely to happen in the dry state. However, upon rehydration membrane structure is immediately lost (20 y-old) or heavily damaged (4°C) (Chapter 6).

The question remains as to how the deesterification takes place – via free radicals or by phospholipase activity. We prefer the explanation of phospholipases being active in the dried state for the following reason. With free radicals being involved one would not expect a large difference between activity at 4°C and -20°C, although the more dense glassy state at -20°C would hamper the lateral diffusion of the free radicals, thus leading to longer life span. With respect to phospholipase activity, the physical condition of the membranes might become important (Oliver *et al.*, 1995). While membranes are rigid at -20°C, they may occur in a mixed liquid crystalline – gel phase condition at 4°C as derived from the ESR data (Chapter 6). It has been shown that during a phase transition from the gel to the liquid crystalline state the activity of phospholipases becomes much greater (Oliver *et al.*, 1995, 1997). The mismatch between gel phase domains and the liquid crystalline matrix gives access to the glycerol backbone, exposing the site of action of the phospholipases. The loss of polar lipids at 4°C is greater than that at 15°C (Chapter 6), which might be explained by the fact that membranes in tropical seeds have coexisting phases at 4°C and are largely in the liquid crystalline phase at 15°C. At −20°C, membranes are in the gel phase, which hampers the activity of phospholipases because of the difficult access to the site of action, and also, the low temperature will reduce the enzyme's activity. The difference between membranes of seeds from the tropics and those from the temperate climate zone lies in the higher T_m of the membranes of the former. When, on cooling, membranes of seeds from temperate climates are half-way a phase transition, the temperature probably is too low to support the additional phospholipase activity to

cause noticeable extra deesterification and faster viability loss. In contrast, membranes of seeds from tropical plants, because of their high T_m, likely reach the mixed phase stage at temperatures that are high enough to support additional enzyme activity.

Conclusions

The current study showed that reasons other than desiccation intolerance and sensitivity to subzero °C storage temperatures may explain the difficult storage behaviour reported for *L. microcarpa* and *S. birrea* seeds. Various types of dormancy obviously are the cause of the difficult seed storage behaviour in *L. microcarpa* and *S. birrea* seeds, whereas in neem seeds imbibitional damage is the major limitation to survival after drying and dry storage. For *K. senegalensis* seeds we suspect that the sensitivity to low above zero °C storage temperatures may be the cause of the sporadic loss of viability sometimes observed in practice.

Furthermore, we think that the use of standard germination as criterion to ascertain desiccation tolerance may not be sufficiently accurate. In addition to germination assays, cellular viability tests must be considered when studying desiccation tolerance. This will help avoid some confusion as to whether death of seeds is induced by desiccation or inappropriate germination protocols, as in the case of neem and *L. microcarpa* seeds. We strongly recommend detailed investigation of the germination requirements, before and after drying, when screening any tropical tree seed for desiccation tolerance.

From the results of this project it appeared that desiccation tolerance in tropical tree seeds is more wide-spread than originally thought. Many other tropical tree species, for which difficult storage behaviour has been reported, are likely to suffer from problems other than sensitivity to desiccation and imbibitional stress. The results obtained in this study will increase and facilitate the use of these species in reforestation and agroforestry programs.

Storage of tropical tree seeds at 4°C appeared to be less effective compared to storage at -20°C or 15-20°C. This observation is of great practical interest because cold rooms are energy consuming and can thus be avoided. Moreover, the low budgets of national tree seeds programs and centres in developing countries raise difficulties in maintaining expensive and sophisticated seed storage facilities. It would therefore be more rational to encourage seed storage at ambient conditions as the life span of many tropical tree seeds appeared to be long enough under these conditions for short and medium term (1-3 years) storage purposes.

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Summary

The tropical trees, *Azadirachta indica* (neem), *Lannea microcarpa*, *Sclerocarya birrea* and *Khaya senegalensis,* are important multipurpose species. Unfortunately, difficult seed storage behaviour limits the utilization of these species in reforestation programs and agroforestry systems. This thesis presents the results of investigations aimed at a better understanding of the seed biology, particularly focussed on the improvement of seed survival after drying and subsequent dry storage. Seeds collected from several stands in Burkina Faso were studied in an attempt to elucidate the causes of viability loss and improve life span. Initially it was thought that the difficulty in storing seeds of these species is due to intolerance of desiccation and sensitivity to low (subzero °C) storage temperatures. However, the results of the current study indicated that other factors are responsible for the difficult storage behaviour of these seeds.

When the moisture content (MC) was reduced from 27 to 5%, neem seeds became sensitive to rehydration temperatures below 25°C, resulting in a noticeable decrease in germination percentage. Rehydration of these dried seeds at elevated temperatures (25-40°C) for one hour prior to further incubation at 30°C substantially improved germination to a level comparable to that of fresh seeds (27% MC). We concluded that neem seed is desiccation tolerant, but that during drying its germination requirements change, which is the main cause of its difficult storage behaviour.

Germination capability of S*. birrea* seeds was low in all seed lots at harvest, but improved substantially during drying and/or dry storage. Improvement of germination with drying is completely in contrast with what would happen in a desiccation-sensitive seed. We interpreted the observed increase in germination during drying and dry storage as resulting from the slow disappearance of an initially present physiological dormancy. Seeds of *S. birrea* thus behave as orthodox seeds, but the irregularity in their germination behaviour has obviously contributed to discrepancies between reports on the species seed biology.

Seeds of *L. microcarpa* harvested in three successive years were extremely variable as to their germinability and storability. Some seed accessions had a generally low germination, while others had an initially high germination. Drying did not affect germination capability of the poorly germinating seed accessions, but did so in the well germinating accessions. These observations would suggest that seeds can be either tolerant or sensitive to desiccation. However, when mechanically scarified prior to germination tests, seeds from all accessions germinated at high percentage (>80%), regardless of MC and age. This indicates that seeds experienced germination constraints upon drying rather than being sensitive to desiccation. Imbibed, intact seeds that failed to germinate displayed a moderate rate of respiration with RQ values

< 1, which indicates that the seed coat is permeable to water and respiratory gases. On scarification, water uptake and respiration steeply rose in narrow association with radicle emergence. Non-scarified seeds lost germination ability and cellular viability after approx 15d of moist incubation at 30°C. Apparently, coat-imposed inhibition of germination does not prolong viability of seeds in the hydrated state. These results suggest that radicle emergence is inhibited mechanically and not by germination inhibitors or physiological dormancy. We conclude that *L. microcarpa* seeds display orthodox storage behaviour, but that physical dormancy explains the difficult seed storage behaviour reported for this species.

In the case of *K. senegalensis*, the MC of seeds at harvest was low (<0.1g H_2O g DM^{-1}) and further drying did not affect the initially high germinability (90-100%). During storage a relatively fast viability loss was observed for seeds stored at 4°C in comparison with seeds stored at -20, 15, and 20°C. The sensitivity to low above-zero (°C) temperatures might be the cause of the erratic loss of viability often observed in these seeds.

We concluded that seeds of all the species investigated in this thesis display orthodox storage behaviour, but that various factors including sensitivity to imbibitional stress, physiological and physical dormancy, and sensitivity to low above-zero (°C) storage temperatures cause the reported difficulties in storing these seeds.

Tropical tree seeds are thought to be particularly sensitive to low imbibition temperatures because of the relatively high gel-to-liquid crystalline phase transition temperature (T_m) of their membranes as compared to the relatively low T_m of membranes of temperate zone seeds. In relation with this, the sensitivity of the four seed species to rehydration at low temperatures was investigated. The results indicated that dried neem seeds are particularly sensitive to imbibitional stress, whereas the seeds of the other species appeared to be less (*K. senegalensis*) or not (*L. microcarpa* and *S. birrea*) sensitive to low imbibition temperatures. This difference in sensitivity to imbibitional stress was observed despite comparable T_m of membranes in the roots of seedlings of *K. senegalensis*, *L. microcarpa* and neem (11.5, 14.5 and 9.6°C, respectively). The hypothesis of tropical tree seeds being particularly sensitive to imbibitional stress because of their high membrane T_m therefore does not have general validity. LTSEM images of cryo-planed *K. senegalensis* seed embryos revealed the presence of a dense peripheral cell layer, which might have slowed water uptake and reduced sensitivity to imbibitional stress. On the other hand, for the sensitive neem seeds, it was observed that the loss of germinability of imbibitional damaged seeds did not involve death of all the cells. An assessment of membrane integrity by ESR and cryo-SEM indicated that seed germination is not supported when approx. 30% or more of axis cells have died. This supposes that there are still on average 70% or less of live cells in a

non-germinating seed. From this observation, we argue that up to this lethal threshold, seeds are able to repair the damage caused by imbibitional stress or survive with a number of damaged cells. We suggest that differences in tolerated number of damaged cells, in intrinsic repair capabilities, or in morphology of the outer cell layers determine the level of sensitivity of seeds to imbibitional stress.

Low temperatures are expected to better maintain viability of seeds in dry storage than do high temperatures. However, we noticed that storage conditions at 4°C were more detrimental for *K. senegalensis* seeds than at -20°C or 15°C, and this was, to a lesser extent, true for *L. microcarpa* seeds. Because viability loss associated with ageing is often attributed to the loss of membrane barrier function, membrane polar lipids (PLs) were analysed in several *K. senegalensis* seed lots differing in age and viability. The data indicated that the content of membrane PLs steadily decreased with ageing. However, it appeared that seeds can withstand the loss of a certain amount of PLs without losing germinability. Based on data of seeds stored dry at 15°C and at 4°C, it was observed that the loss of germinability coincided with a loss of between 15% and 25% of the PLs. With the decrease in PL content, the free fatty acid content rose. Because the free fatty acid also can arise from degradation of triacylglycerols, it was difficult to establish a critical content of accumulated free fatty acids associated with viability loss.

Chemical analysis of membranes in 20 year-old dry-stored *K. senegalensis* seeds, which were already non-germinable for a long period of time, indicated a loss of 80% of the PLs. However, this did not lead to changes in the structure of the plasma membranes in the dried axis as could be observed by high resolution SEM after freeze fracturing. This was interpreted to mean that the deesterified acyl chains were still at the position, at which they originally occurred in the membranes of the fresh seed. This was also the case for the 4°C-stored dried specimens that had just become non-viable. The IMPs (transmembrane proteins) were regularly distributed in the plane of the membrane, indicating the absence of a phase separation in the dry state. The data of the current study suggest that membrane phase separation, thought to occur during viability loss, is unlikely to happen in the dry state. However, at rehydration the plasma membranes suffered considerable damage or ceased existing.

The question as to how the deesterification takes place – via free radical activity or by phospholipase activity – was addressed. With free radicals being involved one would not expect a greater amount of acyl chains to be split off at 4°C than at 15°C. However, since the physical state of the membrane determines the activity of phospholipases, we focussed on the membrane phase issue. The mismatch between gel phase domains and the liquid crystalline matrix at mid-melting temperature gives better access to the site of action, thus increasing the activity of phospholipases. At

either the gel or liquid crystalline phase, accessibility to the site of action is more difficult. An ESR spin label study indicated that membranes in dried and rehydrated axes of *K. senegalensis* seeds were rigid at −20°C, while at 7°C they appeared to be in a mixed liquid crystalline – gel phase condition. The liquid crystalline phase prevailed at 15°C. Thus, it can be expected that during dry storage at 4°C the mixed phase can occur for extended periods of time. Therefore, we suggest that the deesterification of acyl chains leading to the loss of viability of seeds at low above zero (°C) temperatures occurs, at least partly, via phospholipase activity. Because of their high T_m, membranes of tropical seeds are likely to reach mid melting condition at low above zero (°C) temperatures that are high enough to support enzyme activity. By contrast, it is expected that the lower mid-melting temperatures of seeds from the temperate climate zones are too low to effectively support phospholipase activity at sub-zero (°C) temperatures. This special feature of tropical seeds dismisses the use of cold rooms for short and medium term (1-3 years) storage purposes.

The results obtained in this study will increase and facilitate the use of these species in reforestation and agroforestry programs in Burkina Faso and other Sahelian countries.

Résumé

Les arbres tropicaux, *Azadirachta indica* (neem), *Lannea microcarpa*, *Sclerocarya birrea* et *Khaya senegalensis,* sont des espèces à usages multiples. Malheureusement, des difficultés dans la conservation des semences de ces espèces limitent leur utilisation dans les programmes de reforestation et les systèmes d'agroforesterie. Cette thèse présente les résultats d'investigations visant une meilleure compréhension de la biologie de leurs semences, axées en particulier sur l'amélioration de la survie des semences après le séchage et la conservation subséquente en milieu sec. Des semences collectées dans différents peuplements au Burkina Faso ont été étudiées dans le but d'élucider les causes de leur perte de viabilité, et d'améliorer leur longévité. Initialement il était pensé que la difficulté à conserver les semences de ces espèces était due à une intolérance à la dessiccation, et à une sensibilité aux basses températures de conservation (en dessous de zéro °C). Cependant, les résultats de la présente étude ont indiqué que d'autres facteurs sont responsables du caractère difficile de la conservation de ces semences.

Lorsque la teneur en eau (TE) était réduite de 27 à 5%, les semences de neem devenaient sensibles aux températures de réhydratation inférieures à 25°C. Ce qui se traduisait par une baisse notable du pourcentage de germination. La réhydratation de ces semences sèches à des températures élevées (25 à 40°C) pendant une heure suivie d'une incubation à 30°C améliorait substantiellement la germination à un niveau comparable à celui des semences fraîches (27%, TE). Nous avons donc conclu que la semence de neem tolère la dessiccation. Cependant, ses exigences de germination changent pendant le séchage. Ce qui est la principale cause du caractère difficile de sa conservation.

La capacité germinative des semences de S*. birrea* était basse dans tous les lots de semences à la récolte, mais a accru substantiellement au cours du séchage et/ou de la conservation à l'état sec. L'amélioration de la germination au cours du séchage contraste complètement avec ce qui se serait passé pour une semence sensible à la dessiccation. Nous avons interprété cette amélioration de la germination au cours du séchage et la conservation à l'état sec comme étant le résultat de la levée lente d'une dormance physiologique initialement présente dans les semences. Les semences de *S. birrea* se comportent donc comme des semences orthodoxes mais l'irrégularité de leur comportement germinatif a certainement contribué aux contradictions entre les écrits sur la biologie des semences de l'espèce.

Les semences de *L. microcarpa* récoltées pendant 3 années consécutives étaient extrêmement variables en ce qui concerne leur germination et leur pouvoir de conservation. Certaines collections de semences avaient un niveau général de

germination bas, tandis que d'autres avaient un pourcentage initial de germination élevé. Le séchage n'a pas affecté la capacité germinative des semences à faible germination initiale, contrairement aux semences à bonne germination initiale, dont la capacité germinative a été affectée au cours du séchage. Ces observations pourraient laisser penser que les graines peuvent être soit tolérantes ou sensibles à la dessiccation. Toutefois, quand les semences étaient mécaniquement scarifiées avant les tests de germination, tous les lots germaient à des pourcentages élevés (80%), quels que soit la TE et l'âge des semences. Cela montre que les semences faisaient face à des contraintes de germination au cours du séchage plutôt que d'être sensibles à la dessiccation. Les semences intactes et imbibées qui n'avaient pas pu germer, présentaient un taux de respiration modéré avec un quotient respiratoire (QR) <1, ce qui démontre que les téguments de la semence sont perméables à l'eau et aux gaz respiratoires. Lorsque les semences étaient scarifiées, l'absorption d'eau et la respiration augmentaient subitement en relation étroite avec l'émergence de la radicule. Les semences non scarifiées ont perdu leur habileté à germer et leur viabilité cellulaire après approximativement 15 jours d'incubation humide à 30°C. Apparemment l'inhibition de la germination imposée par le tégument ne prolonge pas la viabilité des semences à l'état hydraté. Ces résultats montrent que l'émergence de la radicule est inhibée mécaniquement et non pas par des inhibiteurs de germination ou une dormance physiologique. Nous concluons que les semences de *L. microcarpa* se comportent comme des semences orthodoxes, mais qu'une dormance physique explique les difficultés de conservation rapportées pour les semences de l'espèce.

Dans le cas de *K. senegalensis*, la teneur en eau de ces semences à la récolte était bas (<0.1g H_2O g MS^{-1}) et un séchage poussé n'a pas affecté leur bonne germination initiale (90-100%). Au cours de la conservation, une perte relativement rapide de viabilité a été observée pour les semences conditionnées à 4°C en comparaison avec celles stockées à – 20, 15, et 20°C. La sensibilité aux basses températures au-dessus de zéro °C pourrait être la cause de la perte erratique de viabilité souvent observée dans ces semences.

Nous concluons que les semences de toutes les espèces étudiées dans cette thèse présentent des comportements de semences orthodoxes. Mais divers facteurs notamment la sensibilité au stress d'imbibition, des dormances physiologique et physique, la sensibilité aux basses températures de conservation au-dessus de zéro °C sont les causes de la difficulté rencontrée dans la conservation de ces semences.

Les semences des arbres tropicaux sont supposées être particulièrement sensibles aux basses températures d'imbibition à cause de la température relativement élevée de fusion (T_m) de leurs membranes, comparativement à celle relativement basse des membranes des semences des zones tempérées. En relation avec cette supposition, la

sensibilité des semences des quatre espèces aux basses températures d'imbibition a été investiguée. Les résultats ont indiqué que les semences sèches de neem sont particulièrement sensibles au stress d'imbibition. Tandis que les semences de *K. senegalensis* sont apparues être moins sensibles, et celles de *L. microcarpa* et *S. birrea* non sensibles aux basses températures d'imbibition.

Cette différence de sensibilité au stress d'imbibition a été observée en dépit de T_m des membranes comparables dans les racines de plantules de *K. senegalensis. L. microcarpa* et de neem (11.5, 14.5 and 9.6°C, respectivement). L'hypothèse que les semences des arbres tropicaux seraient particulièrement sensibles au stress d'imbibition à cause de la T_m élevée de leurs membranes n'a donc pas de validité générale. Des images de LTSEM de cryosections d'embryons de semences de *K. senegalensis* ont révélé la présence d'une dense couche périphérique de cellules qui pourrait avoir régulé l'absorption d'eau et réduit la sensibilité aux stress d'imbibition.

D'autre part, pour les semences sensibles de neem, il a été observé que la perte de la capacité germinative des semences au cours du stress d'imbibition n'implique pas la mort de toutes les cellules. Une estimation de l'intégrité des membranes par ESR et cryo-SEM a indiqué que la germination des semences n'est plus soutenue lorsque approximativement 30% ou plus des cellules de l'axe embryonnaire sont endommagées. Cela suppose qu'il y a toujours en moyenne 70% ou moins de cellules vivantes dans l'axe d'une semence qui ne germe pas. De cette observation, nous stipulons qu'en dessous de ce seuil létal, les semences sont capables de réparer les dommages causés par le stress d'imbibition ou de survivre avec un certain nombre de cellules mortes. Nous suggérons que les différences dans le nombre toléré de cellules endommagées, la capacité intrinsèque de réparation, ou la morphologie des couches externes de cellules, déterminent le niveau de sensibilité des semences au stress d'imbibition.

Les basses températures sont supposées mieux maintenir la viabilité des semences au cours de la conservation à l'état sec que les hautes températures. Cependant nous avons observé que les conditions de conservation à 4°C étaient plus destructives pour les semences de *K. senegalensis* que celles à -20°C ou 15°C, et cela était à un degré moindre, aussi le cas pour les semences de *L. microcarpa.* Parce que la perte de viabilité associée au vieillissement est souvent attribuée à la perte de la fonction de barrière de la membrane, les lipides polaires (LPs) ont été analysés dans plusieurs lots de semences de *K. senegalensis* qui diffèrent en âge et en viabilité. Les résultats ont indiqué que les contenus de LPs des membranes décroissaient subitement avec le vieillissement. Néanmoins, il est apparu que les semences peuvent supporter la perte d'une certaine quantité de LPs sans perdre leur capacité à germer.

Sur la base des données obtenues de semences conservées sèches à 15°C et à 4°C, il a été observé que la perte du pouvoir germinatif coïncide avec une diminution de LPs située entre 15 et 25%. Pendant que le contenu en PLs diminuait, celui en acides gras libres (AGLs) augmentait. Mais comme les AGLs peuvent aussi provenir de la dégradation des triacylglycerols, il était difficile d'établir une quantité critique d'accumulation d'AGLs associée à la perte de viabilité.

L'analyse chimique des membranes de semences sèches de *K. senegalensis* conservées pendant 20 ans a indiqué une perte de 80% de leurs LPs. Ces semences avaient perdu leur pouvoir germinatif depuis une longue période. Cependant cette perte en LPs n'a pas conduit à des changements dans la structure des membranes dans les axes embryonnaires à l'état sec comme l'on a pu l'observer par SEM à haute résolution après cryofracture. Cela suppose que les chaînes déestérifiées d'acyles étaient toujours à la position dans laquelle elles se trouvaient originellement dans les membranes de la semence fraîche. Cela était aussi le cas pour les spécimens conservés secs à 4°C qui venaient juste de perdre leur viabilité. Les protéines transmembranaires (PTMs) étaient régulièrement distribuées dans le plan de la membrane, indiquant l'absence d'une séparation de phases à l'état sec. Les données de la présente étude suggèrent que la séparation de phases de la membrane, supposée s'opérer au cours de la perte de viabilité, est invraisemblable à l'état sec. Toutefois, au cours de la réhydratation, les plasmas membranaires ont subi des dommages considérables et ont disparu.

La question à savoir comment la déestérification prend place – via l'activité des radicaux libres ou par l'activité de phospholipases – a été adressée. Avec une implication des radicaux libres, nul ne pourrait espérer un plus grand détachement de chaînes d'acyles à 4°C qu'à 15°C. Mais, surtout que l'état physique de la membrane détermine l'activité des phospholipases, nous nous sommes focalisés sur la question des phases des membranes. Le mélange entre le domaine de la phase gel et la matrice du liquide cristalline à la température de mi-fusion donne un meilleur accès au site d'action et augmente ainsi l'activité des phospholipases. Que se soit dans la phase gel ou liquide cristallin l'accessibilité au site d'action est plus difficile. Une étude par ESR a indiqué que les membranes dans les axes embryonnaires secs et réhydratés des semences étaient rigides à -20°C, tandis qu'à 7°C elles apparaissaient dans une mixture de phases liquide cristallin – gel. La phase liquide cristalline prévalait à 15°C. Ainsi, il peut être supposé que pendant la conservation à l'état sec à 4°C, le mélange de phases se produise pendant des périodes étendues. Par conséquent, nous suggérons que la déestérification des chaînes d'acyles conduisant à la perte de viabilité des semences aux basses températures au-dessus de zéro (°C) se produise au moins partiellement via l'activité de phospholipases.

A cause de leur température de fusion élevée, les membranes des semences tropicales atteignent apparemment la condition de mi-fusion aux basses températures au-dessus de zéro (°C) qui sont suffisamment élevées pour permettre l'activité enzymatique. A l'opposée, il est supposé que les plus basses températures de mi-fusion des membranes des semences des zones de climat tempéré sont très basses pour permettre effectivement l'activité d'une phospholipase aux températures en dessous de zéro (°C). Ce trait spécial des semences tropicales rend désuète l'utilisation des chambres froides pour les besoins de conservation de semences à court et moyen termes (1-3 ans).

Les résultats obtenus dans cette étude vont accroître et faciliter l'utilisation de ces espèces dans les programmes de reforestation et d'agroforesterie au Burkina Faso et dans d'autres pays Sahéliens.

Samenvatting

Azadirachta indica (neem), *Lannea microcarpa*, *Sclerocarya birrea* en *Khaya senegalensis* zijn boomsoorten uit de tropen die van belang zijn vanwege hun veelvoudige toepassingen. Helaas wordt hun gebruik in de bosbouw en herbebossingsprogramma's beperkt doordat de zaden moeilijk te bewaren zijn. Dit proefschrift beschrijft de resultaten van onderzoek dat tot doel had om een beter begrip te krijgen van de zaadbiologie van deze boomsoorten, met als centraal thema de verbetering van overleving na drogen en lange houdbaarheid in droge toestand. Zaden, verzameld op verschillende standplaatsen in Burkina Faso, werden bestudeerd in een poging om de oorzaken van vitaliteitsverlies te achterhalen en de levensduur te verlengen. Het oorspronkelijke idee was dat de problemen met de zaadbewaring van deze soorten het gevolg zijn van gebrekkige tolerantie voor uitdrogen en gevoeligheid voor lage bewaartemperaturen (beneden nul °C). De resultaten van de huidige studie laten echter zien dat andere factoren verantwoordelijk zijn voor het moeizame bewaargedrag van deze zaden.

Bij daling van het vochtgehalte van 27 tot 5%, werden neemzaden gevoelig voor rehydratatie bij temperaturen lager dan 25°C, wat resulteerde in een aanzienlijke daling van het kiempercentage. Rehydratatie bij verhoogde temperaturen (25-40°C) gedurende één uur voorafgaand aan verdere incubatie bij 30°C leidde tot een aanzienlijke verbetering van de kieming tot percentages die vergelijkbaar zijn met die van pas geoogste zaden (vochtgehalte 27%). We hebben dan ook geconcludeerd dat neemzaden uitdroogtolerant zijn, maar dat de vereisten voor kieming veranderen gedurende het drogen. Dit lijkt de hoofdoorzaak te zijn voor het moeizame bewaargedrag van neemzaden.

De kiemkracht van vers geoogste zaden van S*. birrea* was laag in alle zaadpartijen, maar verbeterde aanzienlijk gedurende drogen en/of droge bewaring. Verbetering van de kieming tijdens drogen is precies het omgekeerde van wat kan worden verwacht wanneer de zaden uitdrooggevoelig zijn. We interpreteren de waargenomen verbetering van de kieming gedurende drogen en droge bewaring als het gevolg van het langzaam verdwijnen van een oorspronkelijk aanwezige fysiologische kiemrust. Zaden van *S. birrea* gedragen zich dus als orthodoxe zaden. Echter, de variatie in hun kiemgedrag heeft klaarblijkelijk bijgedragen aan de controverse tussen rapporten aangaande de zaadbiologie van deze soort.

Zaden van *L. microcarpa* die geoogst werden in drie opeenvolgende jaren waren extreem variabel wat betreft hun kiemkracht en bewaarbaarheid. Sommige zaadaccessies hadden een lage kiemkracht, terwijl andere een initieel hoge kiemkracht hadden. Drogen leidde niet tot een verandering van de kiemkracht van de slecht

kiemende zaden, maar wel in het geval van de zaden met een initieel goede kiemkracht. Deze waarnemingen zouden suggereren dat zaden van verschillende accessies ofwel uitdroogtolerant kunnen zijn, ofwel uitdrooggevoelig. Echter, wanneer voorafgaand aan de kiemtest mechanisch insnijden werd toegepast, kiemden veel zaden goed (percentage >80%), ongeacht vochtgehalte en leeftijd. Dit geeft aan dat de zaden door drogen veeleer een inperking van de kieming ondervonden dan dat ze uitdrooggevoelig waren. Intacte zaden die na wateropname niet konden kiemen hadden een bescheiden ademhaling met RQ waarden < 1, hetgeen aangeeft dat de zaadhuid doorlaatbaar is voor water en ademhalingsgassen. Als gevolg van mechanisch insnijden van de zaadhuid stegen de wateropname en de ademhaling sterk, in nauwe samenhang met het tevoorschijn komen van het wortelje. Niet-ingesneden zaden verloren hun kiemkracht en cellulaire levensvatbaarheid na ongeveer 15 dagen vochtige incubatie bij 30°C. Klaarblijkelijk kan de kiemremming die door de zaadhuid wordt veroorzaakt de levensduur van geimbibeerde zaden niet verlengen. Deze resultaten suggereren dat het tevoorschijn komen van het wortelje door de zaadhuid geremd wordt en niet door kiemremmers of fysiologische kiemrust. We concluderen dat *L. microcarpa* zaden orthodox bewaargedrag vertonen, maar dat fysische kiemrust ten grondslag ligt aan het moeizame bewaargedrag dat voor deze soort is gerapporteerd.

In het geval van *K. senegalensis* was het vochtgehalte van pas geoogste zaden laag (<0.1g $H_2O \cdot g$ droge stof $^{-1}$), en verder drogen leidde niet tot vermindering van de oorspronkelijk hoge kiemkracht (90-100%). Gedurende bewaring werd een relatief snel vitaliteitsverlies waargenomen voor zaden bewaard bij 4°C in vergelijking met zaden bewaard bij -20, 15, en 20°C. De gevoeligheid voor lage boven nul (°C) temperaturen zou de oorzaak kunnen zijn voor het onvoorspelbare verlies van levensvatbaarheid dat vaak is waargenomen bij deze zaden.

We concluderen dat de zaden van alle soorten die in dit proefschrift werden bestudeerd orthodox bewaargedrag vertonen, maar dat verschillende factoren waaronder gevoeligheid voor imbibitiestress, fysiologische en fysische kiemrust, en gevoeligheid voor lage boven nul (°C) bewaartemperaturen de oorzaak zijn voor de gerapporteerde moeilijkheden bij hun bewaring.

Tropische boomzaden worden geacht bijzonder gevoelig te zijn voor lage imbibitietemperaturen vanwege hun relatief hoge gel-naar-vloeibaar-kristallijne fase-transitietemperatuur (T_m) van hun membranen; dit in vergelijking mat de relatief lage T_m van membranen in zaden uit de gematigde klimaatzone's. In verband hiermee werd de gevoeligheid van de vier zaadsoorten onderzocht voor rehydratatie bij lage temperaturen. De resultaten wezen erop dat gedroogde neemzaden bijzonder gevoelig zijn voor imbibitiestress, terwijl de zaden van de andere soorten dat minder (*K.*

senegalensis) of niet (*L. microcarpa* and *S. birrea*) zijn. Dit verschil in gevoeligheid voor imbibitieschade werd waargenomen ondanks vergelijkbare T_m van membranen in the wortels van zaailingen van *K. senegalensis*, *L. microcarpa* en neem (respectievelijk 11.5, 14.5 en 9.6°C). De hypothese dat zaden van tropische bomen bijzonder gevoelig zijn voor imbibitiestress vanwege hun hoge membraan T_m heeft dus geen algemene geldigheid. LTSEM plaatjes van cryo-planed, *K. senegalensis* embryos lieten de aanwezigheid zien van een dichte perifere cellaag die wellicht de wateropname kan vertragen en de gevoeligheid voor imbibitiestress kan verminderen. Anderzijds bleek dat het verlies van kiemkracht van door imbibitie beschadigde neemzaden veroorzaakt wordt door de dood van slechts een beperkt aantal cellen. Een beoordeling van de membraanintegriteit met ESR en cryo-SEM gaf aan dat zaadkieming faalt als ongeveer 30% of meer van de cellen in de embryo-as zijn dood gegaan. Dit veronderstelt dat er gemiddeld nog 70% of minder levende cellen zijn in een niet-kiemend zaad. Op grond van deze waarneming beredeneren we dat tot deze lethale drempel zaden in staat zijn om de schade te repareren of te overleven met een aantal beschadigde cellen. We suggereren dat de gevoeligheid van zaden voor imbibitiestress bepaald wordt door verschillen in te tolereren aantal beschadigde cellen, in intrinsieke herstelcapaciteit, of in morfologie van de buitenste cellagen.

Het kan worden verwacht dat lage temperaturen de levensvatbaarheid van zaden in droge bewaring beter kan behouden dan hoge temperaturen. We hebben echter waargenomen dat bewaring bij 4°C schadelijker is voor *K. senegalensis* zaden dan bij -20°C of 15°C, en dit gold in mindere mate ook voor *L. microcarpa* zaden. Omdat vitaliteitsverlies ingevolge veroudering vaak wordt toegeschreven aan verlies van de barrierefunctie van membranen, werden de polaire lipiden (PL) geanalyseerd in verschillende *K. senegalensis* zaadpartijen die verschilden in ouderdom en levensvatbaarheid. De gegevens gaven aan dat het gehalte aan membraan PL geleidelijk daalde met de leeftijd. Het bleek echter dat zaden een verlies van een zekere hoeveelheid PL kunnen tolereren, zonder de kiemkracht te verliezen. Gebaseerd op data van droge zaden bewaard bij 15°C en 4°C bleek het verlies van kiemkracht samen te vallen met een verlies aan PL van tussen de 15% en 25%. Tegelijk met de daling van het PL-gehalte rees het gehalte aan vrije vetzuren. Omdat de vrije vetzuren ook afkomstig kunnen zijn uit degradatie van triacylglycerols was het moeilijk om een kritieke hoeveelheid geaccumuleerde vrije vetzuren te kunnen vaststellen die geassocieerd is met vitaliteitsverlies.

Chemische analyse van membranen in 20-jaar oude, droog opgeslagen *K. senegalensis* zaden die al lange tijd niet meer kiemden, duidde op een verlies van 80% van de PL. Dit leidde echter niet tot veranderingen in de structuur van de plasmamembraan in de gedroogde embryo-assen, zoals kon worden aangetoond met behulp van hoge-

resolutie-SEM na vriesbreken. Dit betekent volgens ons dat de gedeesterifieerde acylketens nog steeds op de plaats waren waar ze oorspronkelijk voorkwamen in de membranen van het verse zaad. Dit was ook het geval voor het bij 4°C bewaarde monster dat juist de kiemkracht had verloren. De IMPs (trans-membraaneiwitten) waren regelmatig verdeeld in het vlak van de membraan, wat aangeeft dat er in de droge toestand geen fasenscheiding is opgetreden. De gegevens van de huidige studie suggereren dat het onwaarschijnlijk is dat fasenscheiding van membraancomponenten optreedt in de droge toestand. Eerder werd gedacht dat dit mogelijk zou zijn gedurende verlies van levensvatbaarheid. Echter zodra water toetreedt lijden de plasmamembranen aanzienlijke schade of ze houden op te bestaan.

De vraag hoe deesterificatie tot stand komt – via vrije-radicaalactiviteit of door phospholipase-activiteit – werd aangepakt. Als het vrije radicalen betreft zou men niet een grotere hoeveelheid afgesplitste acyl ketens verwachten bij 4°C dan bij 15°C. Echter, omdat de fysische staat van de membranen bepalend is voor de activiteit van fosfolipases, hebben we ons gericht op de membraanfase problematiek. De gebrekkige aansluiting tussen gelfase gebieden en de vloeibaar-kristallijne matrix halverwege de fasetransitie geeft een betere toegangkelijkheid tot de plaats van aangrijpen, wat de activiteit van fosfolipases vergroot. Zowel in de gelfase als de vloeibaar kristallijne fase is de toegangkelijkheid tot de plaats van aangrijpen moeilijker. Een ESR spin label studie gaf aan dat de membranen in gedroogde en bevochtigde embryo-assen van *K. senegalensis* zaden star waren bij −20°C, terwijl ze bij 7°C halverwege hun transitie waren. De vloeibaar kristallijne fase had de overhand bij 15°C. Het kan dus worden verwacht dat gedurende bewaring bij 4°C de gemengde fase langdurig kan blijven bestaan. We suggereren daarom dat de deesterificatie van acyl ketens die leiden tot vitaliteitsverlies van zaden bij lage boven nul (°C) temperaturen ten minste gedeeltelijk plaats vindt via fosfolipase-activiteit. Vanwege hun hoge T_m, kan worden verwacht dat membranen van tropische zaden de fasetransitie bereiken bij lage boven nul (°C) temperaturen die hoog genoeg zijn om enzymactiviteit te onderhouden. Het kan worden verwacht dat de algemeen lagere transitietemperaturen van zaden afkomstig uit de gematigde temperatuurszone's te laag zijn om effectief de fosfolipase-activiteit te onderhouden bij temperaturen beneden nul (°C). Deze speciale eigenschap van tropische zaden maakt het gebruik van koude kamers voor de korte en half lange (1-3 jaren) bewaring ongewenst.

De resultaten verkregen in deze studie zullen leiden tot toename en vergemakkelijking van het gebruik van deze soorten in herbebossings- en bosbouwprogramma's in Burkina Faso en andere landen in de Sahel.

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This dream would not have come to my mind without the chance that my father gave me to go to school and without the indefectible support of my mother and my whole family. This thesis is first of all dedicated to you. Thanks for your support.

A great achievement is hardly the result of efforts of only one person, and this PhD project does not make exception. Therefore, I would like to express my gratitude to all people who have contributed in one way or another to the successful work presented in this thesis.

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Neya, O., Lompo, D., Kenny, S. L., Neya, B. and Kaboré, C. (1997) Elaboration d'un tarif de cubage des espèces ligneuses dans la forêt classée du Nazinon. Rapport de stage 3ème année, IDR-Université Polytechnique de Bobo Dioulasso, Burkina Faso.

Neya, O. (1999) Etude des stades de développement des fruits de neem (Azadirachta indica A. Juss.) Memoire de fin de cycle, IDR, Université Polytechnique de Bobo Dioulasso, Burkina Faso.

Neya, O. et Gaméné, C. S. (2001) Méthodes traditionnelles de conservation des semences dans 19 terroirs villageois au Burkina Faso, Rapport d'etude, Centre National de Semences Forestières, Ouagadougou, Burkina Faso.

Neya, O., Gaméné, C. S., Ilboudo, J. B., Ouédraogo, G. L. and Sacandé, M. (2003) Effect of developmental stages on Neem (Azadirachta indica) seed storage, In Seed Conservation: Turning Science into Practice, pp. 151-162. Smith, R.D., Dickie, J.B., Linington, S.H., Pritchard, H.W. and Probert, R.J. eds, The Royal Botanic Gardens, Kew, UK.

Ouédraogo, L. G., Sary, H., Yameogo-Gaméné, C. S. and **Neya, O.** (2003) A participatory approach for conservation of forest genetic resources, In Conserving biodiversity in arid Regions : Best Practices in Developing Nations, pp 62-71. Lemons, J., Victor, R. and Schaffer, D. eds, Kluwer Academic Publishers.

Neya, O., Gaméné, C. S. and Sacandé, M. (2004) Effect of neem (Azadirachta indica) fruit development on seed quality, In Comparative storage biology of tropical tree seeds, pp24-31, Sacandé, M., Jøker, D., Dulloo, M.E. and Thomsen, K.A. (eds), FLD (Forest & Landscape Denmark) - IPGRI (International Plant Genetic Resources Institute).

Sanon, D.M., Gaméné, C.S. and Sacandé, M. **Neya, O**. (2004) Desiccation and storage of Kigelia africana, Lophira lancoelata, Parinari curatellifolia and Zanthoxylum zanthoxyloides seeds from Burkina Faso, In Comparative storage biology of tropical tree seeds, pp 16-23, Sacandé, M., Jøker, D., Dulloo, M.E. and Thomsen, K.A. (eds), FLD (Forest & Landscape Denmark) - IPGRI (International Plant Genetic Resources Institute).

Neya, O., Golovina, E.A., Nijsse, J. and Hoekstra, F.A. (2004) Ageing increases the sensitivity of neem (Azadirachta indica) seeds to imbibitional stress, Seed Science Research **14**, 205-217.

Neya, O., Sacandé, M. and Hoekstra, F.A. Seed coat-imposed constraints induce variability in Lannea microcarpa seed germination. In preparation.

Neya, O., Golovina, E.A., and Hoekstra, F. A. Mechanism of seed coat-imposed restraint of germination in Lannea microcarpa seeds. In preparation.

Neya, O., Golovina, E.A., Nijsse, J., and Hoekstra, F. A. Ageing related changes in membranes composition, structure and integrity of Khaya senegalensis seeds. In preparation.

Curriculum vitae

Oblé Neya was born on the 12th of December 1973 in Boromo, Burkina Faso. He attended high school at the Lycée Provincial of Boromo, and then at the Lycée Ouezzin Coulibaly, Bobo Dioulasso, where he obtained his Baccalaureat - option D - in Mathematics and Natural Sciences in 1993. The same year he joined the Faculty of Sciences and Techniques at the University of Ouagadougou and later in 1996 the Institut de Developpement Rural (IDR) at the Université Polytechnique of Bobo Dioulasso, where he completed his Engineers degree in Rural Development in 1999, specializing in water and forests. As part of his education at the IDR, he conducted researches in the field of forest management and tropical tree seed biology. Since his graduation he has been working for the Centre National de Semences Forestières (CNSF) in Ouagadougou, Burkina Faso. As a worker at the Programme Technologie des Semences at CNSF, he was in charge, from 1999 to 2001, of a collaborative research project entitled "Conservation of Tree Seeds in Rural Areas", using traditional knowledge and techniques of conservation.

In 2001 he was granted a scholarship by the Dutch Organization for the Advancement of Tropical Research (WOTRO) to pursue a PhD programme on the conservation of tropical tree seeds at Wageningen University and Research Centre, the Netherlands. The results reported in this thesis have been obtained from studies conducted jointly at the Laboratory of Plant Physiology, Wageningen University, the Netherlands and at the Centre National de Semences Forestières (CNSF), Ouagadougou, Burkina Faso.

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Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Oblé Neya
Date: 15 November 2006
Group: Plant Physiology, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Imbibitional damage in neem (Azadirachta indica) seeds	Mar 23, 2003
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Neya O et al., (2003) Effect of developmental stages on Neem (*Azadirachta indica*) seed storage	2003
Neya, O. et al., (2004) Effect of neem (*Azadirachta indica*) fruit development on seed quality	2004
► **MSc courses**	
► **Laboratory use of isotopes**	

*Subtotal Start-up Phase 7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
► **EPS theme symposia**	Mar 23, 2003
Theme III Symposium 'Metabolism and Adaptation', Utrecht University	Oct 11, 2002
Theme III Symposium 'Metabolism and Adaptation', Wageningen University	Oct 25, 2004
► **NWO Lunteren days and other National Platforms**	
ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 7-8, 2003
ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 4-5, 2005
► **Seminars (series), workshops and symposia**	
Seminar: Molecular approaches to understand desiccation tolerance in higher plants (Dr D. Bartels) (Nijmegen)	Oct 17 2003
Symposium: Electron Spin Resonance (ESR) in Seed Analysis and Food Science (Wageningen)	Oct 7 2004
Seminars at Plant Physiology	2002-2006
► **Seminar plus**	
► **International symposia and congresses**	
4th Intern. Worksh. 'Desiccation sensitiv. and toler. in seeds & vegetat plant tiss., Blouwaterbaai (South Africa)	Aug 24-31, 2003

	International Workshop on Molecular aspects of seed germination and dormancy (Wageningen)	May 23-24, 2004
	3rd International Symposium on Plant Dormancy: From Molecular level to the whole plant (Wageningen)	May 25-28, 2004
	Intern. Worksh. of Darwin Initiative Research Exercise on Community Tree Seed (Directs), Kumassi (Ghana)	Mar 27-31, 2006
►	**Presentations**	
	Oral: 4th Intern.Worksh. 'Desiccation sensitivity and tolerance in seeds ….. , Blouwaterbaai, South Africa	Aug 24-31, 2003
	Oral: Intern. Worksh. of Darwin Initiative Res. Exercise on Community Tree Seeds (Directs), Kumassi (Ghana)	Mar 27-31, 2006
►	**IAB interview**	Jun 3, 2004
►	**Excursions**	

*Subtotal Scientific Exposure 11.7 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	PhD Course 'Seed Conservation Techniques', Kew, Wakehurst Place (UK)	Sep 9-20, 2002
	PhD course 'Electron Microscopy', Radboud University Nijmegen (NL)	Nov 3-21, 2003
►	**Journal club**	
	Literature study group Plant Physiology	2002-2006
►	Individual research training	

*Subtotal In-Depth Studies 9.5 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Written English course (The Language Centre of Wageningen University), Wageningen, The Netherlands	Mar 6-May 1, 2002
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	

*Subtotal Personal Development 1.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**30.5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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