Responses of fluted pumpkin (*Telfairia occidentalis* Hook. f.; Cucurbitaceae) seeds to desiccation, chilling and hydrated storage

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Abstract

The responses of fluted pumpkin (*Telfairia occidentalis* Hook. f.) seeds to chilling and hydrated storage at 6, 16, and 25 °C, and excised axes to fast flash-drying or slow dehydration, were investigated. Flash- and slow-drying initially enhanced germination by 20% and 7%, respectively, which was sustained despite further water loss to 0.45 g g⁻¹ when axes were flash-dried, but not when slowly dried. Of the seeds stored at 6, 16, and 25 °C, 3.3%, 40%, and 88%, respectively, were discarded within 4 weeks after storage because of germination or fungal proliferation. Nevertheless, axis germination of the non-visibly contaminated seeds after 4 weeks storage at 25 °C was precluded *in vitro* by vigorous fungal proliferation, underscoring the role of the internal fungal inoculum in obviating seedling establishment. In the case of the seeds stored at 6 °C, germinability was lost within 4 weeks, suggesting their chilling-sensitivity. Ultrastructural evidence revealed marked damage associated with chilling, while the ultrastructure of seeds surviving storage for 4 weeks at 25 °C was indicative of enhanced metabolic activity. The results reveal that fluted pumpkin seeds are recalcitrant, being both desiccation- and chilling-sensitive, and that even short-term storage in the hydrated state appears to be unachievable in practice.

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1. Introduction

The fluted pumpkin (*Telfairia occidentalis* Hook f.), a member of the Cucurbitaceae, is a strong climber and short-term perennial (Odiaka and Schippers, 2004), dioecious and diploid – 2n=20 (Okoli, 1987, 1988). Fruits are large, weighing up to 20 kg, and containing 80 seeds on average (Schippers, 2000). The plant is native to Nigeria and also found in the moist coastal areas of West Africa but rarely occurs naturally in East Africa (Akoroda, 1990a,b; Robinson and Decker-Walters, 1997). Leaves, stems, seeds, and roots have high food value and provide a source of oil and raw material for a variety of products (Akubue et al., 1980; Egbekun et al., 1998; Giami and Isichei, 1999; Akwaowo et al., 2000, Giami et al., 2003).

Fluted pumpkin can be propagated only by seeds, but their availability for planting is a major problem and cannot satisfy the widespread interest in the cultivation of the plant (Odiaka and Schippers, 2004). The seeds are difficult to conserve during the intervening period between fruit harvesting at the end of one season and seed planting at the beginning of the next. Because of this short storage life span, Akoroda (1986) classified the seeds as recalcitrant, although the water concentration was not quantified.

Seeds attain maximum physiological quality in terms of germination, vigour and storage reserve accumulation 9 weeks after fruit set, after which vivipary and seed rot set in (Adetunji, 1997) the extent of which is related to the duration of fruit storage after harvesting (Ajayi, unpublished data). The present study reports the first attempt to quantify the responses of fluted...
pumpkin seeds to desiccation and chilling, which constitute fundamental information necessary for storage and long-term conservation strategies for the endangered species (Sarumi, 2001).

2. Materials and methods

2.1. Seeds

Seeds were extracted from mature fluted pumpkin fruits and freighted by air from Nigeria within 24 h after extraction to South Africa where they were immersed for 1 h in a fungicide solution comprising 0.05% Early Impact (active ingredients, flutriafol [triazole] and carbendazim [benzimidazole]; Zeneca Agrochemicals, South Africa) and 0.25% Previcur (active ingredient, propamocarb-hydrochloride; R.T. Chemicals, South Africa) after which they were air-dried at room temperature to the original fresh weight. Thereafter the seeds were dusted with a benomyl fungicidal powder containing the active ingredient, methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate. Germination was assessed (see below) immediately after this treatment, and the remainder of the seeds placed into storage as indicated below.

2.2. In vitro culture of embryonic axes

Viability tests were performed using excised embryonic axes. Prior to culture, embryonic axes were treated in succession with 1% Hibitane [active ingredient, chlorhexidine gluconate, Zeneca, South Africa] followed by the fungicide solution described above, each for 10 min and finally for 5 min with 2.5% calcium hypochlorite, to which two drops of a wetting agent had been added. The explants were then rinsed three times with sterile distilled water and then cultured on full strength MS medium (Murashige and Skoog, 1962) containing 0.8% agar, 3% sucrose, and 1% chloride (Berjak and Mycock, 2004) for 30 min, surface sterilized and cultured as described above.

2.4. Hydrated storage and chilling sensitivity

About 300 seeds were spread in monolayer on a mesh (presoaked in a 3.5% sodium hypochlorite solution for 30 min) and thereafter suspended over wet paper towel in closed translucent buckets maintained at 6, 16 and 25 °C. At 2-week intervals, in vitro germination of excised axes was monitored as described above. At each sampling the numbers of infected or germinated seeds were counted and those seeds were then discarded.

2.5. Fungal isolation

Mycelia of proliferating fungi were isolated from the seed coat initially using colour and other characteristics to distinguish the taxa. They were grown on potato dextrose agar on plates in the dark at 25 ± 3 °C and sub-cultured repeatedly until a pure culture of each type was obtained. The axenic cultures were sent to the Mycology Unit, Biosystematics division of the Agriculture Research Council (ARC) – Plant Protection Research Institute, Pretoria, South Africa for expert identification.

2.6. Transmission electron microscopy (TEM)

The distal root tips were excised from axes that had been rehydrated (see above) into 2.5% phosphate-buffered glutaraldehyde solution (0.1 M, pH 7.2) in which they were maintained overnight and thereafter briefly rinsed in phosphate buffer before and after post-fixation for 1 h in 0.5% aqueous osmium tetraoxide. The specimens were then dehydrated through an ethyl alcohol series followed by infiltration and embedding in a low-viscosity epoxy resin. Sections were post-stained with lead citrate and examined with a Jeol 1010 TEM and the images captured digitally using the SIS Megaview III digital imaging system.

3. Results

3.1. Desiccation sensitivity

Axes excised from the two batches of seeds used for the drying trials were initially at somewhat different initial mean water concentrations, but there was no corresponding difference in germination capacity (Fig. 1). During both flash- and slow-drying, loss of water from excised axes was triphasic (Fig. 1). There was an initial rapid loss of water, about 40% of the initial tissue water content, which had occurred when the first samples were taken in both cases. This water loss was accompanied by enhanced rate and totality of germination. Compared with germination before drying, the rate of germination at the first sampling was up to 1 day faster, and total germination higher by 20% and 7% after flash and slow drying, respectively. During the second phase, there was a less abrupt, but progressively declining, loss of water. In the flash-dried material, the enhanced germinability was sustained over the water concentration range of 1.04 to 0.45 g g⁻¹ (>90% germination at 0.45 g g⁻¹) with rate
and totality of germination declining rapidly thereafter. In contrast, the initial enhanced germination of slowly-dried axes was not sustained. Rather, germination totality progressively decreased from 80% at the first sampling and levelled out to 53% after 48 h of drying (water concentration 0.59 g g$^{-1}$). During the third phase there was no further loss of water, irrespective of flash-drying time, but slightly more water loss occurred from axes that dried slowly. At any particular water concentration there was, on average, 25% more germination after flash (fast), compared with slow, drying. Germinating axes greened and the intensity of greenness was higher for partially-desiccated, compared with non-desiccated axes (results not shown).

3.2. Hydrated storage

The proportion of seeds showing either infection or germinating while in storage was in the order of 25 > 16 > 6 °C. All remaining seeds at 6 °C had lost viability by the 4th week in storage. For 16 and 25 °C storage, seeds that did not show surface fungal proliferation 2 weeks after storage nevertheless had internal fungal contamination. Culture of internal cotyledonary segments from surface-sterilized seeds that showed no external evidence of infection revealed the presence of fungal inoculum. Generally, axes excised from seeds stored at 6 °C showed bacterial rather than fungal contamination, while fungal proliferation in culture predominated in those from seeds stored at the higher temperatures. The dominant fungi isolated from the seeds were Clonostachys roseus and Fusarium solani.

Ultrastructural evidence revealed that the cells of, and contiguous with, the root meristem of axes of freshly-harvested seeds had relatively abundant vacuoles distributed within the cytomatrix, and had spherical nuclei with conspicuous nucleoli (Fig. 2a). The cells showed indications of active metabolism including abundant cristate mitochondria, numerous profiles of rough endoplasmic reticulum, non-membrane-bound polysomes and Golgi bodies (Fig. 2b). Comparable cells were examined from all seeds sampled during storage. After 2 weeks, the axis ultrastructure of hydrated seeds stored at 25 °C was largely maintained (Fig. 2e), while axes from seeds stored at 6 °C and 16 °C showed abnormalities such as clustering of organelles (Fig. 2c), lobed nuclei (inset, Fig. 2d) in some cells, and distended plastids. Storage at 16 °C was accompanied by the occurrence of abnormal nuclear inclusions and an appearance indicative of diminished metabolic activity compared with material that had been stored at 25 °C (Fig. 2d; cf. Fig. 2e). Storage at both 6 and 16 °C was accompanied also by parallel orientation of long endoplasmic reticulum profiles (Fig. 2c, d). After storage for 4 weeks, axis cells from seeds at 6 °C had a completely deranged ultrastructure, with shrunken compacted nuclei (inset, Fig. 2f), and clusters of organelles which had lost all internal structure (Fig. 2f). Cells of axes from seeds stored at 16 °C showed retention of the damage present after 2 weeks of storage, as well as lobed nuclei and electron-translucent plastids and mitochondria (Fig. 2g), while the ultrastructure of seeds stored at 25 °C remained comparatively normal (Fig. 2h). The latter had accumulated starch within the plastids, retained cristate mitochondria, and showed evidence of de novo vacuolation (not illustrated). An unusual, probably abnormal feature, was the diffusivity of the outer nucleolar region, also seen after 4 weeks storage at 16 °C (Fig. 2g).

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Table 1
Influence of storage temperature and duration on wet storage of fluted pumpkin seeds

<table>
<thead>
<tr>
<th>Storage temperature (°C)</th>
<th>Storage duration (weeks)</th>
<th>% seeds visibly infected in storage</th>
<th>% seeds germinating in storage</th>
<th>% seedling establishment in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>73.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0.4</td>
<td>40.0</td>
<td></td>
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<tr>
<td></td>
<td>4</td>
<td>2.9</td>
<td>0*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>26.7</td>
<td>0*</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>1.1</td>
<td>86.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26.8</td>
<td>12.5</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>59.8</td>
<td>6.1</td>
<td>_b</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>57.45</td>
<td>18.9</td>
<td>73.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>46.2</td>
<td>3.1</td>
<td>_b</td>
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<tr>
<td></td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>– _b</td>
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Percentages are expressed in terms of the seeds remaining in storage at the time of sampling. n = 300.

a Associated bacterial proliferation.

b Lack of seedling establishment attributed to fungal proliferation.

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Fig. 1. Changes in water concentration and germination percentage of Telfairia occidentalis embryonic axes dehydrated at different rates. -○- Water content. -■- Germination.
Fig. 2. Ultrastructural aspects of distal axis root tip cells (meristematic or immediate meristem derivatives) of *T. occidentalis* prior to (a,b), and after hydrated storage for 2 (c–e) and 4 weeks (f–h) at 6, 16 and 25 °C. a (bar=5 μm): Cells from axes of fresh seeds were characterised by relatively small, discrete vacuoles and normally-dispersed organelles. b (bar=0.05 μm): Mitochondria had relatively dense matrices and well-developed cristae, polysomes occurred associated with long profiles of rough ER and dispersed in the cytomatrix, and Golgi bodies were prevalent. c, d and e: Cells from material stored hydrated for 2 weeks at 6, 16 and 25 °C, respectively. c (bar=2 μm): Intracellular derangement was already apparent in axes of seeds stored at 6 °C, in terms of an abnormal degree of vacuolation (cf. e, 25 °C storage) and displacement of clusters of organelles (arrowheads). d (bar=2.0 μm): Although axes from 2-week-stored seeds at 16 °C, maintained viability, nuclei commonly contained patches of very dense material (possibly abnormally condensed chromatin) and in some cases, exhibited markedly lobed profiles (inset, bar=2.0 μm). e (bar=5.0 μm): Axis cells from hydrated seeds stored at 25 °C maintained ultrastructural normality. f, g and h: Distal root-tip cells of axes excised after 4 weeks hydrated storage at 6, 16 and 25 °C, respectively. f (bar=2.0 μm): Storage at 6 °C was accompanied by ultrastructural derangement and organelle deterioration, while nuclei were shrunken and showed dense, compacted contents (inset, bar=2.0 μm). g (bar=2.0 μm): After 4 weeks storage at 16 °C, nuclear lobing persisted, and nucleoli were markedly diffuse peripherally. h (bar=5.0 μm): After 4 weeks of hydrated storage at 25 °C, axis cells showed evidence that ongoing metabolism had occurred in terms of starch accumulation. The cells appeared to have maintained general ultrastructural integrity, although nucleoli were peripherally diffuse. ER, endoplasmic reticulum; G, Golgi body; L, lipid; m, mitochondrion; N, nucleus; Nu, nucleolus; p, plastid; v, vacuole.
4. Discussion

Seeds are generally loosely categorised as orthodox, intermediate and recalcitrant based on their post-shedding storage characteristics (Roberts, 1973; Ellis and Hong, 1990). However, accumulating information suggests that seed storage behaviour is unlikely to be so simply categorised, rather constituting a continuum with orthodoxy and recalcitrance being the extremes. Seeds being neither recalcitrant nor orthodox are suggested to fall between these extremes, depending on the degree to which they can tolerate loss of water and the range of time and storage conditions under which viability can be maintained (Berjak and Pammenter, 2004). Recalcitrant seeds do not undergo physiological drying and are consequently shed in a hydrated and metabolically-active, condition. Therefore they cannot be dried for storage nor frozen at the water concentration to which they can tolerate loss of water and the range of time extremes. Seeds being neither recalcitrant nor orthodox are suggested to fall between these extremes, depending on the degree to which they can tolerate loss of water and the range of time and storage conditions under which viability can be maintained (Berjak and Pammenter, 2004). Recalcitrant seeds do not undergo physiological drying and are consequently shed in a hydrated and metabolically-active, condition. Therefore they cannot be dried for storage nor frozen at the water concentrations typifying the newly-shed condition without lethal damage.

The ultrastructure of the embryonic root meristematic and contiguous cells of excised axes from *T. occidentalis* seeds immediately after extraction from the fruit confirmed that they were shed in an active metabolite state. As the viability of fluted pumpkin axes was rapidly lost at a water concentration of \( \sim 0.45 \text{ g g}^{-1} \) (31% wmb), the lowest axis water concentration presently achieved which permitted germination was far too high for dry storage. The marked ultrastructural abnormality and loss of viability within 2 weeks of axes excised from seeds stored at 6 °C confirms that fluted pumpkin seeds are also chilling-sensitive. Microscopic observations revealed the seeds were indeed markedly chilling-sensitive, accumulating some intracellular abnormalities at a temperature as moderate as 16 °C within 2 weeks after being placed in storage, although the effect was not then detected in the germination test. This implies that viability testing alone may be insufficient for characterizing chilling sensitivity in seeds.

Irrespective of the drying rate presently used the water concentration of axes of *T. occidentalis* reached a threshold below which water loss was markedly slower and, in the case of the flash-dried axes, no further dehydration occurred over the time course of the experiment (Fig. 1), from which it is assumed that much of the remaining water was structure-associated (Pammenter et al., 1991; Walters et al., 2001). At, or close to, this threshold water concentration, the axes are suggested to undergo desiccation damage *sensu stricto* (Pammenter et al., 1998; Pammenter and Berjak, 1999; Walters et al., 2001). When desiccation-sensitive axes dry slowly, metabolism becomes increasingly unbalanced, with lethal results at water contents around 0.8 g g\(^{-1}\) (Pammenter et al., 1998). In contrast, according to those authors, rapid dehydration curtails the time during which deleterious metabolism-linked events can occur allowing recalcitrant axes to retain viability at, or close to, the concentration at which all the intracellular water is considered to be structure-associated (\( \sim 0.25 \text{ g g}^{-1} \); Vertucci, 1990). The patterns of viability retention in relation to drying rate for *T. occidentalis* are in agreement with the findings of Pammenter et al. (1991, 1998) and Walters et al. (2001).

Stimulation of germination of fluted pumpkin seeds on initial dehydration, as was observed for excised axes in this study, had earlier been reported by Esiaba (1982) and Nkang et al. (2003). Esiaba (1982) had suggested that fluted pumpkin seeds need ‘curing’, i.e. air-drying under shade for 3–5 days, in order to attain maximum germination and that seeds that were not ‘cured’ before planting either did not germinate well or produced weaker seedlings. Although the scientific basis of this phenomenon is presently being studied (Erdey, personal communication), it has been observed for non-orthodox seeds/axes of a variety of species (Tompsett and Pritchard, 1998; Pammenter and Berjak, 1999). It has been suggested, for seeds of *Aesculus hippocastanum* (Tompsett and Pritchard, 1998), that the promoting effect on germination of a non-damaging degree of dehydration, may be the manifestation of continuing maturation that would naturally have occurred, had the seeds been drier on the parent plant prior to harvest. Although no quantitative measurements of chlorophyll were made in the present study the partially dehydrated axes greened more intensely than did those that had not been subjected to any drying. This is a further indication of the stimulatory effect of a modest degree of dehydration on the axes of a recalcitrant seed-type.

The basic cause of seed germination during storage is well known to be the consequence of ongoing metabolism (Pammenter and Berjak, 1999). In the present case germination may have been accelerated in the storage buckets as condensed water periodically dripped back onto the seeds. This suggests that avoidance of contact of mature seeds with liquid water in hydrated storage is an important consideration in the maintenance of recalcitrant seeds, as presently exemplified by those of fluted pumpkin. In a subsequent experiment (results not shown) in which buckets were not tightly closed and were periodically opened to drain off condensed water, seed germination in storage was considerably reduced but the seeds dried in the storage containers, curtailing the duration for which viability was retained.

The duration of successful hydrated storage is directly related to the stage of embryo development at harvest, and the rate at which germinative metabolism proceeds at the original seed water concentration. In the case of *T. occidentalis* seeds, the incidence of germination when the seeds remain within ripe fruits (Adetunji, 1997) suggests that the embryos of these highly recalcitrant seeds are completely mature, and will rapidly entrain germinative metabolism in hydrated storage. Seeds from unripe fruits (results not shown) had slower rates of germination *in vitro* and in soil, and about 75% less germination and fungal proliferation after 6 weeks in storage compared with seeds from ripe and over-ripe fruits.

The isolation of fungi from internal cotyledon tissues of seeds that did not appear to harbour an external (surface) fungal inoculum, suggests the likelihood of systemic infection prior to storage. Fungi have been recorded as accounting for up to 95% and bacteria 5% loss of fluted pumpkin fruits during storage under ambient conditions (Odiaka and Schippers, 2004). However, the fungi associated with *T. occidentalis* seeds are suggested to be chilling-sensitive, judging from the present

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observation that bacteria rather than fungi were isolated after storage at 6 °C.

Fungal proliferation from endogenous inoculum has been shown to be promoted during hydrated storage in highly recalcitrant *Avicennia marina* seeds, and minimisation of fungal contamination was shown to be associated with significant extension of the storage life span (Calistru et al., 2000). Similarly, fungal contamination was shown to be a major contributory factor in the curtailling storage longevity of *Trichilia dregeana* seeds (Berjak et al., 2004). In the present case, axis ultrastructure of seeds stored at 25 °C for 4 weeks indicated the progress of subcellular activity with no features suggesting deterioration other than the diffuse nucleolar periphery. However, fungal proliferation occurred when the axes were cultured in vitro, and seedlings were not established. Taken together, these observations indicate that even if the condition of axes is apparently unimpaired, proliferating fungi preclude their further development – at least under in vitro conditions. As one of the fungi isolated was a species of *Fusarium*, a genus known to cause seedling damping-off (Agarwal and Sinclair, 1987), it is likely that even if the seeds had been planted out (i.e. axes not excised) after the 4-week storage period, viable seedlings would not have resulted. However, 77% of the axes excised after 2 weeks in storage did establish seedlings, irrespective of the inherent fungal inoculum. This may be explained in terms of the relative vigour of the seeds sampled after 2 weeks, in contrast to that of the longer stored seeds. It has been ascertained that *A. marina* seeds elaborate effective anti-fungal compounds early during hydrated storage, but that the efficacy of such compounds declines as the storage period progresses, with the seeds becoming increasingly susceptible to fungally-mediated deterioration (Anguelova-Merhar et al., 2003).

The high water concentration and actively metabolic condition of mature *T. occidentalis* seeds indicate that they are recalcitrant, which is unequivocally confirmed by their inability to withstand much water loss, and their sensitivity to chilling. *Telfairia occidentalis* is one of the few non-tree species with desiccation-sensitive seeds, and the second cucurbit species, after chayote (*Sechium edule* (Jacq.) Swartz), to be demonstrated as producing recalcitrant seeds (Flynn et al., 2004). The present work has shown that, even if of high quality, fluted pumpkin seeds cannot be stored at low temperatures because of sensitivity to chilling. Furthermore, storage at temperatures above 16 °C is unlikely to be useful for more than 4 weeks because of the germinative metabolism of the seeds on the one hand, and microbial proliferation on the other. Although the lowest water concentration facilitating germination was high for cryopreservation by conventional methods, this could theoretically be achievable using extremely rapid cooling (freezing) rates (Wesley-Smith et al., 1992). However, the size of embryonic axes of *T. occidentalis* far exceeds that at which sufficiently rapid, uniform cooling can be attained (Wesley-Smith et al., 2004). Presently, therefore, other strategies (including the use of somatic embryos and axillary buds for cryopreservation) are under investigation for the conservation of the germplasm, and thus genetic diversity, of *Telfairia occidentalis*.

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