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Oxidative stress, phospholipid loss and lipid hydrolysis during drying and storage of intermediate seeds

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The biochemical and physiological basis of intermediate seed storage behaviour was examined by investigating the effects of equilibrium drying under relative humidities (RHs) of 9–81% and of storage at 20 or 5°C on coffee seed viability and antioxidant, lipid and sugar status. Slow drying induced a significant decrease in the concentrations of the pools of two major antioxidants, glutathione and ascorbate, and an increase in the free fatty acid (FFA) content of seeds, independent of the RH employed. Seeds stored at 81% RH and 20°C lost their viability very rapidly and showed an extensive loss and oxidation of antioxidants, an accumulation of FFA and a selective loss of phospholipids, in particular phosphatidylethanolamine (PE). Interestingly, the changes in PE content were not due to fatty acid de-esterification and the increase in FFA levels resulted from neutral lipid hydrolysis. Decreasing the storage temperature to 5°C considerably slowed both the loss of seed viability and the level of oxidative stress as well as the rates of lipid hydrolysis. No decline in seed viability was observed under storage conditions of 45% RH/20°C. After 1 year under 45% RH/5°C, the loss of seed viability was found to be due to imbibitional damage and could be circumvented by pre-humidifying or pre-heating seeds before sowing.

Introduction

Three categories of seed storage behaviour are generally recognized among plant species: orthodox, intermediate and recalcitrant (Roberts 1973, Ellis et al. 1990). Using coffee (*Coffea arabica*) as a model, Ellis et al. (1990) defined the 'intermediate' category as being for seeds which display the following two main characteristics regarding their level of desiccation tolerance and storage behaviour: (i) seeds that are able to withstand considerable drying [down to a relative humidity (RH) of 30–40%] in comparison with recalcitrant seeds, but which cannot tolerate extreme water loss as is the case in orthodox seeds; (ii) in contrast with orthodox seeds, lowering the storage temperature decreases seed longevity at low water contents (RH < 50%). Therefore, intermediate seeds cannot be stored under conventional genebank conditions (FAO/IPGRI 1994), and for intermediate seed species, cryopreservation is the only technique available for long-term germplasm conservation (Dussert et al. 2001, 2003).

Despite the importance of this category of storage behaviour (Hong et al. 1996), the physiology of intermediate seeds during drying and storage is still largely

Abbreviations – FFA, free fatty acid; GSH, g-glutamylcysteinylglycine; L-AA, L-ascorbic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; PLA2, phospholipase A2; PS, phosphatidylserine; RH, relative humidity; ROS, reactive oxygen species.

unknown. However, significant progress in the understanding of the intermediate storage behaviour has been brought by recent work from Sacandé et al. (2000, 2001) using neem seeds. Comparing seeds desiccated and stored under two RHs (32 and 75%), these authors have shown that the rapid loss of seed viability at 75% RH in comparison with 32% RH was associated with an increase in the free fatty acid (FFA) content and decreases in the phospholipid (PL) content, the total glutathione content and in the percentage of reduced glutathione (γ-glutamylcysteinylglycine, GSH). Therefore, the first objective of the present study was to confirm that oxidative stress, PL loss and lipid de-esterification are key mechanisms of loss of viability in intermediate seeds stored at room temperature and at intermediate hydration levels (around 75% RH), using coffee seeds as the reference system for the intermediate storage category. In addition, for the first time, we investigated the effects of lowering the storage temperature on these three deteriorative processes to understand one of the two main features of intermediate seeds, i.e. the apparent negative effect of low temperature on their longevity at low water contents (Ellis et al. 1990).

Our second objective was to further understand the role of oxidative stress, PL loss and lipid de-esterification in ageing intermediate seeds. To follow the degree of oxidative stress sustained by the seeds, we measured changes in the concentrations of the two major cellular hydrophilic antioxidants, L-ascorbate (L-AA, vitamin C) and GSH. The levels and redox status of these compounds are widely recognized as markers for the degrees of oxidative stress experienced by the cell (Noctor and Foyer 1998, Davey et al. 2000), and L-AA is generally present in higher amounts in desiccation-sensitive than in desiccation-tolerant seeds (Tommasi et al. 1999). Two hypotheses have been proposed for the origin of membrane PL de-esterification in plant systems subjected to drying, freezing or ageing: free radical attack (e.g. Senaratna et al. 1985, Van Bilsen and Hoekstra 1993) or phospholipase A_2 (PLA_2) activity (Oliver et al. 1995, Welti et al. 2002). When FFA production results from PLA_2 activity, then a simultaneous accumulation of lysophospholipids (lyso-PL) is also expected, and to specifically test this hypothesis, we have employed new procedures for the purification and chromatography of FFA, PL and lyso-PL in oily seeds. Additionally, for the first time in intermediate seeds, we have investigated whether PL loss occurs in all PL classes.

GSH and disaccharide contents have been reported to increase in neem seeds during the 3 weeks of equilibrium drying (Sacandé et al. 2000), and this has been interpreted as being due to the post-harvest maturation of seeds during slow drying. The beneficial role of disaccharides in desiccation tolerance has indeed been well established (Crowe et al. 1992, Hoekstra et al. 2001), and slow drying has been shown to be necessary for the survival of carrot somatic embryos to desiccation, by avoiding lipid hydrolysis and extensive leakage at imbibition (Tetteroo et al. 1996). By contrast, Walters et al. (2001) showed that in pea and tea embryonic axes, desiccation tolerance is positively correlated with the drying rate and negatively correlated with O_2 consumption. These authors assumed that the oxidative stresses induced by slow drying are due to the longer time spent in a partially dehydrated state, where cells continue to respire but cannot scavenge reactive oxygen species (ROS) and other toxic by-products (Leprince et al. 1999, 2000). To resolve these conflicting reports, the third objective of this study was to verify whether physiological changes could be detected during the course of drying in coffee seeds and whether they could be correlated with either post-harvest maturation or oxidative damage.

Finally, the fourth objective of the present work was to test whether oxidative stress, PL loss and lipid de-esterification could be also involved in the second main characteristic of intermediate seeds, i.e. their immediate loss of viability when dried below a certain threshold hydration level (between 20 and 80% RH depending on the intermediate seed species). This loss of viability during desiccation constitutes a considerable problem for the long-term germplasm conservation by means of seed cryopreservation (Dussert et al. 2001, 2004, Hor et al. 2005), and although the polygenic origin of seed desiccation tolerance in coffee has clearly been demonstrated (Dussert et al. 2004), the underlying molecular and physiological mechanisms to desiccation sensitivity still remain elusive. In the present work, we have therefore investigated whether changes in antioxidants, sugars and lipids can be detected in coffee seeds dried under various RHs, chosen to cover the hydration levels below which seeds die because of desiccation and desiccation-related effects.

Materials and methods

Plant materials

Fresh mature seeds of *C. arabica* L. (variety Caturra) were provided from CICAFE, San Jose, Costa Rica. Three seed lots were employed corresponding to three consecutive years of production at CICAFE (2001, 2002 and 2003). Fruits were always harvested on trees grown under the same conditions, and seeds were extracted, processed and shipped to IRD, Montpellier, France, using the same procedure. Seed viability and water content (WC) upon receipt in the laboratory were very similar for the three consecutive years: the proportion of

seeds germinating was 98–100% and seed initial WC was 0.49–0.58 g H_2O g^{-1} DW. Desiccation and subsequent trials were always performed within 3 weeks after receipt of the seed lots. The effect of drying under 81% RH was investigated in the three seed lots. Storage behaviour was studied using the seed lot harvested in 2001. Desiccation and LN exposure sensitivity was analysed in the seed lot 2003.

Desiccation, storage, cooling, thawing, rehydration and germination procedures

Seeds were desiccated by equilibration over various saturated salt solutions for 20 days at 25°C in the dark, as previously described by Dussert et al. (2001, 2003, 2004). The WC of seeds at equilibrium (expressed in g H_2O g^{-1} DW) was always estimated using 10 replicates of one seed and their dry weight (DW) was measured after 1 day of desiccation in an oven at 105°C. For the measurement of seed desiccation sensitivity, seeds were desiccated over K acetate (23% RH), K_2CO_3 (45% RH), KOH (9% RH), $MgCl_2$ (32% RH), NH_4NO_3 (62% RH) and (NH4)2SO4 (81% RH) saturated solutions. Tolerance to LN exposure of seeds dehydrated as a function of the equilibration time under 81% RH was investigated by placing seeds in 10 ml of polypropylene tubes prior to immersion in LN. Thawing was carried out by plunging the tubes in a 37°C water bath for 30 min. Storage behaviour at 5 and 20°C was investigated using seeds desiccated over K_2CO_3 and $(NH4)_2SO_4$ saturated solutions. Batches of 100 seeds were sealed hermetically in aluminium foil–polyethylene bags and were stored for 0, 3 and 12 months in the dark. For each condition tested, seed viability was assessed using three replicates of 36 seeds. Seed pre-humidification, osmoconditioning and pre-heating treatments were performed as described in Dussert et al. (2003). For germination, batches of nine seeds were placed above 18 g of vermiculite fully imbibed with 45 ml of sterile water in closed plastic boxes (©Magenta, Chicago, IL). Seed viability was assessed using the criterion of normal seedling development, i.e. emergence of the hypocotyl, radicle geotropic growth and opening of cotyledonary leaves, after 6 weeks of culture at 25°C in the dark. Zygotic embryos were extracted from seeds after desiccation and cultured in vitro according to the method described by Dussert et al. (2001).

Sample preparation before biochemical analyses

For each seed lot and each condition tested, several batches of 15 seeds were rapidly immersed and stored in LN (−196°C) until analysis. Frozen seeds were ground in the presence of LN in a pre-cooled mill, and the frozen powder was immediately transferred to a plastic 15-ml centrifuge tube floating in Dewar vase filled with LN; they were then directly freeze-dried. Dry samples were stored at −80°C until use. All analyses were performed in at least triplicate.

L-ascorbate and glutathione

Simultaneous analysis of L-AA and GSH contents was carried out by ion-suppression RP-HPLC essentially as described in Davey et al. (2003, 2004). Determination of the oxidized forms (DHA and GSSG) was by the subtractive method after reduction of extracts with DTT (Davey et al. 2003).

Lipid extraction

Total lipids were extracted from 800 mg samples of freeze-dried powder using a modified Folch method (Folch et al. 1957) with methylene chloride replacing chloroform, as described in Dussert et al. (2001).

Phospholipid high-performance liquid chromatography

Lipid extracts were fractionated by reverse-phase Solid Phase Extraction (SPE) using 2 g Isolute C8 EC (octyl endcapped) columns supplied by Argonaut IST (Mid Glamorgan, UK) and a vacuum manifold (Vac Master, Argonaut IST). A small amount of anhydrous sodium sulfate was deposited onto the column. The column was then conditioned with 10 ml of methanol, four times. Lipid extract (100–150 mg) was dissolved in 1 ml of chloroform/methanol (2/1, v/v) and loaded onto the column. PL elution was carried out by adding 16 ml of methanol. The flow rate was adjusted to 1 ml min^{-1}. Methanol was evaporated and PL dissolved in 1 ml of CH_2Cl_2/MeOH (2:1). The PL fraction was then analysed by high-performance liquid chromatography (HPLC) with an evaporative light-scattering detector (ELSD). The HPLC system consisted of the following components: injector LC 508 (Beckman Coulter, Fullerton, CA), pump LC 126 (Beckman Coulter), oven (Croco-Cil, Cluzeau Info Labo, Puteaux, France) and ELSD Chromachem (Eurosep, Cergy Pontoise, France). The HPLC column was a Lichrospher 100 Diol 5 µm (25 cm × 4.6 mm i.d.) from Merck (Darmstadt, Germany). Column temperature was maintained at 30°C. The ELSD was set at 40°C for nebulization temperature, 50°C for evaporation temperature and at 1.5 bar pressure for nebulization gas (nitrogen). A binary gradient was used: solvent A, hexane/isopropylic alcohol/acetic acid (82/18/1.3, v/v/v), and solvent B, isopropylic alcohol/water/acetic acid (86/14/1.3, v/v/v).

Flow rate was 1 ml min^{-1}. Changes in the percentage of solvent A in the binary gradient were 95, 60, 50, 50, 95 and 95 at 0, 23, 24, 25, 35 and 40 min, respectively. All solvents were filtered prior to analysis. Standard curves were run with the commercial standards of PE, PC, PG, PS, LPE, LPC and phosphatidylinositol (PI) from Sigma (Saint Quentin Fallavier, France).

FFA purification and analysis

FFAs were purified using a 2 g NH2 SPE (Argonaut IST) cartridge. The flow rate was adjusted to 1 ml min^{-1} using a vacuum manifold (Vac Master, Argonaut IST). The column was conditioned four times with 10 ml of hexane. Lipid extract (100–150 mg) was dissolved in 1 ml of chloroform and loaded onto the column. Neutral lipids and FFAs were separated by successive elution with 18 ml of chloroform/isopropyl alcohol (2/1, v/v) and 16 ml of a 2% (v/v) solution of acetic acid in diethyl ether, respectively. The FFA fraction containing heptadecanoic acid (17:0) as internal standard (100 μg) was dried under nitrogen at 37°C and methylated with 5 ml of 14% BF_3 methanolic solution at 90°C for 1 min. FAMEs were extracted from methanol by adding 1 ml of hexane and about 20 ml of saturated NaCl solution. The sample was shaken manually for 30 s and completed until 50 ml with the saturated NaCl solution. After collecting the hexane fraction, FAMEs were directly injected in GC. GC analyses were performed using an HP 5890 system with flame ionization detection (FID). A Famewax capillary column (RESTEK, Evry, France), 25 m × 0.25 mm × 0.25 μ, was used. The analyses were carried out in programme temperature mode from 185 to 225°C at 4°C min^{-1} and the isothermal for 10 min. Carrier gas was helium at 40 cm s^{-1}. Both injector and detector were at 230°C. FAMEs were identified by comparison with known standards (Sigma) and were quantified as a percent of total fatty acids (w/w). FFA content was determined by comparison with the internal standard area.

Sugar extraction and analysis

Forty milligram of freeze-dried powder was used for extraction of sugars in 3 ml of ethyl alcohol 80% (V/V) containing 0.5 mg ml^{-1} of lactose as internal standard. Extracted samples were heated for 20 min at 80°C before centrifugation at 4000 *g* and 4°C for 15 min. The supernatant was collected and freeze-dried. Dried extracts were then dissolved in 9 ml of distilled water and filtered (0.22 μm pore diameter) before analysis. Sugars were quantified by high-performance anion exchange chromatography (Dionex Chromatography Co., Sunnyvale, CA) using a CarboPac PA-1 (Dionex) HPLC column. Sugars were resolved isocratically with 150 m*M* NaOH at 1 ml min^{-1}. Mono-, di- and oligosaccharide contents were determined by comparison with retention times and calibration curves obtained with sugar standards.

Results

Biochemical status of fresh seeds

In the three seed lots studied, GSH was the major antioxidant present in fresh seeds with levels about 10-fold higher than L-AA (Table 1, Fig. 1). About 20% of GSH and almost 100% of L-AA of seeds was oxidized upon receipt of the seed lots (Table 1). Fresh seeds also contained significant amounts of FFA (about 1 mg g^{-1} of lipids), while only traces of lyso-PL were detected (Table 1, Fig. 1). Three different PLs were identified in fresh coffee seeds, independent of the year of harvest (Table 1). Phosphatidylcholine (PC) was always the major PL, accounting for 75% of PL, while PE and PI represented 6 and 19%, respectively. The fresh seed PL content did not vary between the seed lots studied, always representing 0.41% of the total lipids. Sucrose was the major soluble sugar in fresh seeds of all seed lots studied. Its content was always very high, as it represented about 7% of the seed DW. Glucose, fructose, stachyose and raffinose were always present in low amount. Among all metabolites studied, only GSH and L-AA contents differed in fresh seeds of the three seed lots analysed – e.g. seed GSH content before drying varied from 1250 in seed lot 2001 to 1900 nmol g^{-1} DW in seed lot 2003.

Effect of equilibrium drying under 81% RH

The effects of equilibrium drying under 81% RH were identical in the three seed lots studied (Table 1). Specifically, desiccation induced a significant decrease in total GSH and total L-AA contents, an increase of the FFA content and a significant change in the proportions of PE and PC (Table 1, Fig. 1). For GSH and L-AA, the effect of drying was not only qualitatively but also quantitatively equivalent between the three seed lots studied, with losses of approximately 500 nmol g^{-1} DW GSH during equilibrium (Fig. 1). By contrast, the accumulation of FFA induced by drying was two times higher in seeds of the seed lot 2001 than in the two other seed lots. A significant decrease of the percentage of PE and a reciprocal increase in the percentage of PC were observed, while PI did not vary significantly. In all seed lots, drying did not influence either the sugar composition of seeds or the redox status of seed L-AA and GSH pools. Importantly, the significant increase in FFA contents was not accompanied by an increase in the levels of lyso-PL.

Table 1. Probability *P* of significance of the effects of the seed lot and of equilibrium drying (20 days under 81% RH at 25°C) on the antioxidant, lipid and sugar composition of *Coffea arabica* seeds as determined by two-way ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$). Mean values in fresh and dried seeds of the three seed lots studied (2001, 2002 and 2003) are given for parameters showing no significant effect of the seed lot. For all parameters showing significant effect of the seed lot and of drying, mean values are given in Fig. 1. [a]Seed lot 2002 not studied.

	Fresh seeds	Dried seeds	Seed lot effect, *P*	Drying effect, *P*	SL × D, *P*
Antioxidants					
Total glutathione (nmol g^{-1} DW)	Fig. 1		0.007**	0.002**	0.91
Glutathione redox status (% GSSG)	16.7	19.0	0.57	0.76	0.22
Total L-ascorbate (nmol g^{-1} DW)	Fig. 1		0.046*	0.012*	0.42
L-ascorbate redox status (% DHA)	96.9	93.4	0.72	0.73	0.43
Lipid classes (mg g^{-1} lipid)					
Free fatty acids	Fig. 1		0.000**	0.011*	0.008**
Phospholipids[a]	4.14	4.54	0.50	0.49	0.83
Lyso-phospholipids[a]	Traces		–	–	–
Phospholipid composition[a] (%)					
PE	6.4	3.6	0.25	0.000***	0.61
PC	74.7	79.6	0.16	0.046*	0.92
PI	18.6	16.6	0.24	0.25	0.79
Sugars (mg g^{-1} DW)					
Sucrose	73.1	69.9	0.06	0.31	0.14
Glucose	2.4	2.4	0.83	0.93	0.87
Fructose	2.1	2.2	0.28	0.52	0.85
Raffinose	0.7	0.8	0.31	0.86	0.33
Stachyose	0.5	0.5	0.80	0.93	0.41

Seed desiccation sensitivity

The relationship between the RHs employed for equilibrium drying and whole seed viability followed a typical sigmoidal pattern as expected (Fig. 2). Warm pre-humidification of seeds after desiccation significantly improved their tolerance to desiccation when compared with seeds placed directly under germination conditions and the viability of seeds equilibrated under 23% RH increased from 38 to 88% with this treatment. Whatever the RH employed for drying, 99–100% of embryos extracted from seeds after drying developed into normal seedlings (Fig. 2), confirming that desiccation-induced loss of seed viability was due to damage to the endosperm only. The loss of viability induced by desiccation was not associated with a change in either the antioxidant status or the sugar composition of the seeds (Table 2). There was also no significant correlation between the RH employed for drying and seed FFA ($P = 0.054$) and PL ($P = 0.319$) contents (Fig. 3). Finally, the percentage of PE, PC and PI did not vary significantly with the drying RH (Fig. 4, $P = 0.119$, 0.293 and 0.125, respectively).

Seed storage behaviour at 5 and 20°C after drying under 45 and 81% RH

Seeds dried under 81% RH lost their viability very rapidly when stored at 20°C, with only 30% developing into normal seedlings after 3-month storage, and a complete loss of viability after 1 year (Fig. 3). None of the three controlled rehydration procedures tested (pre-heating, pre-humidification and osmoconditioning) improved the percentage of seedling recovery after storage under these conditions (Table 3). By contrast, no change in viability was observed throughout 1 year of storage at 20°C in seeds dried under 45% RH, again independent of the rehydration procedure employed after storage (Fig. 5, Table 3). Storing seeds at 5°C considerably slowed the loss of viability of seeds dried under 81% RH, and after 1 year of storage, almost half of the initial viability was maintained, regardless of the rehydration procedure employed (Fig. 5, Table 3). By contrast, the percentage of seedlings recovered from seeds stored at 5°C after drying under 45% RH was significantly influenced by the rehydration procedure, and pre-humidifying seeds for 24 h or pre-heating seeds in a 40°C water bath increased seed viability from 39 to 80% (Table 3).

Physiological basis of seed storage behaviour

The seed antioxidant and lipid contents analysed were significantly modified by the three factors controlling the viability of seeds in storage, namely the hydration level, the storage temperature and the time (Table 4, Figs 5 and 6). The number of significant factors and

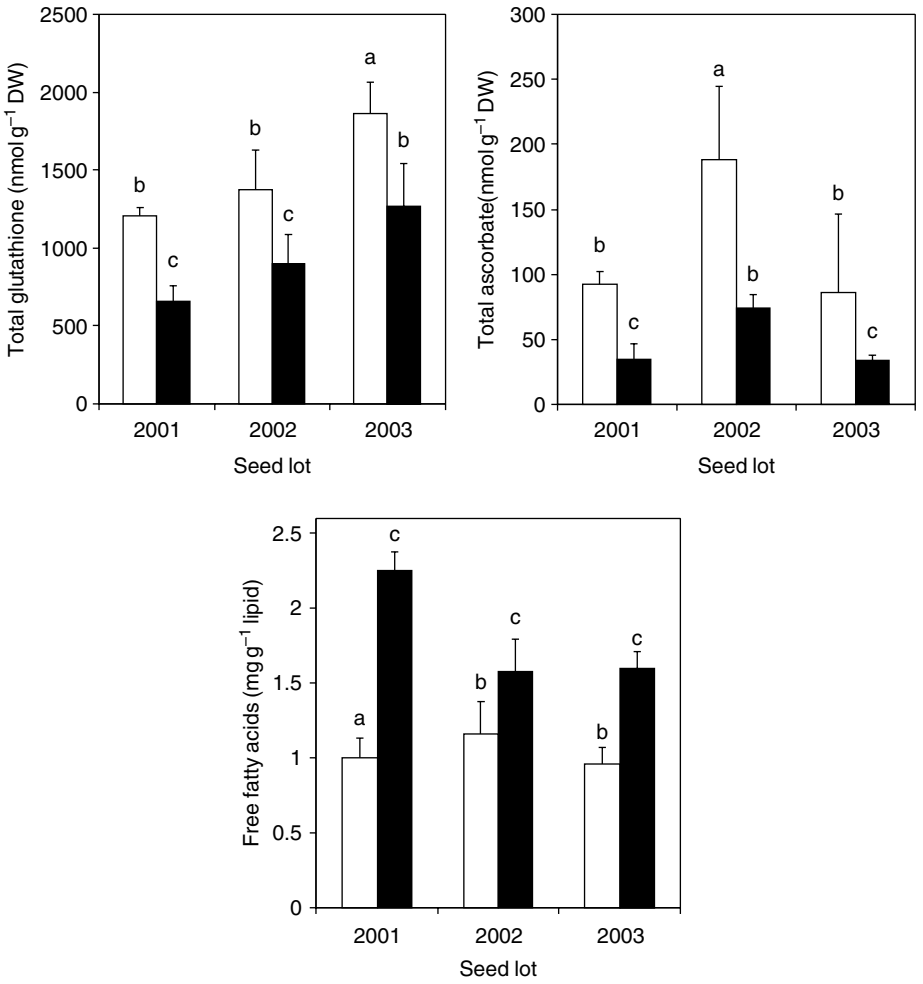

Fig. 1. Seed glutathione L-ascorbate and free fatty acid (FFA) content in three seed lots of *Coffea arabica* (harvested in 2001, 2002 and 2003) before (white bars) and after (black bars) equilibrium drying (20 days under 81% RH at 25°C). Bars showing the same letter are not significantly different at $P = 0.05$ according to the Newman and Keuls test. RH, relative humidity.

interactions varied between the different antioxidant and lipid traits analysed, showing the complexity of the underlying mechanisms (Table 4).

The seed GSH pool followed a pattern very similar to that of seed viability remaining almost constant in seeds dried under 45% RH, independent of the storage temperature, while in seeds dried at 81% RH, its loss was more rapid at 20°C than at 5°C (Fig. 5). Oxidation of glutathione was also considerably more rapid in seeds dried under 81% RH than under 45% RH, again independent of the storage temperature (Table 4). As a result, after 1-year storage, the low residual glutathione pool was completely oxidized, while the percentage of GSSG remained moderate (25–50%) in seeds dried under 45% RH (Fig. 5). L-AA, which was present in low amounts in fresh seeds and only in its oxidized form, declined very rapidly at both temperatures and both hydration levels, suggesting that this antioxidant does not play a major role in the long-term preservation of seed viability or that it is rapidly consumed.

The accumulation of FFA was also very closely correlated with the evolution of seed viability: the FFA content increased much more rapidly in seeds dried under 81% RH than in those dried under 45% RH, and more rapidly as well in seeds stored at 20°C than in those at 5°C (Fig. 5). The final FFA content of seeds stored at 20°C after drying under 81% RH was very high, i.e. 9 mg g^{-1} of lipid, representing therefore nearly 1% of total lipids. This final amount of FFA was higher than that of PL, indicating that PL hydrolysis could not be the major source of FFA.

Although there was a clear overall relationship between the loss of PL and seed viability decline, the three PL classes exhibited different behaviours (Fig. 6). After drying at 81% RH, the loss of PE was highly correlated with the decline in seed viability (Fig. 6). The loss of PC and PI was observed only under the most detrimental conditions for seed viability (Fig. 6). However, the decrease in the PC and PI appeared to be much delayed in comparison with the loss of PE,

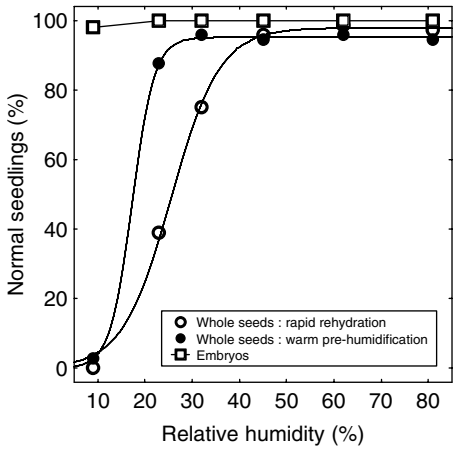

Fig. 2. Viability of *Coffea arabica* (Caturra seed lot 2003) seeds desiccated by equilibration under various RHs (20 days at 25°C) and placed directly under germination conditions after desiccation (o) or pre-humidified in water-saturated air for 24 h at 37°C prior to culture under germination conditions (●) and germination of zygotic embryos (□) extracted from desiccated seeds after 2 days of imbibition in water at 25°C. Solid lines correspond to the fitted patterns of the desiccation sensitivity model. RH, relative humidity.

suggesting that the mechanism underlying PL loss is selective.

Lyso-PLs were not detected in seeds before and immediately after the 3 weeks of desiccation (Tables 1 and 2), and after 3 and 12 months of storage, whatever the conditions, lyso-PC was only present in very low amounts. As PC was the major PL in coffee seeds, this result could indicate that this low PL de-esterification process was non-selective. There was therefore apparently no relationship between the storage behaviour of coffee seeds (Fig. 5) and the pattern of accumulation of lyso-PC (Fig. 6). The evolution of the seed FFA and lyso-PC contents were also qualitatively and quantitatively very different, indicating that PLA_2 activity was certainly not the main cause of lipid hydrolysis in seeds losing their viability. Lyso-PC accumulation was maximal in seeds dried under 45% RH at 20°C where after 1 year FFA represented 0.2% of total lipids. Assuming that hydrolysis was not selective regarding the lipid class attacked (PL or neutral lipids), 0.2% of the PL should be hydrolysed, i.e. 0.002×4.4 mg = 8.8 μg g^{-1} of lipids. The order of magnitude of this theoretical content was very similar to that measured in seeds (25 μg g^{-1} of lipid), showing that de-esterification of lipids could also be not selective, i.e. irrespective of their class.

The hydration status of seeds, the temperature of storage and the time had no significant effect on the content in the five mono-, di- and oligosaccharides detected in coffee seeds (Table 4), suggesting that these sugars play a minor or no role in their storage behaviour at the hydration levels and the temperatures studied.

Influence of the time in equilibrium under 81% RH on seed tolerance to LN exposure

The initial WC of seeds of the seed lot 2003 was 0.49 g H_2O g^{-1} DW. At this WC, seed tolerance to LN exposure was nil (Fig. 7). After 8 days of desiccation, seed WC dropped down to 0.25 g H_2O g^{-1} DW without any significant increase in the proportion of seeds developing into normal seedlings after cryopreservation. By day 10, tolerance to LN exposure increased

Table 2. Effect of the relative humidity (RH) employed for equilibrium drying (20 days at 25°C) on *Coffea arabica* (seed lot 2003) seed water content (WC) at equilibrium and biochemical composition. Results of one-way ANOVA: *P*.

	Equilibrium RH			
	23%	45%	81%	
WC at equilibrium (g/g)	0.07	0.11	0.23	*P*-value
Antioxidants				
Total glutathione (nmol g^{-1} DW)	1086.1	977.1	1269.8	0.34
Glutathione redox status (% GSSG)	15.4	14.3	15.7	0.41
Total L-ascorbate (nmol g^{-1} DW)	36.5	28.7	34.1	0.75
L-ascorbate redox status (% DHA)	96.0	98.3	86.2	0.47
Sugars (mg g^{-1} DW)				
Sucrose	77.4	73.5	72.0	0.59
Glucose	2.9	2.3	2.8	0.70
Fructose	3.0	1.7	2.6	0.15
Raffinose	0.6	0.5	0.2	0.98
Stachyose	0.7	0.7	0.5	0.27

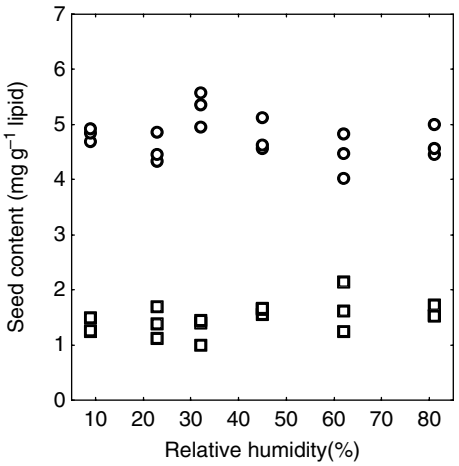

Fig. 3. Phospholipid (○) and free fatty acid (□) contents of *Coffea arabica* (Caturra seed lot 2003) seeds desiccated by equilibration under various relative humidities (20 days at 25°C). Each point corresponds to one measurement.

significantly corresponding to a decrease in seed WC to 0.24 g H_2O g^{-1} DW. After 13 days of desiccation, the equilibrium point was reached and the percentage of seedlings recovered from frozen seeds reached a maximum of 44%. Maintaining the seeds for 7 more days in equilibrium under 81% RH considerably decreased seedling recovery after LN exposure to 26%.

Discussion

The present study clearly demonstrates that oxidative stress, lipid hydrolysis and PL loss are involved in the ageing of coffee seeds stored at high temperature (20°C) and at intermediary RHs (81%). Similar cellular damage has been identified in neem seeds stored under the same conditions (Sacandé et al. 2001). These changes could therefore constitute a shared response in the intermediate seed category. However, the fact that GSH pool reduction, PL loss and lipid hydrolysis are also seen in orthodox tomato seed (De Vos et al. 1994) and pollen storage (Van Bilsen and Hoekstra 1993) suggests that these mechanisms are common to the ageing processes of a panel of organisms much broader than intermediate seeds alone.

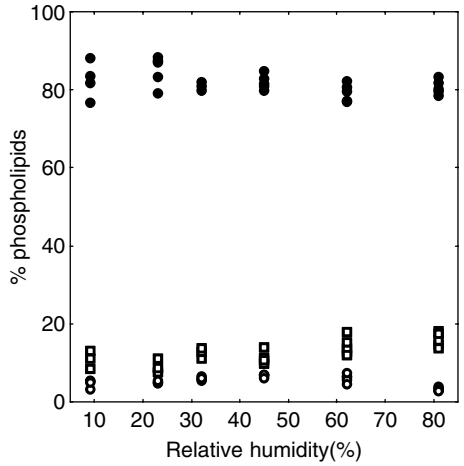

Fig. 4. Phospholipid composition of *Coffea arabica* (Caturra seed lot 2003) seeds desiccated by equilibration under various relative humidities (20 days at 25°C): percentages of PE (□), PC (●) and PI (○). Each point corresponds to one measurement.

Table 3. Effect of rehydration procedure on the percentage of normal seedlings recovered from *Coffea arabica* seeds stored for 1 year at 5 and 20°C after equilibrium drying under 45 and 81% RH. Means followed by the same letter were not significantly different at $P = 0.05$ according to the Newman and Keuls test

	Storage at 5°C		Storage at 20° C	
	45% RH	81% RH	45% RH	81% RH
Rapid rehydration	39 a	49 a	72 b	0
Osmoconditioning	35 a	32 a	65 b	0
Pre-humidification	81 b	35 a	82 b	0
Pre-heating	79 b	38 a	78 b	0

In addition, the present work offers an important new perspective on the effect of low temperature on the storage behaviour of intermediate seeds. In the initial studies that led to the definition of the intermediate category, Ellis et al. (1990) observed that decreasing the storage temperature led to a decreased longevity of dry coffee seeds, and this characteristic still represents one of the main criteria to distinguish orthodox from intermediate seed storage behaviours. In the experiments of Ellis et al. (1990), the hydration levels tested corresponded to seeds dried between 10 and 50% RH. According to our work, the apparent decrease in longevity of seeds stored at low RH and temperature is due to imbibitional damage. Indeed, we have observed that after drying under 45% RH and storage at 5°C for 1 year, the percentage of seedling recovery was 40% only if seeds were rapidly rehydrated, but viability did not decrease when seeds were pre-heated or pre-humidified before germination.

We have not only shown the importance of the rehydration procedure after storage at 5°C but also that decreasing the storage temperature slows down all the deteriorative processes observed at 20°C, i.e. lipid hydrolysis, oxidative stress and PL loss. This important result was relevant at both hydration levels tested and may offer important applied perspectives for germplasm

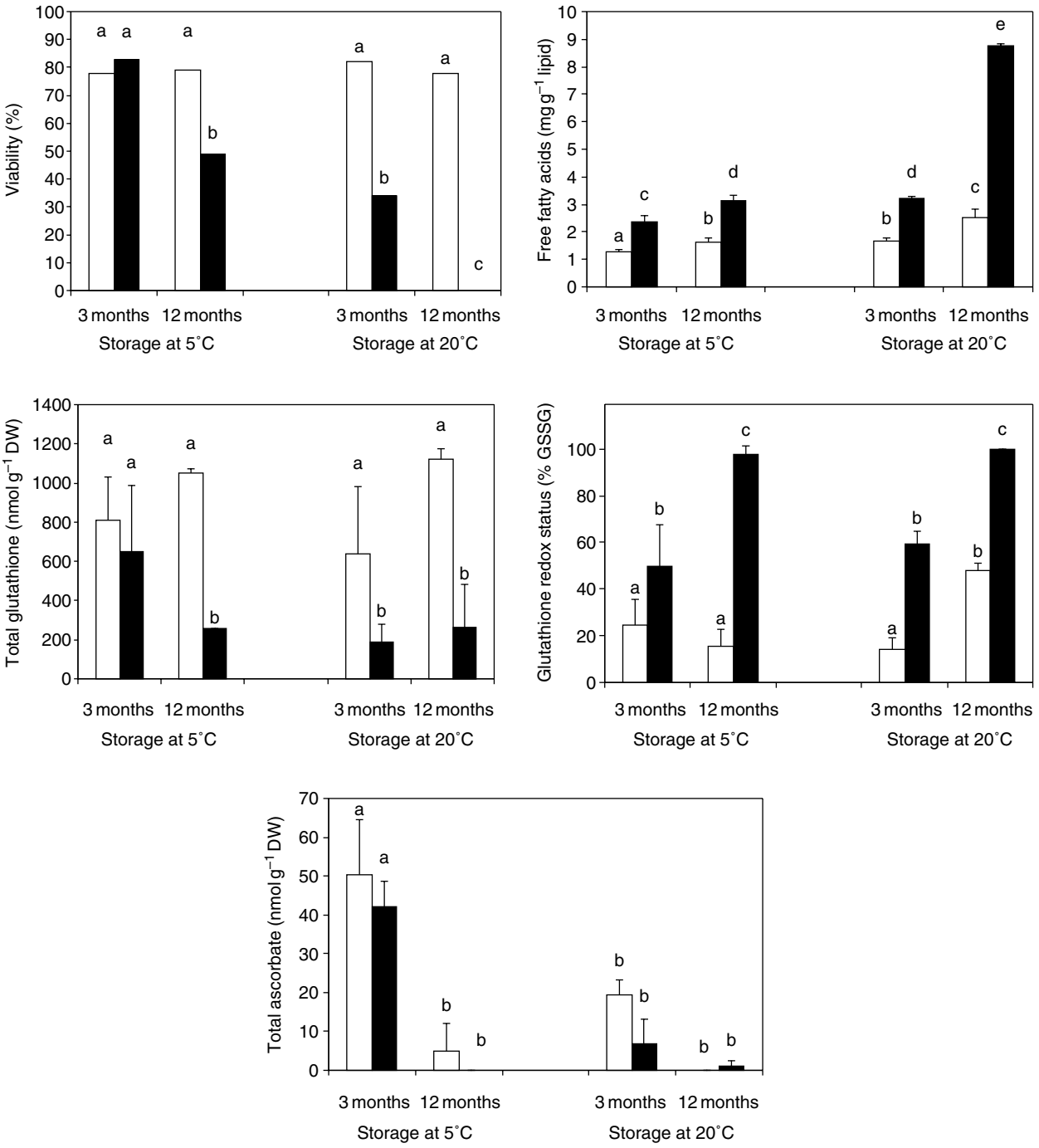

Fig. 5. Effect of the time of storage at 5°C and 20°C on viability (under optimal rehydration conditions), free fatty acid (FFA) content, glutathione and L-ascorbate contents, and redox status of glutathione in *Coffea arabica* (seed lot 2001) seeds after drying under 45% RH (white bars) and 81% RH (black bars). Bars showing the same letter were not significantly different at $P = 0.05$ according to the Newman and Keuls test. RH, relative humidity.

conservation. It also questions one of the two criteria retained by Ellis et al. (1990) in their definition of the intermediate category, i.e. the negative effect of lowering the storage temperature on seed longevity. The main difference between intermediate and orthodox seeds would thus be their differing levels of desiccation tolerance and not the mechanisms underlying their storage behaviour.

Table 4. Probability *P* of significance of the effects of the hydration level (45 and 81% RH), time (3 and 12 months) and storage temperature (5 and 20°C) on the antioxidant, lipid and sugar composition of *Coffea arabica* seeds as determined by three-way ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

	Time	Hydration	Temperature	T × H	T × t	H × t	T × H × t
Antioxidants							
Total glutathione (nmol g^{-1} DW)	0.35	0.001**	0.21	0.034*	0.12	0.41	0.58
Glutathione redox status (% GSSG)	0.000***	0.000***	0.08	0.005**	0.07	0.59	0.018*
Total L-ascorbate (nmol g^{-1} DW)	0.000***	0.10	0.000***	0.236	0.001**	0.91	0.45
Lipids (mg g^{-1} lipid)							
Free fatty acids	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
PE	0.000***	0.000***	0.33	0.000***	0.000***	0.99	0.000***
PC	0.032*	0.004**	0.17	0.08	0.08	0.01	0.33
PI	0.046*	0.000***	0.007**	0.003**	0.032*	0.13	0.16
Lyso-PC	0.049*	0.21	0.24	0.10	0.016*	0.018*	0.18
Sugars (mg g^{-1} DW)							
Sucrose	0.18	0.31	0.22	0.86	0.14	0.05	0.66
Glucose	0.31	0.18	0.27	0.56	0.12	0.49	0.12
Fructose	0.37	0.07	0.62	0.42	0.11	0.75	0.35
Raffinose	0.99	0.52	0.73	0.52	0.26	0.16	0.76
Stachyose	0.1	0.80	0.61	0.55	0.73	0.98	0.30

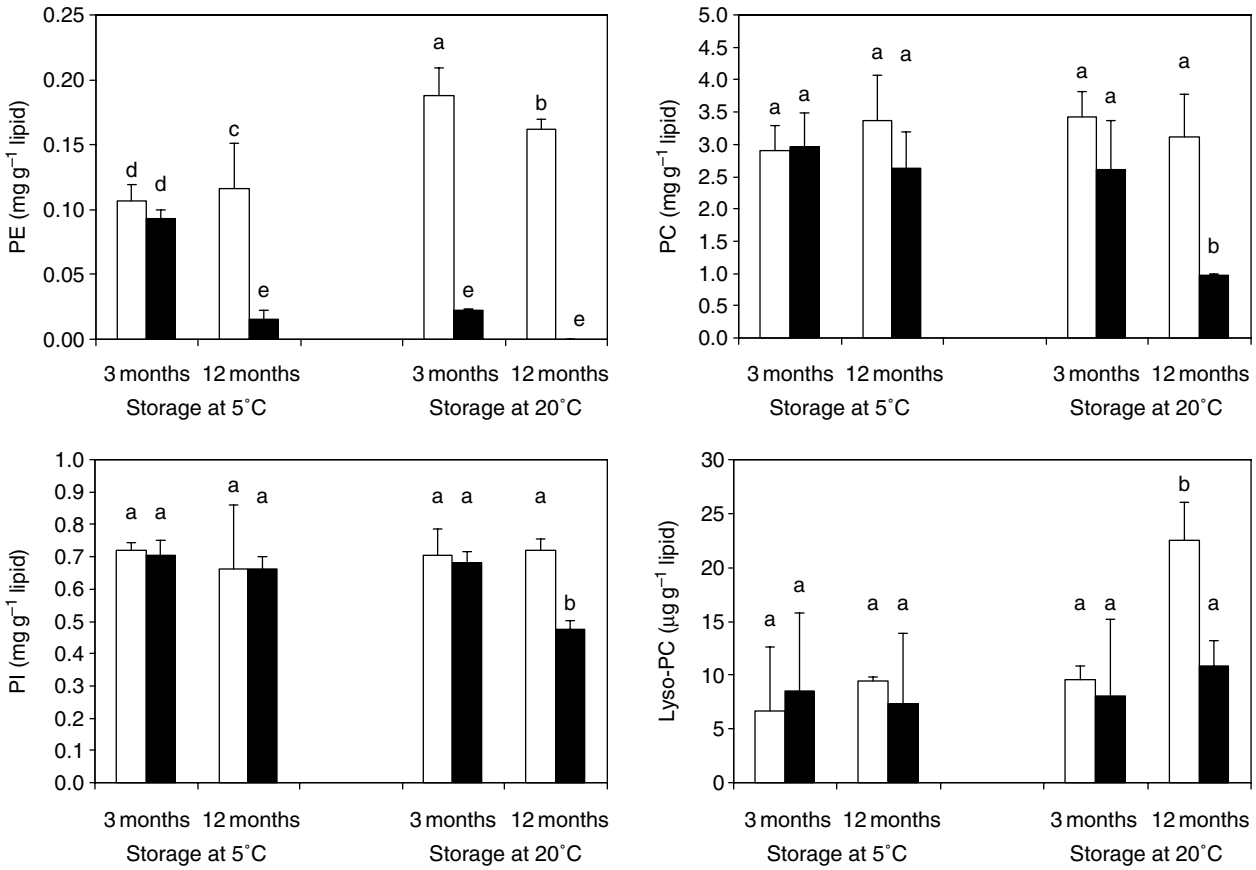

Fig. 6. Effect of the time of storage at 5°C and 20°C on phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and lyso-PC contents in *Coffea arabica* (seed lot 2001) seeds after drying under 45% RH (white bars) and 81% RH (black bars). Bars showing the same letter were not significantly different at $P = 0.05$ according to the Newman and Keuls test. RH, relative humidity.

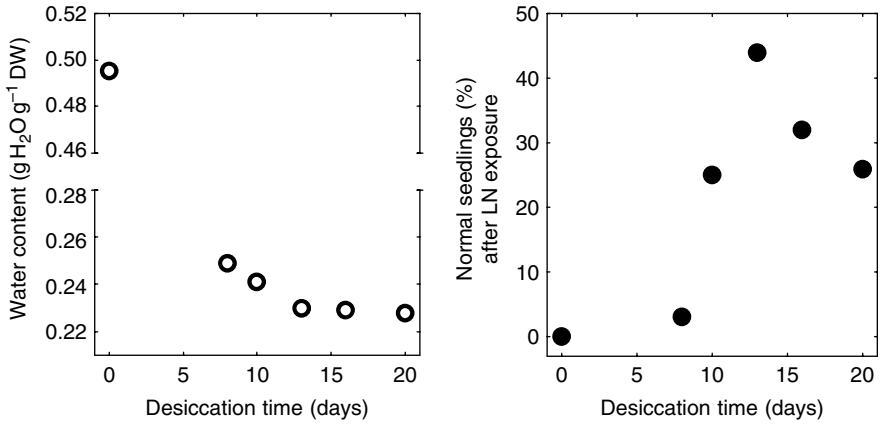

Fig. 7. Water content (WC) and viability after liquid nitrogen exposure of *Coffea arabica* seeds (seed lot 2003) during the 20 days of equilibration under 81% RH. RH, relative humidity.

The origin of oxidative stress in dehydrating organisms is likely to be due to an uncontrolled formation of ROS as a result of the impairment of the electron transport chains (Oliver et al. 2001) and is expected to occur within an interval of intermediary hydration levels where the downregulation of metabolism becomes unco-ordinated (Leprince et al. 1999, 2000). The water potential boundaries of this interval were estimated to be −5 and −15 MPa, i.e. in equilibrium with 90 and 97% RH at 25°C, respectively (Walters et al. 2001). This assumption is in accordance with our results in coffee that the reduction of the antioxidant pool during drying was independent of the final hydration level and that the primary oxidative stress occurred when seeds crossed the interval of hydration levels where ROS are produced. However, we have shown that in seeds dried under 81% RH, oxidative stress was not restricted to the drying step but also continued for several months after drying. According to the above lower boundary, the continuing oxidative stress at 81% RH cannot be explained by mitochondrial ROS release, as at this hydration level, respiration has been reported to have ceased (Walters et al. 2001). Therefore, the most likely explanation for oxidative stress continuing at 81% RH is the occurrence of free radical-based chain reactions, initiated at higher hydration levels. In coffee seeds, drying seeds under 45% RH lowered or even stopped these chain reactions. It can be speculated that the increase in cytoplasmic viscosity with drying (Leprince and Hoekstra 1998) plays an important role in the decrease of the ageing reactions in coffee seeds at 45% RH.

FFAs are well-established membrane destabilizing agents (Crowe et al. 1989, Mckersie et al. 1989, Zuidam et al. 1995) and their accumulation was clearly associated with ageing in coffee seeds. Moreover, we also clearly demonstrated that FFAs arise from the hydrolysis of neutral lipids and not from PLA_2 activity, as proposed by Oliver et al. (1995, 2002) and Welti et al. (2002), as there was no corresponding accumulation of lyso-PL. Although the PLA_2 hypothesis can be discarded, an enzymatic origin of FFA cannot be completely excluded. In coffee seeds, triacylglycerols (TAGs) are stored in oleosin-rich oil bodies (Leprince et al. 1998), and many plant lipases can efficiently hydrolyse the TAG content of oil bodies without pre-hydrolysis of the PL monolayer and of the oleosin coat (Beisson et al. 2001). Therefore, TAGs are not isolated in oil bodies, and as lipases are active at very low RH (<10%, Leopold 1990), the accumulation of FFA observed in ageing coffee seeds could result from lipase-mediated TAG hydrolysis. However, the strong correlation between changes in GSH levels and the accumulation of FFAs suggests that the hypothesis that de-esterification results from free radical attack (e.g. Senaratna et al. 1985, Van Bilsen and Hoekstra 1993) is at least as important.

Importantly, we show here that there is a selective loss of PL during storage and that PE levels decreased very rapidly while PC and PI loss was observed only after 1 year of storage. Again, this loss of PE was not due to fatty acid de-esterification, which would have led to the formation of lyso-PE in significant amounts. Because the presence of PE was apparently strictly required to maintain seed viability, this point deserves further investigations. The formation of *N*-acylphosphatidylethanolamine (NAPE) could have contributed to this rapid and selective loss of PE (Chapman et al. 1999, Rawyler et al. 2002). NAPE is synthesized from PE and FFA and has been identified in seeds of numerous species (Chapman et al. 1999). NAPE is now considered as a new stress-related lipid characteristic of plant cells under oxygen deprivation (Rawyler et al. 2002). Although our HPLC procedure was not set up for measuring NAPE, an unidentified peak, whose retention time corresponds to that observed elsewhere for NAPE under similar HPLC conditions (Néron et al. 2004), was highly negatively correlated with the PE content of seeds (data not shown). In order to

test this attractive hypothesis, we are currently developing a new HPLC method for NAPE quantification in coffee seeds.

The present work also demonstrates that slow drying by equilibration under controlled RH induced significant deteriorative processes in coffee seeds. Indeed, in the three seed lots studied, equilibrium drying led to a considerable reduction in the size of the GSH and L-AA pools and an increase in the FFA content. Slow drying of coffee seeds did not influence directly viability as is the case in recalcitrant seeds (Pammenter et al. 1998), but it did induce oxidative stress and lipid hydrolysis at levels high enough to be detected. This new finding represents an important validation of the view of Walters et al. (2001) that slow drying induces oxidative stress because of the longer time spent within the interval of intermediary hydration levels and where the downregulation of metabolism is unco-ordinated (Leprince et al. 1999, 2000). From the present work, we can thus conclude that the slow drying-induced post-harvest maturation reported in neem seeds by Sacandé et al. (2000) is not a general trait of intermediate seeds. As unfavourable changes were detected in coffee seeds during slow drying, the possibility that maintaining seeds at equilibrium in 81% RH for very short periods (a few days) could alter their level of tolerance to LN exposure was also tested. We found a rapid decline in cryoability within the first week following equilibration. This result shows that seed tolerance to ultra-low temperature is strongly dependent on both the antioxidant status and the lipid integrity of seeds. From a practical standpoint, equilibrium drying remains our preferred method for whole-seed cryopreservation, because it provides the better reproducibility and precision to reach the optimal hydration level in species where the hydration window for seed cryopreservation is extremely narrow (Dussert et al. 2001, Hor et al. 2005). However, the present work shows that it is particularly important to cryopreserve intermediate seeds as soon as the equilibrium point is reached.

None of the physiological parameters studied changed significantly at RH $< 23\%$, the point at which coffee seeds lose their viability. However, seed prehumidification before sowing dramatically increased the level of desiccation tolerance of seeds between 23 and 40% RH, suggesting a pivotal role for the physical state of the membrane (Hoekstra and van der Wal 1988) within this interval of hydration levels. It is then possible that desiccation injury in coffee seeds was associated with irreversible structural changes, such as demixing and the formation of non-lamellar domains in membranes, which could not be detected by the experimental approach employed in the present study because they do not necessarily lead to changes in the biochemical composition of the cell components.

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