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# Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains

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Soluble sugar contents and antioxidant enzyme activities were measured in the embryo of wheat (*Triticum aestivum* L., cv Charger) seeds throughout their storage under two conditions (45 °C and 100% relative humidity (RH), and 30 °C and 75% RH) to investigate whether grain deterioration during ageing was related to lipid peroxidation resulting in a decrease in the efficiency of the antioxidant defence system, and in changes in sugar metabolism. The half-viability period ( $P_{50}$ ) was only 6 d at 45 °C and 100% RH against about 3.75 months at 30 °C and 75% RH. However, sensitivity of grains to accelerated ageing carried out at 45 °C and 100% RH increased during their pre-storage at 30 °C and 75% RH. Loss of grain viability at 45 °C and 100% RH was associated with an accumulation of H<sub>2</sub>O<sub>2</sub> which was concomitant with a progressive decrease in catalase (CAT) and superoxide dismutase (SOD) activities, and with an increase in glutathione reductase (GR) activity. However, malondialdehyde content did not change, suggesting that there was no lipid peroxidation during such an ageing. In return, ageing of grains at 30 °C and 75% RH was not associated either with strong changes in CAT, SOD and GR activities or with an accumulation of H<sub>2</sub>O<sub>2</sub>. When there was no correlation between CAT, SOD and GR activities and seed viability, there was a linear correlation between CAT activity and seed vigour evaluated by the  $T_{50}$  during ageing at 45 °C and 100% RH. Sugar metabolism in wheat embryo largely depended on ageing conditions. Loss of grain viability at 45 °C and 100% RH was associated with a marked decrease in sucrose (Su) and a slight increase in raffinose (Ra), and subsequently in the Ra/Su ratio. At 30 °C and 75% RH, seed ageing was associated with an increase in both sugars but with no strong increase in Su/Ra ratio. Our results suggest that ageing was associated with various mechanisms depending on the conditions of ageing, and that accelerated ageing at 45 °C and 100% RH was not the only model to consider in order to understand the mechanisms involved in seed deterioration.

**Keywords:** Wheat; Ageing; Soluble carbohydrates; Antioxidant enzymes; Lipid peroxidation

## 1. Introduction

During storage of seeds, ageing leads to a progressive decrease in their vigour, and then in a loss of their viability

*Abbreviations:* AOS: active oxygen species; CAT: catalase; DW: dry weight; GR: glutathione reductase; MC: moisture content; MDA: malondialdehyde;  $P_{50}$ : half viability period; RFO: raffinose family oligosaccharides; RH: relative humidity; Ra: raffinose; ROS: reactive oxygen species; SD: standard deviation; SOD: superoxide dismutase; Su: sucrose;  $T_{50}$ : time to reach 50% of the germination percentages obtained after 14 d

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(McDonald, 1999; Priestley, 1986; Smith and Berjak, 1995). Seed susceptibility to ageing varies among the species, but it is also dependent on the maturity stage and the conditions of development (Hay and Probert, 1995; Priestley, 1986). As in pearl millet (Kameswara Rao et al., 1991), tomato (Demir and Ellis, 1992) and bean (Bailly et al., 2001), seed sensitivity to accelerated ageing decreases during the maturation drying phase of wheat grain development (Ellis and Pieta Filho, 1992; Lehner et al., 2006). However, the rate of loss of seed viability is mainly a function of temperature and seed water content (McDonald, 1999; Roberts and Ellis, 1989). In orthodox seeds (seeds that are tolerant to desiccation, such as wheat

grains), there is a positive relationship between viability loss and both factors (Ellis and Roberts, 1980a, b; Priestley, 1986; Roberts and Ellis, 1989).

Seed deterioration during ageing is associated with numerous cellular and biochemical alterations including loss of membrane integrity, impairment of RNA and protein syntheses, and DNA degradation (Gidrol et al., 1988; Gutiérrez et al., 1993; McDonald, 1999; Vazquez-Ramos et al., 1988). Although the exact mechanisms of loss of viability are by no means completely elucidated, reactive oxygen species (ROS) accumulation and lipid peroxidation are often invoked to be the major causes of seed deterioration (Bailly, 2004; Hendry, 1993; Priestley, 1986). Lipid peroxidation has been demonstrated to occur during ageing in soybean (Sung, 1996), Norway maple (Pukacka, 1991), peanut (Sung and Jeng, 1994), beech (Pukacka and Ratajczak, 2005) and sunflower seeds (Bailly et al., 1996; Kibinza et al., 2006), but it does not seem to be involved in other species, such as pigeon pea (Kalpana and Madhava Rao, 1994), maize (Linn and Pearce, 1990) and wheat (Girard and Le Meste, 1992). These contradictory results might be explained partly by the differences in the conditions of ageing performed in these studies. Indeed, oxidative reactions are largely dependent on the seed moisture content (MC). At very low MC, it is likely that they are involved in the deterioration processes. Increasing MC facilitates enzymatic oxidations such as that by lipoxygenase (Vertucci and Farrant, 1995), and leads to faster cellular damage. At MC higher than 0.25 g H<sub>2</sub>O g<sup>-1</sup> dry matter (DM), respiration is estimated to occur (Vertucci and Farrant, 1995) and might be considered to be one of the major sources of production of ROS (Staniek and Nohl, 2000).

The main enzymes involved in cell detoxification are superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT) and peroxidases such as ascorbate peroxidase (APX), glutathione peroxidase (GPX) and thioredoxin peroxidase (TPX) (Bailly, 2004; Mittler, 2002; Scandalios, 1997). The ability of seeds to scavenge ROS is considered to be involved in seed storability (Bailly, 2004; Priestley, 1986). In addition, ageing is associated with a depletion of antioxidant enzyme activities in soybean (Murthy et al., 2002), cotton (Goel et al., 2003), beech (Pukacka and Ratajczak, 2005) and sunflower seeds (Bailly et al., 1996, 1998; De Paula et al., 1996; Kibinza et al., 2006; Torres et al., 1997).

Previous studies on pea, maize and rape seeds assumed that soluble sugars, mainly the raffinose family oligosaccharides (RFO), might also be involved in the protection against the deleterious effects occurring during dehydration and/or ageing, suggesting that they are directly linked to storage ability (for review see: Obendorf, 1997). Moreover, various authors (Bernal-Lugo and Leopold, 1992; Horbowicz and Obendorf, 1994; Piotrowicz-Cieslak, 2005; Sinniah et al., 1998; Steadman et al., 1996) have demonstrated that sugar content, particularly the ratio of oligosaccharide to sucrose contents, might be used as an

indicator of seed storability or ageing. In maize, for example, a value of this ratio higher than 0.2 corresponds to a good seed storability (Bernal-Lugo and Leopold, 1995). Oligosaccharides might contribute to the stabilization of intracellular glasses by increasing cytoplasm viscosity and the glass-to-liquid transition temperature, which are likely to slow down ageing alterations (Bernal-Lugo and Leopold, 1995; Leopold et al., 1994; Sun, 1997).

Most of the studies on the cellular and biochemical deterioration during seed ageing have been performed under accelerated ageing conditions, i.e., at high temperature and in saturating humidity (McDonald, 1999). Although accelerated ageing has been recognized as a good predictor of seed storability, the mechanisms involved in seed deterioration under such extreme conditions differ from those occurring under ambient conditions. In particular, the contribution of lipid peroxidation, sugar hydrolysis and Maillard reactions in seed ageing depended on water content and temperature (Kibinza et al., 2006; Murthy et al., 2003; Walters, 1998).

The aims of the present work were: (1) to characterize the sensitivity of wheat grains to ageing conditions, (2) to investigate whether seed deterioration is associated with H<sub>2</sub>O<sub>2</sub> accumulation and lipid peroxidation depending on temperature and seed MC during ageing, and (3) to determine whether soluble sugars and the activities of the main antioxidant enzymes might be appropriate indicators of the loss of seed viability. For this purpose, wheat seeds were artificially aged for different periods of time under two conditions: 45 °C at 100% relative humidity (RH) conditions which were generally used for accelerated ageing (Priestley, 1986), and 30 °C at 75% RH, conditions which were closer to those of storage during summer months in seed companies.

## 2. Experimental

### 2.1. Plant material

Experiments were carried out with the cultivar Charger of wheat (*Triticum aestivum* L.), which was one among the most cultivated cultivars in France. Plants were grown in the experimental fields of Syngenta-Agro located in Gaillon (Eure, France) in 2004. Ears were collected at the end of the maturation drying phase, i.e., when grain water content reached 10–12% fresh weight basis. Seeds were removed from the ears and placed at 20 °C and 70% RH for 5 months in order to break their dormancy. Experiments were carried out with non-dormant grains and no sprouting injury was observed.

Germination tests were performed with whole non-dormant caryopses. Biochemical measurements were carried out on isolated embryos cut off the seeds with as little endosperm as possible, though still covered dorsally by the seed coat.

## 2.2. Water content determination

Embryos were isolated, weighed and then dried by oven drying at 105 °C for 48 h for determination of the DM. MC was calculated on DM basis. Results are expressed as  $\text{g H}_2\text{O g}^{-1}$  DM and correspond to the means of the values obtained in 3 replicates of 10 embryos  $\pm$ SD.

## 2.3. Germination assays and seed viability determination

Germination assays were performed by placing whole caryopses (4 replicates of 25 grains each) in 9-cm Petri dishes on a layer of cotton wool moistened with deionized water, in darkness at 20 °C. A grain was regarded as germinated when the radicle had pierced the seed coat. Germination counts were made daily up to 14 d.

The results presented are the means of the germination percentages obtained in 4 replicates  $\pm$ SD. They are expressed as the germination percentages obtained after 14 d, or as the time to reach 50% of the germination percentages obtained after 14 d ( $T_{50}$ ) estimated graphically from the cumulative germination curves.

Topographical tetrazolium testing was also used to verify that seeds which did not germinate at 20 °C were dead. For this purpose, seeds were cut longitudinally and were incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (Moore, 1973) for 3–4 h at 25 °C in darkness. Seeds of which the embryo exhibited no overall carmine red staining were scored as non-viable.

## 2.4. Accelerated ageing treatment

Ageing treatments were carried out by placing seeds for 1–7 d at 45 °C in tightly closed boxes with free water at the bottom in order to obtain 100% RH and for 1–4.5 months at 30 °C in tightly closed boxes with saturated NaCl solution at the bottom in order to obtain 75% RH. After ageing, seeds were dried back to their original water content by placing them for 1 week at 20 °C and 70% RH, and germination was tested at 20 °C for 14 d.

## 2.5. Enzyme extraction and assays

Fifty isolated embryos (about 150 mg FW) were ground in liquid nitrogen and then homogenized in 1.5 ml of a potassium phosphate buffer (0.1 M, pH 7.8) containing 2 mM  $\alpha$ -dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1.25 mM polyethylene glycol 4000, and 10% (w/v) of polyvinylpyrrolidone. The homogenate was then centrifuged for 30 min at 14 000g and the supernatant was desalted on a PD 10 column (Amersham Biosciences, Sweden).

SOD (EC 1.15.11) and CAT (EC 1.11.16) activities were determined at 25 °C according to Bailly et al. (1996). The results are expressed as units  $\text{mg}^{-1}$  protein (SOD) and  $\text{nmol H}_2\text{O}_2$  decomposed  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein (CAT).

GR (EC 1.6.4.2) activity was determined at 25 °C as described previously by Esterbauer and Grill (1978), by following the rate of NADPH oxidation at 340 nm. The assay mixture contained 0.5 mM NADPH, 10 mM oxidized glutathione, 3 mM  $\text{MgCl}_2$  in 0.1 M potassium phosphate buffer (pH 7.8), and 100  $\mu\text{l}$  of enzyme extract in a total volume of 400  $\mu\text{l}$ . GR activity was expressed as  $\text{nmol of NADPH oxidized min}^{-1}$   $\text{mg}^{-1}$  protein.

The results presented correspond to the means  $\pm$ SD of the values obtained with three different extracts and three measurements per extract (i.e. nine measurements). Protein contents of the extracts were determined using the Bio-Rad protein assay kit with bovine serum albumin as calibration standard.

## 2.6. Hydrogen peroxide content

$\text{H}_2\text{O}_2$  contents of isolated embryos were determined according to O'Kane et al. (1996). Fifty embryos were ground in a mortar and homogenized with 5 ml of perchloric acid (0.2 N). After 15 min of centrifugation at 13 000g at 4 °C, the resulting supernatant was neutralized to pH 7.5 with 4 N KOH and then centrifuged for 3 min at 1000g and 4 °C. The supernatant was immediately used for spectrophotometric determination of  $\text{H}_2\text{O}_2$  at 590 nm using a peroxidase-based assay. The reaction mixture contained 12 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 1.3 mM 3-methyl-2-benzothiazolidone hydrazone, 20  $\mu\text{l}$  (0.25 units) horseradish peroxidase (Sigma, USA) and 50  $\mu\text{l}$  of the supernatant for a total volume of 1.5 ml. The reaction started with the addition of peroxidase. Increase in absorbance at 590 nm was monitored after 5 min at 25 °C and compared with the absorbance obtained with known amounts of  $\text{H}_2\text{O}_2$ . Results are expressed as  $\text{nmol H}_2\text{O}_2 \text{g}^{-1}$  DW and correspond to the means of the values obtained with five different extracts  $\pm$ SD.

## 2.7. Malondialdehyde measurements

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content from 0.5 g FW of isolated embryos, according to Heath and Parker (1968). Results are expressed as  $\mu\text{mol g}^{-1}$  DW and correspond to the means of measurements carried out with five different extracts  $\pm$ SD.

## 2.8. Soluble sugar measurements

Soluble sugars were extracted according to Black et al. (1996). Ten isolated embryos were ground at room temperature in a mortar in 1 ml 80% aqueous ethanol containing melezitose (1.5  $\text{mg ml}^{-1}$ ) as internal standard, and the extract was heated for 30 min at 80 °C. After centrifugation for 15 min at 14 000g and removal of the supernatant, the pellets were resuspended and reextracted twice, in 0.5 ml and then 0.35 ml 80% aqueous ethanol, at

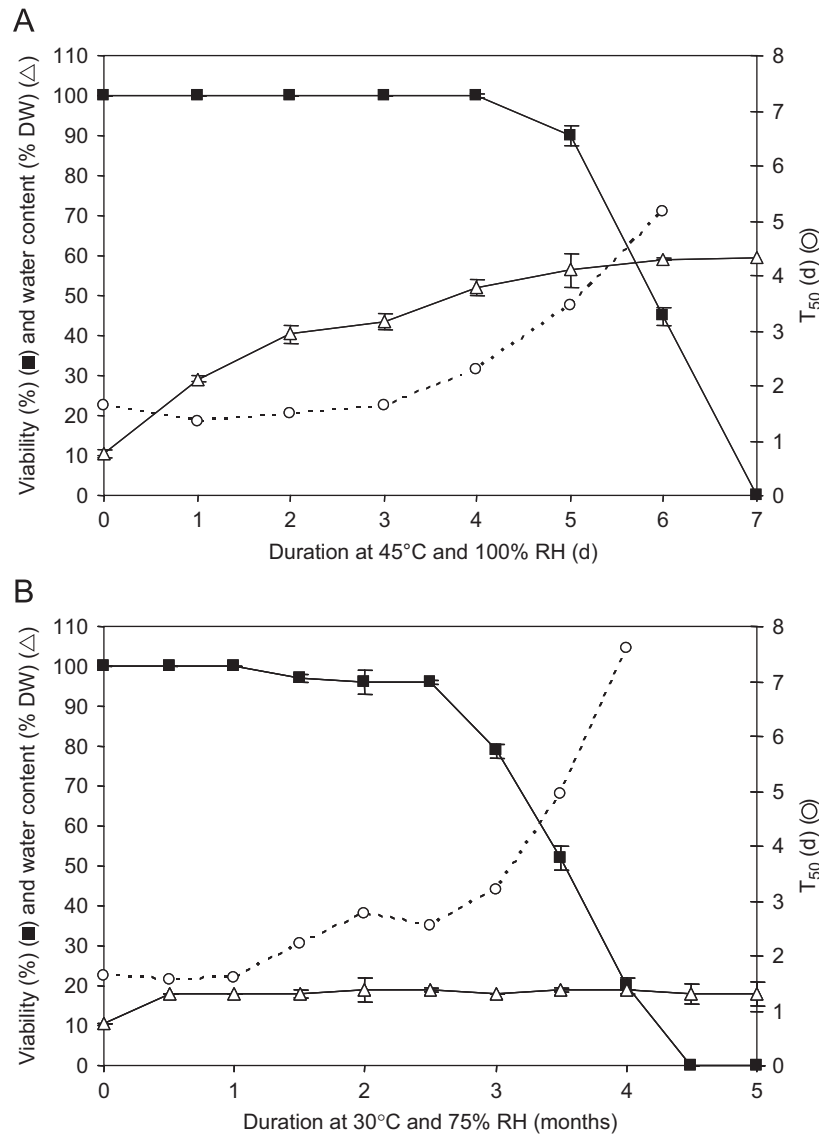


Fig. 1. Changes, during accelerated ageing at 45 °C and 100% RH (A) and at 30 °C and 75% RH (B), in grain viability (■), embryo moisture content expressed on dry weight basis (△) and the time to reach 50% of the germination percentages obtained after 14 d ( $T_{50}$ ) (○). Means of 3 replicates  $\pm$  SD. Where no bars are shown, the spread of SD is less than the size of the symbols.

room temperature and centrifuged again. The three supernatants were combined and reduced to dryness in a centrifugal evaporator (RC 10-22, Jouan, France). The dry extracts were dissolved in 100  $\mu$ l of ultra-pure water and then filtered through an acetate filter (0.45  $\mu$ m pore size, Nalgene, USA) before being analysed by HPLC. Ten  $\mu$ l samples were injected onto a Spherisorb-NH2 column (Thermo Separation Products, France) and eluted with 70/30 (v/v) acetonitrile/H<sub>2</sub>O at a flow rate of 1 ml min<sup>-1</sup> using a Spectra Physics 8700 pump (Spectra Physics, France). The eluents were analysed with a differential refractometer (Spectra Physics 8430) and the peak areas were integrated by a Spectra Physics 4290 integrator. Fructose, glucose, sucrose and raffinose were identified by co-elution with standards (Sigma, USA).

Results are expressed as mg of sugars g<sup>-1</sup> DW of embryos and correspond to the means of three measurements  $\pm$  SD.

### 3. Results

#### 3.1. Effect of ageing treatments on embryo water content and seed germination

Fig. 1 shows the changes in embryo water content, seed viability and the time to obtain 50% of the maximal germination percentages ( $T_{50}$ ) during accelerated ageing performed at 45 °C and 100% RH (Fig. 1A) and at 30 °C and 75% RH (Fig. 1B).

Before ageing treatment, the embryo MC was around 10% DW, all the grains were able to germinate at 20 °C within 3 d, and the  $T_{50}$  was around 1.6 d. The final germination percentage was markedly reduced after ageing for 4 d at 45 °C and 100% RH, and for 2 months at 30 °C and 75% RH. The longer the treatment and the higher the temperature and the RH, the greater was the reduction of seed viability. The half-viability period ( $P_{50}$ ) of wheat grains, i.e., the time taken for germination to drop to 50% of the initial viability, was only 6 d when they were placed at 45 °C and 100% RH (Fig. 1A) as against about 3.75 months at 30 °C and 75% RH (Fig. 1B).

Ageing at 45 °C and 100% RH was associated with a progressive increase in embryo water content which reached about 40% and 60% DW after 2 and 6 d of treatment, respectively (Fig. 1A). It resulted also in a decrease in seed vigour and then in seed viability from 4 d of treatment. Forty-five % of the seed population remained able to germinate after 6 d of ageing and no germination occurred after 7 d.  $T_{50}$  remained at around 1.5–1.8 d during the first 3 d of ageing, and then regularly increased, reaching 5.2 d after 6 d of treatment.

At 30 °C and 75% RH, the embryo water content reached 17% DW after 2 weeks and then remained constant throughout the 5 months of ageing (Fig. 1B). Seed viability started to decline from 2 months, and then decreased linearly as a function of the duration of storage, all the grains being dead after 4.5 months.  $T_{50}$  increased regularly from 1 month of storage. It was twice as high (i.e. 3.2 d) than the  $T_{50}$  of control non-aged seeds and reached 7.7 d after 3 and 4 months of storage, respectively.

The susceptibility of grains to accelerated ageing at 45 °C and 100% RH increased when they were previously treated at 30 °C and 75% RH (Table 1), and the longer the duration of the treatment the stronger was their loss of viability. All control grains placed directly at 45 °C and 100% RH remained viable after 4 d of ageing (Fig. 1A and Table 1), whereas viability of those pretreated for 1.5, 2 and 2.5 months at 30 °C and 75% RH, fell below 50% after 4, 3 and 2 d of accelerated ageing at 45 °C and 100% RH, respectively (Table 1). After 3 months of treatment at 30 °C and 75% RH, almost all the grains died when they were aged at 45 °C and 100% RH for only 2 d.

Table 1

Effect of the duration of pretreatment at 30 °C and 75% RH on the viability of seeds subsequently aged for 2–4 d at 45 °C and 100% RH

Duration of treatment at 30 °C and 75% RH (months)	Seed viability (%) after ageing at 45 °C and 100% RH for (d)			
	0	2	3	4
0 (control)	100	100	100	99 ± 0.7
1.5	100	80 ± 2.0	76 ± 1.4	26 ± 2.8
2	100	64 ± 6.3	40 ± 1.4	18 ± 0.7
2.5	100	40 ± 2.1	8 ± 2.1	12 ± 1.4
3	92 ± 1.1	4 ± 1.9	4 ± 1.8	6 ± 2.3
3.5	86 ± 2.1	2 ± 0.7	8 ± 2.2	2 ± 1.0

Control seeds were placed directly at 45 °C and 100% RH. Means of 4 replicates ± SD.

### 3.2. Hydrogen peroxide content and lipid peroxidation

$H_2O_2$  content of the embryo of untreated control grains was 3.29  $\mu\text{mol g}^{-1}$  DW. It remained close to this initial value, ranging from 3.10 to 3.42  $\mu\text{mol g}^{-1}$  DW, during ageing performed at 30 °C and 75% RH (Table 2). In contrast, when grains were aged at 45 °C and 100% RH, the  $H_2O_2$  content of the embryo decreased during the first 3 d of the treatment, reaching a minimal value of 1.04  $\mu\text{mol g}^{-1}$  DW, and then increased regularly up to 5.54  $\mu\text{mol g}^{-1}$  DW at 6 d of ageing (Table 2).

MDA content of the embryo did not significantly change during either ageing treatments. Its value remained between 0.38 and 0.45  $\mu\text{mol g}^{-1}$  DW, i.e., close to its initial value before ageing (0.42  $\mu\text{mol g}^{-1}$  DW) (Table 2).

### 3.3. Antioxidant enzyme activities

Before ageing, the activities of CAT, SOD and GR in the embryo were 7.3 nmol  $H_2O_2$  decomposed  $\text{min}^{-1} \text{mg}^{-1}$

Table 2

Effect of the duration of ageing on hydrogen peroxide ( $H_2O_2$ ) and malondialdehyde (MDA) contents in wheat embryos

Ageing treatment		$H_2O_2$ ( $\mu\text{mol g}^{-1}$ DW)	MDA ( $\mu\text{mol g}^{-1}$ DW)
Conditions	Duration		
45 °C 100% RH	0 d	3.29 ± 0.02	0.42 ± 0.03
	1 d	2.56 ± 1.20	0.41 ± 0.03
	2 d	1.64 ± 0.51	0.39 ± 0.05
	3 d	1.04 ± 0.00	0.43 ± 0.01
	4 d	1.42 ± 0.20	0.43 ± 0.02
	5 d	2.42 ± 0.80	0.42 ± 0.03
6 d	5.54 ± 0.97	0.39 ± 0.04	
30 °C 75% RH	0 month	3.29 ± 0.02	0.42 ± 0.03
	1 month	–	0.43 ± 0.05
	2 months	3.12 ± 0.10	0.45 ± 0.05
	2.5 months	–	0.43 ± 0.02
	3.5 months	–	0.41 ± 0.10
	4 months	3.42 ± 0.12	0.43 ± 0.03
4.5 months	3.10 ± 0.25	0.39 ± 0.01	

Embryos were isolated from grains aged for various times at 45 °C and 100% RH and at 30 °C and 75% RH. Means of 5 replicates ± SD.

protein, 13.2 units  $\text{mg}^{-1}$  protein and 235 nmol  $\text{NADPH min}^{-1} \text{mg}^{-1}$  protein, respectively. Table 3 shows that the changes in antioxidant enzyme activities in the embryo during loss of seed viability depended on the conditions of ageing.

Accelerated ageing at 45 °C and 100% RH induced an increase from 7.3 to 9.2 nmol  $\text{H}_2\text{O}_2$  decomposed  $\text{min}^{-1} \text{mg}^{-1}$  protein in CAT activity during the first 2 d and then a decrease to 3.0–3.9 nmol  $\text{H}_2\text{O}_2$  decomposed  $\text{min}^{-1} \text{mg}^{-1}$  protein (i.e. 41–53% of its initial value) after 5–6 d (Table 3). SOD activity decreased markedly during the

Table 3  
Effect of the duration of ageing on catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) activities in wheat embryos

Ageing treatment		Activity of		
Conditions	Duration	CAT	SOD	GR
45 °C 100% RH	0 d	7.3 ± 1.1	13.2 ± 2.6	235 ± 49
	1 d	9.6 ± 3.2	6.6 ± 2.1	296 ± 7
	2 d	9.2 ± 1.0	5.4 ± 0.7	649 ± 100
	3 d	6.7 ± 3.1	3.8 ± 0.8	401 ± 40
	4 d	6.1 ± 0.5	1.6 ± 1.2	505 ± 50
	5 d	3.0 ± 1.5	0	1150 ± 360
	6 d	3.9 ± 1.2	0	1140 ± 200
30 °C 75% RH	0 month	7.3 ± 1.1	13.2 ± 2.6	235 ± 49
	0.5 month	5.9 ± 0.6	9.8 ± 1.1	130 ± 30
	1 month	6.6 ± 0.7	9.7 ± 2.6	115 ± 60
	2 months	6.5 ± 0.7	8.1 ± 1.8	–
	2.5 months	5.9 ± 0.1	7.2 ± 1.9	200 ± 60
	3.5 months	6.3 ± 0.3	11.2 ± 3.1	125 ± 40
	4 months	5.8 ± 0.5	9.9 ± 2.2	160 ± 32
4.5 months	6.4 ± 1.0	10.1 ± 2.0	180 ± 21	

Embryos were isolated from grains aged for various durations at 45 °C and 100% RH and at 30 °C and 75% RH. CAT activity: nmol  $\text{H}_2\text{O}_2$  decomposed  $\text{min}^{-1} \text{mg}^{-1}$  protein. SOD activity: units  $\text{mg}^{-1}$  protein. GR activity: nmol  $\text{NADPH min}^{-1} \text{mg}^{-1}$  protein. Means ± SD of the values obtained with three different extracts and three measurements per extract.

treatment performed at 45 °C and 100% RH; it was about 50% and 28% of its initial value after 1 and 3 d, respectively, and became nil after 5 d (Table 3). Contrary to CAT and SOD activities, GR activity increased regularly during ageing and became 4.8 times higher than its initial value (1140 nmol  $\text{NADPH min}^{-1} \text{mg}^{-1}$  protein) after 6 d (Table 3). There existed no clear relationship between the antioxidant enzyme activities and grain viability (data in Fig. 1A and Table 3). In contrast, a negative sub-linear correlation seemed to exist between CAT activity, expressed as the percentage of its initial value measured in non-aged seeds, and the time to obtain the germination of 50% of the viable seeds ( $T_{50}$ ) (Fig. 2), but such a correlation was not observed for SOD and GR.

As early as 0.5 month of ageing at 30 °C and 75% RH, the activity of the antioxidant enzymes studied decreased, representing only about 81%, 75% and 55% of the initial activities of CAT, SOD and GR, respectively (Table 3). These activities then remained almost unchanged until 4.5 months of ageing, i.e., when complete loss of seed viability occurred (cf. Fig. 1B). For example, CAT activity remained between 5.8 and 7.1 nmol  $\text{H}_2\text{O}_2$  decomposed  $\text{min}^{-1} \text{mg}^{-1}$  protein, i.e. between 79% and 97% of its initial value. SOD activity varied between 7.2 and 11.2 units  $\text{SOD mg}^{-1}$  protein, and GR activity fluctuated between 115 and 200 nmol  $\text{NADPH mg}^{-1} \text{protein min}^{-1}$ . There then existed no correlation between the enzyme activities and germination of seeds aged at 30 °C and 75% RH. Interestingly, Table 4 shows that the decrease in CAT activity during accelerated ageing at 45 °C and 100% RH was faster in embryos from seeds previously placed at 30 °C and 75% RH than in those from seeds aged directly at 45 °C and 100% RH. After 3 d of ageing at 45 °C and 100% RH, CAT was between 30.1% and 37% of its initial activity when seeds were previously aged at 30 °C and 75% RH. In contrast, it remained at about 92% of its initial activity when the grains were placed directly at 45 °C and 100% RH.

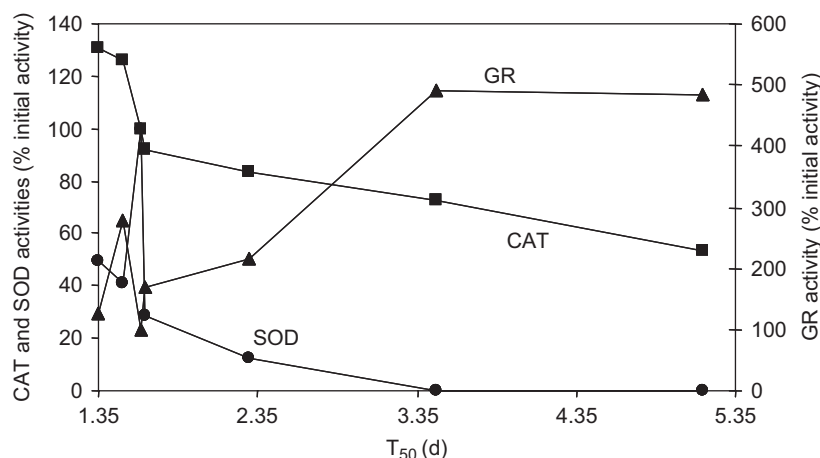


Fig. 2. Relationships between the time to obtain 50% germination ( $T_{50}$ ) at 20 °C with grains aged for various times at 45 °C and 100% RH, and catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) activities measured in the embryos. Values from Fig. 1A and Table 3.

Table 4

Effect of the duration of pretreatment at 30 °C and 75% RH on CAT activity in the embryo of seeds subsequently aged at 45 °C and 100% RH for 2–4 d

Duration of ageing at 30 °C and 75% RH (months)	CAT activity (% initial value) after subsequent ageing at 45 °C and 100% RH for (d)			
	0	2	3	4
0 (control)	100	126.1	91.8	83.6
1.5	87.7	103.6	37	26.5
2.5	80.8	91.8	30.2	20
3	80.8	80.8	30.1	17.8
3.5	86.3	94.5	31.5	17.8

Control seeds were placed directly at 45 °C and 100% RH. Means of the values obtained with three different extracts and three measurements per extract. CAT activity in the embryo of untreated control seeds was  $7.3 \pm 1.1$  nmol H<sub>2</sub>O<sub>2</sub> decomposed min<sup>-1</sup> mg<sup>-1</sup> protein.

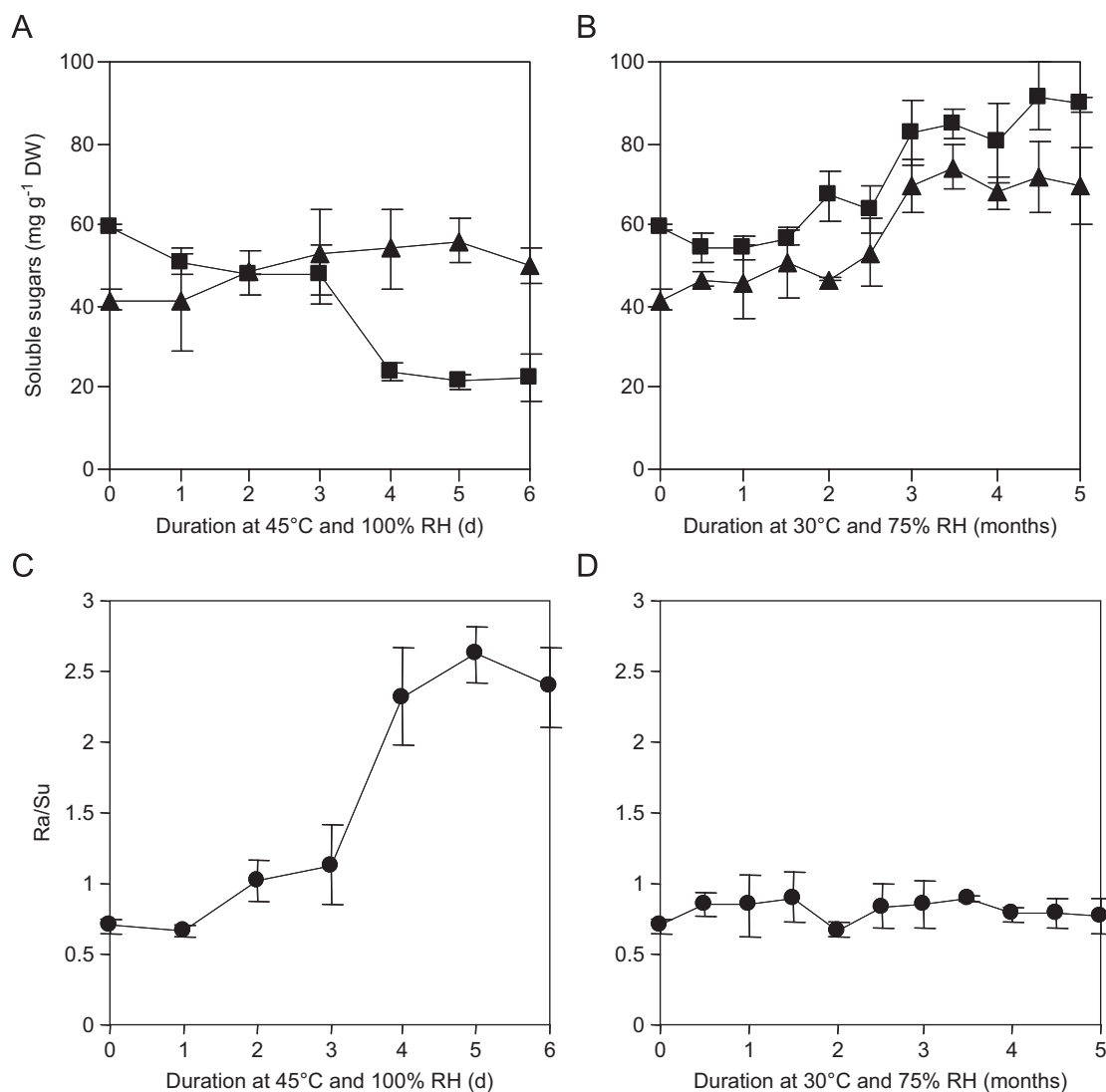


Fig. 3. Changes in sucrose (■) and raffinose (▲) contents (A, B) and in the Ra/Su ratio (C, D) in embryos from grains aged at 45 °C and 100% RH (A, C) and at 30 °C and 75% RH (B, D). Means of three measurements  $\pm$  SD. Where no bars are shown, the spread of SD is less than the size of the symbols.

However, there was no correlation in the duration of the pretreatment at 30 °C and 75% RH and the decrease in CAT activity during subsequent ageing at 45 °C and 100% RH (Table 4).

### 3.4. Soluble sugars

Fig. 3 shows the changes in soluble sugar contents and in the Ra/Su ratio in embryos excised from seeds aged for

various times at 45 °C and 100% RH (Fig. 3A and C) and at 30 °C and 75% RH (Fig. 3B and D). In embryos of untreated control seeds, soluble sugars were mainly sucrose (Su, 59.5 mg g<sup>-1</sup> DW) and raffinose (Ra, 49.4 mg g<sup>-1</sup> DW), hexoses (glucose + fructose) being almost absent.

During ageing at 45 °C and 100% RH, the Ra content of the embryos remained almost constant, ranging from around 40 to 50 mg g<sup>-1</sup> DW (Fig. 3A). Su content decreased between the third and the fourth day of the treatment, and then remained almost constant at around 21–23 mg g<sup>-1</sup> DW. Consequently, the Ra/Su ratio increased progressively during ageing, reaching the value of 2.5 after 6 d as against 0.7 for the non-aged control seeds (Fig. 3C).

When ageing was performed at 30 °C and 75% RH, both sugars increased similarly during the treatment. After 4.5–5 months, Su content was around 90 mg g<sup>-1</sup> DW, and that of Ra reached 70 mg g<sup>-1</sup> DW (Fig. 3B). The increase in both soluble sugars resulted in the Ra/Su ratio varying between 0.6 and 0.9, i.e. close to its initial value, during the 5 months of ageing (Fig. 3D).

#### 4. Discussion

As previously shown by other authors (McDonald, 1999; Priestley, 1986), seed ageing is characterized by a progressive decrease in seed vigour followed by a loss of seed viability, the rate of which is a positive function of temperature and water content. As expected, wheat seeds were more sensitive to accelerated ageing carried out at 45 °C and 100% RH, when water content of the embryo was 50–60% DW, than to a treatment performed at 30 °C and 75% RH, when the embryo water content was only 17% DW (Fig. 1).

Susceptibility of seeds to accelerated ageing is a good indicator of their vigour in numerous species (McDonald, 1999). In wheat, previous studies (Ellis and Pieta Filho, 1992; Lehner et al., 2006) have shown that this susceptibility decreases during the maturation-drying phase of seed development, suggesting an increase in seed vigour after the end of seed filling. The present work demonstrates that storage of grains at 30 °C and 75% RH resulted in an increase in wheat seed sensitivity to accelerated ageing at 45 °C and 100% RH, i.e., in a decrease in seed vigour, as early as after 1.5 months of storage, whereas a significant increase in  $T_{50}$  was observed only after 2–3 months (Fig. 1B).

Lipid peroxidation and oxidative stress have been widely indicated as the major causes of seed deterioration during ageing (McDonald, 1999; Priestley, 1986). Previous data obtained with various species, such as sunflower (Bailly et al., 1996; Kibinza et al., 2006), soybean (Sung, 1996), Norway maple (Pukacka, 1991), watermelon (Chiu et al., 1995) and peanut (Sung and Jeng, 1994), have demonstrated that loss of seed viability during accelerated ageing is associated with an accumulation of MDA. On the contrary, the results presented here with wheat (Table 2)

and those obtained by Pearce and Abdel-Samad (1980) with peanut, by Priestley and Leopold (1979) with soybean and by Kalpana and Madhava Rao (1994) with pigeon pea show that loss of seed viability is not related to an increase in lipid peroxidation evaluated by MDA content. Furthermore, Girard and Le Meste (1992) did not observe any correlation between free radical content and the viability of wheat grains. Although loss of seed viability results from cellular damage, different mechanisms might be involved in mortality depending on ageing conditions, mainly on water content (Kibinza et al., 2006; Murthy et al., 2003; Walters, 1998), which might explain why the lipid peroxidation model has often been the subject of debate. In sunflower seeds, for example, Kibinza et al. (2006) showed that loss of seed viability at 35 °C is associated with lipid peroxidation when MC of the embryo axis is higher than 29.9% DW, whereas below this critical MC it results from accumulation of H<sub>2</sub>O<sub>2</sub> without lipid peroxidation. In wheat grains placed at 45 °C and 100% RH, i.e., at embryo MC close to 50–60% DW, loss of viability might also be attributed to H<sub>2</sub>O<sub>2</sub> production without MDA accumulation (Table 2). H<sub>2</sub>O<sub>2</sub>, or its derivatives such as the hydroxyl radical, are known to react with other molecules than lipids, such as proteins and nucleic acids (Beckman and Ames, 1997). These oxidative attacks can lead to inactivation of key enzymes and/or damage to transport protein receptors or ion channels, thus resulting in extensive cellular dysfunction (Halliwell and Gutteridge, 1999). At 30 °C and 75% RH, i.e. at low seed MC, loss of viability occurred without accumulation of H<sub>2</sub>O<sub>2</sub> or MDA, but we cannot exclude a possible role of secondary products, such as free radicals, aldehydes and hydroperoxides, produced through autooxidation reactions, in cell deterioration (McDonald, 1999).

In addition, our results show that loss of wheat seed viability at 45 °C and 100% RH is concomitant with a progressive impairment of the efficiency of the antioxidant defence system (Table 3). It is in particular associated with a decrease in CAT and SOD activities and in an increase in GR activity (Table 3), the latter being not marked enough to limit H<sub>2</sub>O<sub>2</sub> accumulation from the fifth day of the treatment (Table 2). Depletion of antioxidant enzyme activities was also observed during seed ageing in soybean (Murthy et al., 2002), cotton (Goel et al., 2003), beech (Pukacka and Ratajczak, 2005) and sunflower (Bailly et al., 1996, 1998; De Paula et al., 1996; Kibinza et al., 2006; Torres et al., 1997). However, the enzymes involved in the antioxidant defence system exhibit different sensitivities to ageing depending on the species and the conditions of storage. In wheat seeds, SOD was more sensitive than CAT to a treatment at 45 °C and 100% RH (Table 3), whereas in sunflower seeds placed in the same conditions CAT activity was more sensitive than that of GR, SOD being almost unchanged (Bailly et al., 1996). Alteration of SOD activity was also observed during ageing of seeds of *Acer platanoides* (Pukacka, 1991) and soybean (Sung, 1996), but an increase in GR activity during ageing treatment is in disagreement with data obtained by some authors

(Bailly et al., 1996; De Paula et al., 1996; De Vos et al., 1994; Kibinza et al., 2006). Such an increase in GR activity measured *in vitro* probably did not reflect the *in vivo* activity since H<sub>2</sub>O<sub>2</sub> strongly accumulated after 4 d of ageing. Although H<sub>2</sub>O<sub>2</sub> did not accumulate during the first day of ageing at 45 °C and 100% RH (Table 2), there existed a good correlation between seed vigour, evaluated by T<sub>50</sub> at 20 °C, and CAT activity, but such a correlation between T<sub>50</sub>, or seed viability, and both SOD and GR activities was less obvious (Fig. 2). CAT activity was also suggested to be a good indicator of seed vigour in sunflower (Bailly et al., 1998) and rice (Tanida, 1996).

After 0.5 month of accelerated ageing performed at 30 °C and 75% RH, the activities of CAT, GR and SOD were lower than those measured in control non-aged seeds. Thereafter, they remained almost unchanged (Table 3). There was no correlation between the enzyme activities measured *in vitro* and seed viability. The ability of wheat embryos to maintain the enzyme activities was not then a good indicator of ageing, which was in disagreement with the suggestion of Bernal-Lugo et al. (2000) with maize caryopses. However, we cannot exclude the possibility that the antioxidant machinery became unable to remove the ROS produced during ageing and by resumed metabolism during the imbibition phase of germination (Bailly, 2004). In addition, our results show that the sensitivity of CAT to accelerated ageing at 45 °C and 100% RH increased with the duration of previous treatment at 30 °C and 75% RH.

Different authors have demonstrated that soluble sugars, mainly oligosaccharides, and the oligosaccharides/sucrose ratio are positively correlated with potential seed longevity (Bernal-Lugo and Leopold, 1995; Horbowicz and Obendorf, 1994; Lin and Huang, 1994; Sinniah et al., 1998; Steadman et al., 1996) and/or are good indicators of seed ageing (Bernal-Lugo and Leopold, 1992; Petruzelli and Taranto, 1989; Piotrowicz-Cieslak, 2005; Yaklick, 1985). It has been suggested that oligosaccharides inhibit crystallization of sucrose during desiccation, and thus permit formation of a glassy state which is likely to slow down ageing alterations (Bernal-Lugo and Leopold, 1995; Buitink et al., 1998b; Leopold et al., 1994; Leprince and Walter-Vertucci, 1995; Walters, 1998). There is, for example, a good correlation between the longevity of pollen of *Typha latifolia* or pea seeds and the molecular mobility in the cytoplasm (Buitink et al., 1998a). This seems to be confirmed for wheat seeds when pre-harvest sprouting occurs, since a decrease in raffinose is associated with a loss of storage ability (Lehner, 2005). According to previous data (Black et al., 1996; Lehner et al., 2006), raffinose and sucrose are the main soluble sugars in the embryo of wheat and the value of the raffinose/sucrose ratio is about 0.7 in embryo of mature grains (Fig. 3). Our results demonstrate clearly that sugar metabolism in wheat embryo largely depends on ageing conditions (Fig. 3). Loss of seed viability at 45 °C and 100% RH was associated with a marked increase in the Ra/Su ratio from the third day of accelerated ageing (Fig. 3C), due to a decrease in Su

content (Fig. 3A). Decrease in soluble sugars which has already been observed in wheat (Petruzelli and Taranto, 1989), maize (Bernal-Lugo and Leopold, 1992) and soybean seeds (Yaklick, 1985) during accelerated ageing, indicates that activity of hydrolytic enzymes might occur during the treatment. In fact,  $\alpha$ -galactosidase and invertase are active as soon as the water activity (W<sub>a</sub>) is higher than 0.50 and 0.58, respectively (Drapon, 1985; Silver and Karel, 1981). In addition, soluble sugars can be metabolized through respiration which occurs when the water content is higher than 25% DW (Bruni and Leopold, 1991; Vertucci and Farrant, 1995) and the rate of Maillard reactions increases as water content rises above 0.14 g H<sub>2</sub>O g<sup>-1</sup> DW (Murthy et al., 2003). In wheat embryo, a value of the Ra/Su ratio exceeding 1, due to a decrease in Su, was associated with the outset of loss of viability at 45 °C and 100% RH. The marked decrease in sucrose content observed after 3 d of ageing might reflect an increase in respiratory metabolism leading to an increase in H<sub>2</sub>O<sub>2</sub> production (Table 2). It is well known that mitochondria participate for a large part in the production of AOS (Kowaltowski and Vercesi, 1999), and Kibinza et al. (2006) showed in sunflower embryo that the higher the energy metabolism during ageing the lower was the seed viability.

In contrast, loss of viability of wheat grains placed at 30 °C and 75% RH was associated with a progressive increase in both sucrose and raffinose contents in the embryo (Fig. 3B). Increase in sucrose might result from the hydrolysis of starch which is present in the embryo of mature grains at harvest (Black et al., 1996). Increase in sucrose was also observed in lupin seeds stored for several years under laboratory conditions (about 20 °C), but it was concomitant with a decrease in oligosaccharide content (Piotrowicz-Cieslak, 2005). Such data suggest that biochemical (enzymatic) changes can occur at low MC. Reducing sugars are also known to play an important role in the Amadori and Maillard reaction (Wettlaufer and Leopold, 1991), the products of which are involved in various alterations during ageing (Kristal and Yu, 1992; Monnier, 1990; Murthy et al., 2003). However, no increase in fructose, glucose or galactose has been noticed in wheat embryo during either type of ageing (data not shown).

In conclusion, our results demonstrate that ageing of wheat grains (cv Charger) is associated with changes in the antioxidant defence system and in soluble sugar content in the embryo. Similar results were obtained with other cultivars such as Trémie and Apache (data not shown). They give evidence that these biochemical events largely depend on the conditions of ageing and underline the likely key role of the embryo water content in the mechanisms involved in seed deterioration or protection against ageing damage. Consequently, accelerated ageing cannot be used as the only model to understand the mechanisms involved in seed ageing, and we cannot establish a clear relationship between either active oxygen species scavenging, or soluble sugars and seed ageing independently on the conditions of

storage. This absence of correlation might result from the fact that all measurements were carried out in embryos isolated from aged seeds before their imbibition. Further investigations are required for elucidating the effects of storage conditions on the subsequent efficiency of the antioxidant system during the critical step of imbibition.

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