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Antioxidants, Free Radicals, Storage Proteins, and Proteolytic Activities in Wheat (*Triticum durum*) Seeds during Accelerated Aging

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Accelerated aging was performed by incubation of wheat seeds at 40 °C and 100% relative humidity for 3, 4, 6, 10, and 14 days. The effects of the treatment on seed germinability and on several biochemical characteristics of flour (carotenoids, free radical and protein contents, and proteolytic activity) and gluten (free radical content and flexibility) were evaluated. A decrease of germinability was found during aging, the germination being completely inhibited after 14 days. The lutein content decreased gradually, without going to zero, while that of free radicals increased. A reduction of soluble proteins and a degradation of glutenins and gliadins were observed, associated with a substantial increase of protease activity and a decrease in gluten flexibility. The results were discussed in reference to those previously obtained by natural aging of wheat seeds of the same species and cultivar.

KEYWORDS: ***Triticum durum*; seed aging; free radicals; spin labeling EPR; lutein; proteases; storage proteins**

INTRODUCTION

Seeds deteriorate and lose their viability during periods of prolonged storage. The two most important environmental factors influencing the rate of deteriorative processes in seed aging are the relative humidity of the air, which controls seed moisture content, and the temperature (*1*−*4*). Considerable research has occurred to achieve a better understanding of the physiology of seed deterioration. However, a common interpretation has not been formulated yet, due to many reasons related to both the complexity of seed physiology and the variety of methods employed (*4*). In fact, studies performed on seed deterioration show that this process has devastating consequences on almost every physiological event in a normally functioning cell. In particular, DNA and RNA are somehow degraded leading to impaired transcription and translation, cellular membranes show an increased permeability, and changes in carbohydrate reserves and antioxidants occur (*1*−*4*). Most evidence suggests that free radical peroxidative attacks to membrane lipids could cause these degenerative events (*5*). However, at present, this model of seed deterioration remains speculative (*1*−*4*).

Accelerated aging is a method using high temperature and relative humidity that permits controlled deterioration of seeds, and is employed to foresee their storage potential (*6*). However, this artificial aging process might be physiologically different from natural aging. In fact, some authors questioned whether physiological events occurring during accelerated aging reflected those found during natural aging, whereas others concluded that physiological changes in seeds subjected to accelerated aging were the same as natural aging, with the only difference being the rate at which they occur (see ref *4* and references therein). The answer to this dilemma is further complicated by the fact that, to the best of our knowledge, only few comparative studies of natural and accelerated aging of seeds of the same species and cultivar are reported in the literature.

In a previous study performed by our research group (*7*), the effects of natural aging on some physiological and biochemical properties of wheat (*Triticum durum* cv. Cappelli) seeds were

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investigated. Germination tests were performed on seeds, whereas organic free radicals content was measured in flour and gluten by electron paramagnetic resonance (EPR) and carotenoids in flour were determined by RP-HPLC. Moreover, gluten flexibility was investigated using the spin labeling EPR technique (*8*, *9*), which has been revealed to be particularly useful for achieving information on the structure and dynamics of gluten proteins (*10*−*12*). In the present work, the same parameters were measured on wheat seeds of the same cultivar during accelerated aging and the results were compared to those previously obtained to assess whether physiological and biochemical processes governing accelerated aging and dry storage of seeds were identical.

Findings on gluten from accelerated aged seeds prompted us to better investigate the effects of artificial aging on endosperm proteins by analyzing both electrophoretic profiles of storage proteins and proteolytic activities in flour. In particular, we were interested in understanding whether the changes in proteins and enzymes of accelerated aged seeds were similar to those occurring during seed germination as previously stated by Chauan et al. (*13*).

MATERIALS AND METHODS

Materials. Seeds of durum wheat (*Triticum durum* cv. Cappelli) were cultivated in the experimental fields of the Department of Botanical Sciences of Pisa University (Italy). Seeds were harvested in 1999 and stored in sealed glass containers at 10 °C in the dark.

trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), phenylmethanesulfonyl fluoride (PMSF), pepstatin A, hemoglobin (Hb), azocasein, *N*-carbobenzoxy-L-phenylalanyl-L-alanine (CBZ-Phe-Ala), and 3-maleimidoproxyl (3-MAL) were purchased from Sigma (St. Louis, MO).

Vitavax 200FF was purchased from Uniroyal Chemical Co. Inc. (Middlebury, CT).

All reagents and solvents were of analytical grade and used without further purification.

Accelerated Aging. Accelerated aging was performed by storage of seeds at 40 °C and 100% RH according to Delouche and Baskin (*6*). Before treatment, seeds of uniform size were selected by sieving, surface disinfected in 1% NaClO for 5 min and washed 10 times with sterile distilled water. A lot of 90 g of seeds (approximately 1400 caryopses) was used for each incubation period. All seeds were surface treated with fungicide Vitavax 200FF. Every lot was suspended over 3 L of distilled water on a plastic mesh tray within a closed plastic box (25 × 25 × 14 cm). Boxes were maintained at 40 °C in a growth chamber for 3, 4, 6, 10, and 14 days, respectively. After treatment, seeds were rapidly air-dried under a laminar flow hood until they reached their original moisture content (10.9%). The moisture content was controlled by weighing.

Control seeds were subjected to the same treatments except for accelerated aging.

Germination Tests. For germination tests, four replicates of 25 seeds from each lot were uniformly spread into Petri dishes with filter paper placed on the top and on the bottom and imbibed with 6 mL of distilled water. Germination was carried out at 23 °C in the dark for 24, 48, and 72 h. A seed was considered germinated when the primary root was at least 1-mm long.

Results were expressed by means of two parameters (*7*), i.e., germination capacity, defined as the percentage of completely germinated seeds after 72 h, and germination rate, determined according to the following equation:

$$\text{germination rate} = \frac{nt_1 \times 100 + nt_2 \times 50 + nt_3 \times 33.3}{N_{tot}}$$

where nt_1 is the number of seeds germinated after 24 h, nt_2 and nt_3 are the number of seeds germinated between 24 and 48 h and between 48 and 72 h, respectively, and N_{tot} is the number of seeds used in the test.

Coleoptile and primary root were measured after 72 h of seed incubation in germination conditions.

Preparation of Flour and Gluten. Seeds were ground in a break roller-mill (Labormill 4 RB, Italy) and flour was stored at −20 °C. Gluten was manually extracted from flour according to the following procedure. Three milliliters of distilled water was added to 5 g of flour, mixed with a glass rod and the so obtained dough was washed drop by drop with 300 mL of distilled water. Gluten was separated from flour and water using a thin mesh net, frozen, lyophilized, homogenized in dry conditions with a pestle and a mortar, and stored at −20 °C. Gluten had a moisture content of ∼6% and reconstituted readily with water at room temperature. Flour from seeds aged for 14 days was unable to give gluten.

Extraction of Carotenoids and Determination of Lutein. Carotenoids were extracted from flour with CH_3OH following the procedure of Pinzino et al. (*7*).

Lutein content in the extracted solutions was determined by isocratic RP-HPLC using a Water apparatus model 510 equipped with two pumps and a spectrophotometric detector (Water model 441) and a Nucleosil 300-7 C-18 column (4 × 250 mm). Extracts were eluted at 30 °C using 96% CH_3OH as mobile phase at a flow rate of 0.5 mL/min and detected at 445 nm. In these conditions, retention time for lutein was 15 min.

Front-Surface Absorbance Measurements. Front-surface absorbance spectra of flour samples were obtained following the method of Zandomeneghi et al. (*14*). A Jasco FP770 spectrofluorometer was used in the reflectance experiments. The samples, about 0.6-mm thick, were enclosed in a homemade cell, suitably designed to avoid specular reflections in measurements on powder (*15*). The cell windows were 30° tilted with respect to the incident beam. Powdered $BaSO_4$ was used as a reference light scatterer (*16*).

EPR Measurements. EPR measurements were performed using a Varian (Palo Alto, CA) E112 spectrometer (X-band) equipped with a Varian E257 temperature control unit. The spectrometer was interfaced to a 100 MHz personal computer by means of a homemade data acquisition system consisting of an acquisition board (*17*) and a software package especially designed for EPR and ENDOR experiments (*18*). The content of free radicals was measured at room temperature on dry gluten and flour powders inserted in a quartz tube with internal diameter of 4 mm. Spectra were recorded using a standard EPR cavity, at a field set of 3265 G, a scan range of 100 G, a microwave power of 10 mW, a time constant of 0.125 s, and a modulation amplitude of 1.25 G. Quantification of organic radicals was performed by comparison of the double integrals of the spectra with that of the standard Varian Weak Pitch measured under identical instrumental conditions.

The 3-MAL spin label (Sigma Chemical, St. Louis, MO) was used to label the sulfydryl groups of cysteine residues in the gluten samples. Spin labeling was performed following the method reported by Capocchi et al. (*12*). For EPR measurements, about 30 mg of the fully hydrated labeled gluten was inserted into a quartz tube closed at both ends to avoid sample loss. EPR spectra were recorded between 0 and 50 °C with 5 °C increments using a standard EPR cavity, at a field set of 3265 G, a scan range of 100 G, a microwave power of 2 mW, a time constant of 0.125 s, and a modulation amplitude of 1.25 G.

Soluble Proteins Extraction and Evaluation. Albumins and globulins are commonly referred to as soluble proteins in flour (*19*). They were extracted from flour with 0.1 M Tris-HCl buffer (pH 7.4) (ratio 1:3, w/v). Their concentration in flour was evaluated using the Lowry method as modified by Besandoun and Weinstein (*20*). Bovine serum albumin was used as a standard, and the measurements were performed in a Perkin-Elmer 550S UV−vis spectrophotometer.

Storage Proteins Extraction and Analysis. Gliadins and glutenins, the two most abundant classes of storage proteins in wheat seeds were extracted as reported by Capocchi et al. (*21*) and freeze-dried.

Analyses were performed by discontinuous SDS−PAGE according to Laemmli (*22*), using a 12% resolving gel and a 4% stacking gel. Freeze-dried proteins were dissolved in 62 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.1% Bromophenol Blue to a final concentration of 1 mg of protein/mL, boiled for 8 min and loaded (4 μL) on SDS gels. Molecular weight

Table 1. Germination Parameters of Unaged and Aged Wheat Seeds

duration of accelerated aging (days)	germination capacity (%)	germination rate	root length (mm)	shoot length (mm)
0	98 ± 2	76 ± 4	31 ± 1	15 ± 1
3	82 ± 3	50 ± 9	24 ± 1	12 ± 1
4	42 ± 4	41 ± 6	15 ± 3	7 ± 1
6	26 ± 6	13 ± 4	13 ± 2	6 ± 1
10	6 ± 3	2 ± 2	4 ± 1	3 ± 1
14	0	0	0	0

standards used included β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), ovotransferrin (77.0 kDa), bovine serum albumin (66.25 kDa), glutamate dehydrogenase (55.5 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30.0 kDa), myoglobin (17.2 kDa), and cytochrome *c* (12.3 kDa). The gels were stained overnight with Coomassie Brilliant Blue R250 (*23*), destained with 7% (v/v) acetic acid, scanned and processed by a digital image analysis program (SigmaGel, Jandel Corp.).

Enzymatic Assays. Proteases were extracted from flour following the procedure reported by Bottari et al. (*24*) using 2 g of flour and 10 mL of 0.2 M sodium acetate buffer (pH 5) containing 5 mM 2-mercaptoethanol.

The proteinase activity was measured by using 2% (w/v) Hb dissolved in McIlvaine buffer (pH 3). The flour extract (15−30 μL) was diluted to 1 mL with McIlvaine buffer (pH 3) and the reaction was started by adding 1 mL of Hb, incubated at 30 °C for 2−24 h and stopped by adding 1 mL of 24% (w/v) trichloroacetic acid (TCA) and vortexing. After standing at 4 °C for 10 min, the obtained suspension was cleared by centrifugation (10 min at 4000*g*) and 0.5 mL of the supernatant was utilized to determine the TCA-soluble products by the ninhydrin method (*25*). The hemoglobinase activity was measured both in the absence and in the presence of pepstatin A in final concentration of 1.46 μM.

The proteinase activity was also measured by using 0.2% (w/v) azocasein dissolved in 0.2 M sodium acetate buffer. The flour extract (20−100 μL) was diluted to 1 mL with 0.2 M sodium acetate buffer (pH 5.4) containing 2-mercaptoethanol (final concentration 2.5 mM) and the reaction was started by adding 1 mL of azocasein, incubated at 30 °C for 2−24 h and stopped by adding 1 mL of 24% (w/v) TCA and vortexing. After standing at 4 °C for 10 min, the obtained suspension was cleared by centrifugation (20 min at 4000*g*) and the supernatant was utilized to measure absorbance at 330 nm. The azocaseinase activity was measured both in the absence and in the presence of PMSF, pepstatin A and E-64, utilized in final concentrations of 1 mM, 1.46 μM, and 20 μM, respectively. One unit of azocaseinase activity was defined as the amount of enzyme required to produce an absorbance change of 1.0 in a 1-cm cuvette under standard assay conditions (*26*).

The carboxypeptidase activity was measured with 5 mM *N*-carbobenzoxy-L-phenylalanyl-L-alanine (CBZ-Phe-Ala) dissolved in 0.2 M sodium acetate buffer (pH 5). The flour extract (15−30 μL) was diluted to 1 mL with 0.2 M sodium acetate buffer (pH 5) and the reaction was started by adding 1 mL of CBZ-Phe-Ala. After 1 h of incubation at 30 °C, the reaction was stopped by adding 1 mL of 24% (w/v) TCA and vortexing. The TCA-soluble products were determined by the ninhydrin method (*25*) as above-described. The activity was measured both in the absence and in the presence of PMSF in final concentration of 1 mM.

At least three replicates were performed for each analysis utilized in this paper.

RESULTS

Germination Tests. Accelerated aging resulted in a decreased germination capacity. In fact, the percentage of seeds completing germination after 72 h rapidly decreased when the aging treatment time was prolonged, reaching ~30% after 6 days and ~0% after 14 days of treatment (see **Table 1**). The germination rate followed an analogous trend (see **Table 1**). The growth of shoot and primary root was affected by seed aging, the average length of both organs being ~50% that of the control seeds after 4 days and ~0% after 14 days of treatment (see **Table 1**). It should be noted that nongerminated seeds were not included in the mean shoot and root length measurements.

Moreover, it is worth noting that fungi growth was observed on the surface of seeds treated from 10 to 14 days and the use of a fungicide (Vitavax 200FF) did not eliminate this problem. No further treatment with fungicide was performed and visible damaged seeds were not discarded.

Table 2. Lutein, Free Radicals and Soluble Protein Content in Flour and Free Radicals Content in Gluten from Unaged and Aged Wheat Seeds

duration of accelerated aging (days)	lutein in flour (ppm)	protein in flour (mg/g)	free radicals in flour (spins/g)	free radicals in gluten (spins/g)
0	2.6 ± 0.1	3.48 ± 0.08	$(3 \pm 1) \times 10^{14}$	$(4 \pm 1) \times 10^{15}$
3	2.28 ± 0.05	3.1 ± 0.1	$(3 \pm 1) \times 10^{14}$	$(7 \pm 1) \times 10^{15}$
4	2.04 ± 0.06	2.14 ± 0.08	$(3 \pm 1) \times 10^{14}$	$(7 \pm 1) \times 10^{15}$
6	1.59 ± 0.03	2.11 ± 0.03	$(3 \pm 1) \times 10^{14}$	$(8 \pm 1) \times 10^{15}$
10	1.25 ± 0.08	1.62 ± 0.02	$(4 \pm 1) \times 10^{14}$	$(11 \pm 1) \times 10^{15}$
14	1.04 ± 0.03	1.34 ± 0.01	$(8 \pm 1) \times 10^{14}$	

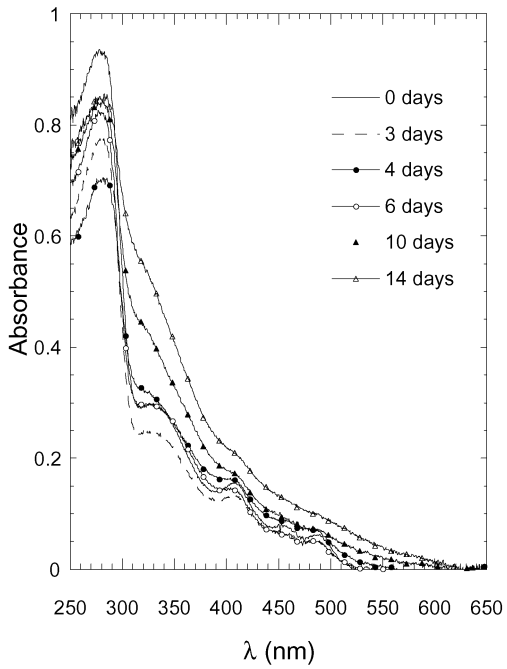

Figure 1. Front-surface absorbance spectra of flour from seeds of durum wheat unaged or aged for 3, 4, 6, 10, and 14 days.

Moreover, it is worth noting that fungi growth was observed on the surface of seeds treated from 10 to 14 days and the use of a fungicide (Vitavax 200FF) did not eliminate this problem. No further treatment with fungicide was performed and visible damaged seeds were not discarded.

Carotenoids in Flour. As reported in **Table 2**, lutein content in flour gradually decreased during the aging treatment, going from 2.63 ppm in the unaged sample to 1.04 ppm in the sample treated for 14 days.

These results were consistent with changes observed in the front-surface UV−Vis absorbance spectra of flours (see **Figure 1**). In fact, flours obtained from seeds subjected to aging showed a progressive loss of the typical vibrational structure of the absorption band between 430 and 530 nm due to lutein (*14*). At 10 and 14 days, the unstructured absorption at $\lambda > 300$ nm that is present in all spectra and that is due to other chromophores, significantly increased, showing a deep change of chromophores present in flour aged for at least 10 days.

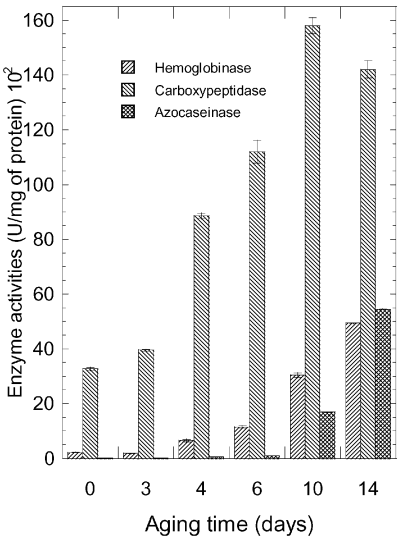

Figure 2. Proteolytic activities in flour from unaged and aged wheat seeds. Each value is an average of three replicates of at least two extractions.

Table 3. Inhibition Percentages of Proteolytic Activities

duration of accelerated aging (days)	CBZ-Phe-Ala	haemoglobin	azocasein		
	PMSF	pepstatin A	E-64	pepstatin A	PMSF
0	92	97		42	23
3	97	93		47	23
4	91	84		39	32
6	97	81		36	46
10	86	72			88
14	89	74			95

Free Radicals in Flour and Gluten. EPR spectra of flour samples were quite weak. They showed a signal with g value of 2.004−2.005 and peak to peak line width of 9−12 G, which can be attributed to organic free radicals. As reported in **Table 2**, levels of free radicals in the examined samples were in the order of 10^{14}/g. When radical contents in flour obtained from aged and control seeds were compared, no appreciable changes were found up to 6 days of treatment, while the radical content slightly increased after 10 days and was 2.5 times that of the control after 14 days.

Organic free radicals in gluten samples showed EPR spectra in which a main signal with $g \cong 2.006$ and peak to peak line width from 7 to 9 G was present, with a shoulder on the left (data not shown). Radical levels were in the order of 10^{15}/g and, as reported in **Table 2**, increased with seed aging duration, becoming almost 3 times higher than that of the gluten from unaged seeds after 10 days of treatment.

Proteins and Protease Activities in Flour. The soluble protein content in flour showed a progressive decrease during the aging treatment (see **Table 2**); after 14 days, the content was almost half of that of the control.

Among proteins extracted from flours, three proteolytic activities were detected by utilizing exogenous substrates, i.e., Hb, CBZ-Phe-Ala, and azocasein (see **Figure 2**). The activity degrading CBZ-Phe-Ala was strongly inhibited by PMSF, while that hydrolyzing hemoglobin was inhibited by pepstatin A. The azocaseinase activity was inhibited by pepstatin A and PMSF for samples aged up to 6 days and only by PMSF for samples aged for 10 and 14 days (see **Table 3**). E-64 had no effect on azocaseinase activity indicating that no proteinase activity of cysteine type was present in artificially aged seeds. These properties allowed to assign the proteolytic enzymes degrading Hb and CBZ-Phe-Ala as the aspartic proteinase and carboxypeptidase previously isolated from wheat seeds (*27, 28*). As far as enzymes degrading azocasein are concerned, they could be identified as aspartic and serine proteinases. The last activity was observed in germinating soybean (*29*) and mungbean (*30*) seeds, and its involvement in the first step of storage protein degradation is a common feature in germinating legume seeds (*30*). On the other hand, to the best of our knowledge this activity is here observed for the first time in wheat seeds.

As shown in **Figure 2**, for all the proteolytic activities an increase occurred with prolonging the duration of seed aging, especially marked for the azocaseinase one after 10 and 14 days. In particular, after 14 days of treatment, the specific activity of carboxypeptidase, aspartic proteinase and serine proteinase reached values about 5, 22 and 328 times higher than that of the control.

SDS−PAGE analysis of seed storage proteins extracted from flour (*21*) revealed that high and low molecular weight glutenin subunits (HMW-GS and LMW-GS) disappeared almost completely after 10 days of aging (see **Figure 3**b), while gliadins, present at least in traces up to 10 days, reduced dramatically after 14 days of treatment (see **Figure 3a**). These changes were accompanied by the appearance, between 10 and 14 days, of bands due to low molecular weight (lower than 17.2 kDa) proteins.

Spin Labeling EPR of Gluten. Gluten obtained from flours of seeds unaged or aged for 3−4 days was yellow and abundant (at least 0.1 g of dry gluten per g of flour). On the other hand, less colored gluten was obtained from flour of seeds aged for 10 days, with yields decreasing by increasing the aging time (down to 0.02 g of dry gluten per g of flour). Flour from seeds aged for 14 days did not give gluten.

EPR spectra of fully hydrated spin labeled gluten were of a composite type with line shapes resulting from the superposition of at least two sub-spectra each belonging to spin labels differing in their mobility (see **Figure 4**), as observed in previous studies (*7, 10−12*). Slow moving spin labels (immobile) gave broad lines in the spectra, while sharp lines were attributed to fast moving spin labels (mobile), the mobility of spin labels being determined by the flexibility of the protein segments to which they are bound and the steric hindrance of their environments (*8*). The mobile component could be thus attributed to spin labels bound to sulfhydryl groups which allow the labels to retain a high degree of rotational freedom, i.e., shallow, solvent-exposed protein groups. On the other hand, the slow moving component was due to labels bound to sulfhydryl groups localized in somewhat restrictive, crevice-like regions of the proteins.

Small but significant differences were observed between the EPR spectra recorded at the same temperature on gluten obtained from control and aged seeds (see **Figure 4**). At different temperatures similar spectra, but with different intensity and mobility of the two components, were observed (see **Figure 4**). Two parameters were calculated from the spectra to obtain quantitative information on spin labels dynamics and, in turn, on gluten flexibility. The first one is the rotational correlation time (τ) of the mobile spin labels, determined according to the Freed and Fraenkel equation (*31*):

$$\tau = (6.65 \times 10^{-10})\Delta h_{+1}[(I_{+1}/I_{-1})^{1/2} - 1] \qquad (1)$$

where Δh_{+1} is the peak-to-peak width of the low-field line, in Gauss, and I_{+1} and I_{-1} are the amplitudes of the low- and high-

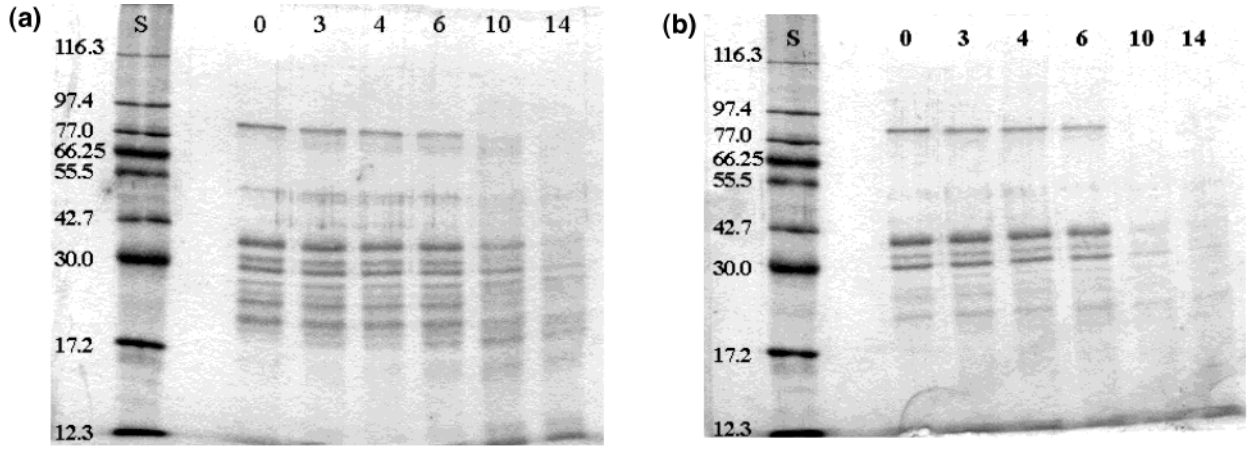

Figure 3. SDS–PAGE of gliadins (a) and glutenins (b) extracted from flour of control seeds (lanes 0) and seeds aged for 3, 4, 6, 10, and 14 days. S represents molecular weight standards.

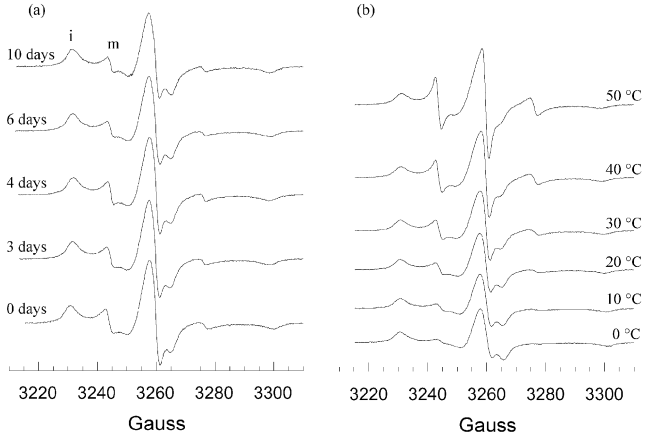

Figure 4. EPR spectra of fully hydrated gluten spin labeled with 3-MAL. (a) Spectra recorded at 25 °C on gluten from seeds unaged and aged for 3, 4, 6, and 10 days; (b) Spectra recorded on gluten from control seeds at the indicated temperatures. "i" and "m" indicate slow and fast moving spin label lines, respectively.

field lines, respectively. The second parameter that can be calculated from the EPR spectra is *R* (*10*, *11*), defined as the ratio

$$R = i/m \qquad (2)$$

where *i* and *m* are the amplitudes of the low-field peaks of the slow moving and fast moving spin labels, respectively (see **Figure 4**). *R* reflects the ratio between slow and fast moving spin labels populations. Both *R* and τ decreased with increasing the temperature (see **Figures 5** and **6**), indicating that a progressive transfer of less mobile radicals to a more mobile population occurs and that the rotational diffusion of mobile spin labels becomes faster. The trends of *R* and τ as a function of temperature were described in terms of the Arrhenius equation and activation energies were determined. As shown in **Table 4**, very small differences were found between values determined in the different samples.

Slightly higher values of *R* were found at each temperature for gluten from artificially aged seeds with respect to gluten from unaged ones (see **Figure 5**). On the other hand, τ values were similar in samples treated up to 4 days, whereas lower values were found for sample treated for periods of 6 and 10 days (see **Figure 6**).

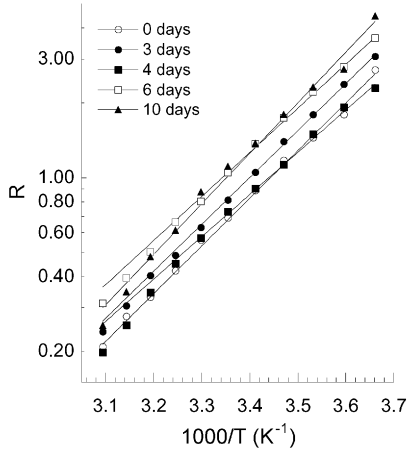

Figure 5. R ratios vs 1000/*T*. Lines represent fits of experimental values in terms of the Arrhenius equation.

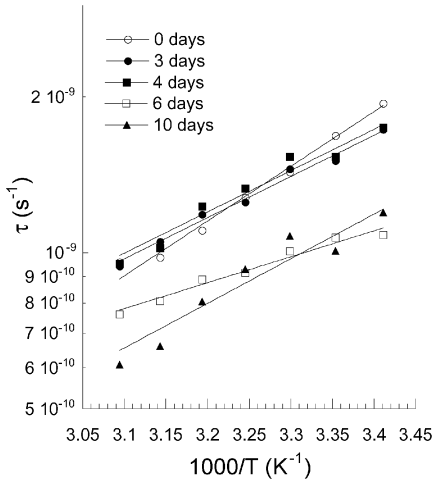

Figure 6. Rotational correlation time τ vs 1000/*T*. Lines represent fits of experimental values in terms of the Arrhenius equation.

DISCUSSION

Accelerated aging finally resulted in a loss of seed viability preceded by a progressive reduction of both germination ability and root and shoot growth (*32*−*34*). Comparing the present results with that obtained by Pinzino et al. (*7*) on naturally aged wheat seeds of the same cultivar, we found that seeds artificially aged for 3, 4, 6, 10, and 14 days have germination capacities similar to those of seeds naturally aged for about 15, 30, 33,

Table 4. Activation Energy of R (E_a^R) and τ (E_a^τ) in Gluten from Unaged and Aged Wheat Seeds

duration of accelerated aging (days)	E_a^R (kJ/mol)	E_a^τ (kJ/mol)
0	36.8 ± 1.3	20.2 ± 0.9
3	36.2 ± 0.4	15.2 ± 0.8
4	32.6 ± 1.0	15.3 ± 1.4
6	34.0 ± 0.5	9.6 ± 0.8
10	39.0 ± 2.0	16.5 ± 2.4

35, and 36 years, respectively. However, different correlations between germinability and lutein content in flour were found for artificially aged seeds with respect to naturally aged ones. In fact, the content of lutein was almost zero in flour from wheat seeds naturally aged for more than 30 years, whereas the lowest content observed in accelerated aging experiments for unviable seeds was about 1 ppm. Anyway, during the accelerated aging treatment a gradual reduction of lutein content in flour was revealed by HPLC measurements. In addition, front-surface absorbance spectra of flour samples showed the progressive loss of vibrational structure in the 430−530 nm spectral region, a feature ascribable to a decrease of lutein content, and a dramatic increase in the absorbance of samples aged for 10 and 14 days at $\lambda > 300$ nm, where measured variations are significant. Reasonably, this increase can be connected to the appearance of new compounds resulting from molecular degradation processes.

The loss of antioxidants was accompanied by an increase of organic free radicals in flour and gluten, in agreement with the findings of Priestley et al. (*35*) and of Buchvarov and Gantcheff (*36*) in the axes of soybean seeds subjected to accelerated aging. The content of organic radicals in gluten obtained from seeds artificially aged for 6 days was comparable with that of gluten from seeds naturally aged for 15 years (*7*), corresponding to the highest radical content found in natural aging experiments. In fact, after 20 years of natural aging the content of free radicals progressively decreased reaching the lowest value after 35 years (*7*). On the contrary, the free radical level in gluten and flour from accelerated aged seeds progressively increased with prolonging the treatment time. These differences indicate that no relationship can be found between the quantity of free radicals and germination capacity during natural and accelerated aging, as previously stated by Girard and Le Meste (*37*), probably due to the recombination of free radicals into substances undetectable by EPR analysis. Moreover, it must be noticed that radical level in gluten is 1 order of magnitude higher than in the corresponding flour, indicating that free radicals are mainly associated with flour protein, whereas starch molecules do not undergo aging induced scissions or produce radicals that decay or react too rapidly for detection by EPR. In agreement with these findings, the dominant feature of the EPR spectra of gluten samples (data not shown) was a line with g value typical of nitrogen-centered radicals delocalized on proteins (*38*); shoulders were observed to the left of the peak ascribable to peroxyl radicals (*39*). This suggests that bond breakage has occurred in endosperm proteins during seed aging and predicts the likelihood of molecular degradation or cross-linking.

Other findings support the involvement of proteins in seed deterioration. In particular, a decline in soluble protein content of flour was observed during accelerated aging (see **Table 2**), in agreement with the results found in pigeonpea (*40*), bamboo (*41*), tomato (*42*), sal (*43*), watermelon (*44*), maize (*45*), French bean (*46*), soybean and barley (*13*) and, in particular, in wheat (*47*) seeds. Moreover, a degradation of wheat storage proteins was observed during accelerated aging (see **Figure 3**), which resulted in a loss of the capability of flour to form gluten and in a decrease in gluten flexibility, as observed by spin labeling EPR (see **Figures 4** and **5**). These findings, similar to those obtained during in vitro gluten degradation by proteases from dry and germinating wheat seeds (*12*), confirm the hypothesis that macromolecules essential for seed germination are degraded during aging (*3*−*4*). In this regard, a marked increase of three proteolytic enzymes (i.e., carboxypeptidase, aspartic and serine proteinase) of dry wheat seeds was found in the present study during accelerated aging (see **Figure 2**). A rise in proteinase activity during accelerated aging of seeds was also reported by other authors in sorghum (*48*), maize (*45*), pigeonpea (*40*), barley, and wheat (*49*).

All together, our results indicate that biochemical events produced by accelerated aging seem not to be the same occurring in wheat seeds during natural aging. Our findings suggest that accelerated aged seeds underwent some biochemical changes needed for the process of germination and resulted in the loss of their viability. Nevertheless, we cannot agree with Chauan et al. (*13*) statement that seed deterioration biochemically mimics seed germination because fundamental biochemical changes undergoing during wheat seed germination, such as synthesis of cysteine proteinase (*50*, *51*), were not observed during accelerated aging.

Finally, the contribution of microorganisms to the deterioration of seeds artificially aged for 10 and 14 days cannot be ruled out. In fact, pathogens produce different classes of proteases (i.e., aspartic proteinases, aminopeptidases, carboxypeptidases, and prolyl dipeptidyl peptidases) (*52*) that greatly increase the rate of deterioration of the host's cellular structures. Unfortunately, the fact that conditions which favor seed deterioration (i.e., high moisture content and temperature used in accelerated aging) also favor microbial growth makes difficult to ascertain which aspects of deterioration are microbially induced and which are self-inflicted by seeds (*1*).

ABBREVIATIONS USED

EPR, electron paramagnetic spectroscopy; 3-MAL, 3-maleimidoproxyl; CBZ-Phe-Ala, *N*-carbobenzoxy-L-phenylalanyl-L-alanine; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; Hb, hemoglobin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid.

SAFETY

Precautions were taken to prevent any contact of 3-MAL and labeled gluten with skin and eyes.

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