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Protein deterioration and longevity of quinoa seeds during long-term storage

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ABSTRACT

Seed protein stability and seed deterioration during storage were studied in seeds of two different quinoa (*Chenopodium quinoa* Willd.) cultivars, i.e., cv. Ollagüe and cv. Baer II. Germination and viability tests proved that cv. Baer II was more longevous than cv. Ollagüe. Protein insolubilisation was detected during storage and correlated with longevity. However, protein solubility was restored by priming in both cultivars, disregarding their germination capability. Extremely high contents of advanced glycation end-products (AGEs), assessed by carboxymethyllysine (CML) quantification, were obtained from stored seeds as viability was reduced; primed seeds showed slightly lower AGE content, indicating a strong association between quinoa seed ageing and Maillard products accumulation. High intensity fluorescence values were observed in seeds stored in the gene bank bearing 100% germination values, which indicate that the detection of fluorescence is not by itself an appropriate indicator of protein damage by Maillard-type product accumulation in quinoa seeds. This work establishes for the first time a direct association between seed viability and AGE accumulation, due to Maillard reaction, in quinoa seeds.

1. Introduction

Chenopodium quinoa (Willd.) is a pseudocereal native to the Andean regions of South America. Quinoa seeds have a high nutritional value and their protein composition meets and surpasses World Health Organization (WHO) recommendations. Quinoa has been an important grain crop for many centuries, grown by poor peasant communities with scarce economic resources, many of whom are engaged in subsistence agriculture. Nowadays, quinoa is gaining popularity because of its excellent characteristics and its adaptability to different agroclimatic conditions. Several quinoa cultivars are being exploited for their different characteristics (i.e. grain size and low bitterness, tolerance to drought) (Rojas, Soto, & Carrasco, 2004). Extensive breeding of quinoa, as well as international commerce merits the study of genetic diversity, *in situ* and *ex situ* conservation and seed conservation properties. In this way, the preservation of the nutritional as well as the organoleptic characteristics of the grain is of leading interest.

Quinoa seeds have an orthodox behaviour (Ellis, Hong, & Roberts, 1988) and mature seeds can be stored for relatively long periods at an appropriate temperature and humidity range without losing viability. Seed damage occurring in orthodox seeds during storage can be overturned by pre-germinative metabolic events which take place during imbibition, since increasing seed moisture contents hastens the repair processes (McDonald, 1999). Those pre-germinative events generally involve nucleic acids, and protein repair and synthesis, as well as membrane repair. However, the molecular processes that become operative in the seeds, as well as their timing, are species-specific. Preliminary studies using gas chromatography (GC) in quinoa seeds showed fatty acids stability during storage. The oxidative stability of quinoa seed lipids was established using free fatty acids, conjugated diene hydroperoxides and hexanal as indicators of lipid oxidation in seeds subjected to accelerated ageing conditions (Ng, Anderson, Coker, & Ondrus, 2007).

Water content is an important factor affecting the rate of seed deteriorative reactions and ageing during storage (Justice & Bass, 1978; Priestley, 1986). In a previous study, no frozen water was detected in quinoa seeds at water contents below 20% (Matiacevich, Castellión, Maldonado, & Buera, 2006), due to restricted water molecular mobility. Hence, it is critical to define storage conditions. In dry seeds, enzymatic reactions play little role in seed ageing, also because of mobility restrictions. However, certain non-enzymatic events, such as the Maillard reaction, can occur

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even at very low water content (Priestley, 1986; Sun & Leopold, 1995; Wettlaufer & Leopold, 1991). Maillard reaction refers to a series of complex reactions, as a consequence of which proteins become aggregated and lose solubility. These modifications can take place by non-enzymatic glycosylation with reducing sugar or by reaction with aldehydes produced from free radical-mediated lipid peroxidation (Murthy & Sun, 2000; Priestley, 1986; Priestley & Leopold, 1983; Sun & Leopold, 1995; Wettlaufer & Leopold, 1991). The Maillard reaction may contribute to seed ageing through the chemical alteration of functional proteins, thus depressing metabolic capability, and reducing the ability of the metabolic system to limit free radical damage and to repair the damage during germination (Murthy, Liang, Kumar, & Sun, 2002).

N^{ε}-(carboxymethyl)lysine (CML) is considered an advanced Maillard reaction marker and is used as an indicator of protein damage in foods, so is widely used in the nutritional evaluation of severely and even mildly heat-treated foods. CML represents "blocked" lysine as it is nutritionally unavailable for higher organisms (Ahmed, Thorpe, & Baynes, 1986; Erbersdobler & Somoza, 2007). Protein fluorescence analysis has regularly been used to study protein modification by this reaction during seed storage. Several authors have previously reported the development of a fluorescence peak attributed to the presence of advanced glycosilation end-products (AGEs) in aqueous solutions of seed proteins, yet fluorescence spectra of various AGE products slightly differ between species, i.e., excitation/emission wavelengths of 375/440 nm for soybean (Sun & Leopold, 1995), 370/440 nm for onion, tomato and cauliflower (Baker & Bradford, 1994), 350/420 nm for mungbean (Murthy, Kumar, & Sun, 2003; Murthy & Sun, 2000; Murthy et al., 2002). Nevertheless, Baker and Bradford (1994) did not find correlation between fluorescence development and seed deterioration in onion, cauliflower, tomato, carrot, broccoli and lettuce, probably because of interference from other fluorescent compounds, such as polyphenols, present in seed coats. There are no previous reports of Maillard reaction or protein deterioration in quinoa seeds.

In this paper we test the hypothesis that protein deterioration and insolubilisation occur during seed ageing and is one of the main causes of viability loss. In addition, seed viability, protein solubility and protein resolubilisation by pre-germinative reparation through a humidification treatment (priming) were evaluated in stored seeds of two different quinoa cultivars. Our second hypothesis proposes that seed protein damage is due to Maillard reaction and so we have used different complementary methods to determine protein stability. The relationship between protein insolubilisation, Maillard products accumulation, fluorescence development and loss of germination capability during storage was also studied in seeds stored at ambient laboratory conditions or preserved in a gene bank, in order to prove if fluorescence development is an adequate marker to assess the extent of Maillard reaction in quinoa seeds. The purpose of this study was to analyze protein instability, due to storage effects in different cultivars of quinoa seeds, in order to provide novel and alternative tools for predicting quality changes during seed storage. This work is part of a comprehensive study on seed conservation in *C. quinoa*.

2. Materials and methods

2.1. Seed material

Quinoa (*C. quinoa* Willd., *Chenopodiaceae*) seeds of two different cultivars were studied. Cv. Baer II, an improved line obtained from cv. Baer, is an original from the lowlands near Temuco in southern Chile, where rain falls up to 1200 mm annually. Cv. Baer II belongs to the "sea-level" adaptation group. Cv. Ollagüe is original from the homonymous town in the northern Chilean altiplano next to the border with Bolivia at 4000 m altitude, with less than 100 mm of annual accumulated precipitations. It belongs to the "altiplano" adaptation group. Seeds were obtained from experimental greenhouses at the School of Sciences of the University of Buenos Aires in Buenos Aires, Argentina and stored in gene bank conditions at −20 °C and 6% water content (wc), or at 14% relative humidity (RH) at room temperature for 12 months until used. Seeds in all experiments were pre-sorted by hand; discarding excessively small, large and damaged seeds.

2.2. Experimental storage conditions

According to our previous studies (Matiacevich & Buera, 2006; Matiacevich et al., 2006) storage conditions were chosen between the glass transition temperature (T_g) of quinoa embryos and the water content at which frozen water is easily detected. Between those limits, in the supercooled state, the water content is high enough to overcome the molecular mobility restrictions imposed on the material in the glassy state, but lower enough to avoid microbial spoilage or enzymatic activity. The selected conditions for controlling ageing were 14% RH and ambient laboratory temperature (around 25 °C) for 3 and 6 months. Seeds stored in the gene bank were maintained at −20 °C and 6% wc (which corresponds to approximately 20% RH, as calculated by sorption isotherms, unpublished data). The water content was evaluated gravimetrically after drying in an oven at 100 °C for 4 days until constant weight.

2.3. Seed testing

Briefly, three replicates of 50 seeds each were pre-incubated at 100% RH and 5 °C for 3 h, in order to avoid imbibition damage. The surfaces were disinfected in a sodium hypochlorite solution (5.5 g Cl/l) for 10 min, washed thoroughly with distilled water and set to germinate over wet paper in Petri dishes at 24 ± 1 °C for 72 h. Germination was evaluated according to ISTA Rules (ISTA, 2005). Tetrazolium test for viability was conducted on quinoa seeds. Seeds were cut longitudinally in halves. Seed halves were embedded in a 1% 2,3,5-triphenyl-2H-tetrazolium chloride (Merck, Darmstadt, Germany) solution and incubated at 25 °C for 24 h in darkness. Results were evaluated according to ISTA Rules (ISTA, 2005).

2.4. Protein extraction

Proteins were extracted from stored seeds with and without previous incubation in chambers at 100% RH and 6 °C for 20 h, with the aim of inducing pre-germinative events affecting protein solubility of deteriorated seeds (priming). The water content achieved after pre-incubation was 21.5% for cv. Baer II and 31.0% for cv. Ollagüe. Approximately 30 mg of ground seeds were incubated in 1.5 ml of a series of sequential extraction solutions as follows: 50 mM Tris–HCl (Sigma, St. Louis, MO), 200 mM NaCl (Merck) buffer (pH 8.3) (Low Salt, LS) for 20 min on ice (twice); 50 mM Tris–HCl, 1 M NaCl buffer (pH 8.3) (High Salt, HS) for 20 min on ice; 70% ethanol (Merck) for 30 min at 65 °C (E); and 0.1 M NaOH (Merck) solution (Alkali, H) for 30 min on ice. In each extraction stage, 0.6 μl of protease inhibitor cocktail for general use (Sigma) were added to the solutions. Supernatants were obtained after 10 min centrifugation at 15,000*g* and 5 °C and saved for further analysis. Finally, pellets obtained after all the protein extraction procedure were saved for further insoluble protein content analysis.

2.5. SDS–PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970), using a

15% separating gel with a 4% stacking gel (30% T, total acrylamide-bisacrylamide monomer concentration, 2.67% C, crosslinker concentration). Samples were prepared by trichloroacetic acid (TCA)–acetone precipitation. Briefly, three volumes of a chilled (−20 °C) solution of 13.3% (v/v) TCA (Sigma) and 0.093% (v/v) of 2-mercaptoethanol in acetone (Merck) were added to each sample and incubated overnight at -20 °C. Samples were centrifuged for 15 min at 4000*g* at 6 °C and pellets were resuspended in acetone containing 0.07% (v/v) 2-mercaptoethanol. Samples were centrifuged for another 15 min at 4000*g* at 6 °C, and pellets were dried and resuspended in a buffer containing 2% (w/v) SDS (Merck), 10% glycerol (v/v) (Sigma), 5% (v/v) 2-mercaptoethanol and 0.01 mg/ml bromophenol blue (Sigma) in 62.5 mM Tris–HCl buffer (pH 6.8). Broad-range SDS–PAGE molecular weight standards (Bio-Rad Laboratories, Hercules, CA) and invertase from *Saccharomyces cerevisiae* (Solvay SA, Buenos Aires, Argentina), as a control glycosylated protein, were resuspended in the same buffer. All samples were heated at 99 °C for 6 min and loaded (20 μg, except for saline-soluble humidified seeds fractions where 100 μg of proteins were loaded). Electrophoresis was run at 20 mA (0.75 mm thickness) for 75 min in a Mini-Protean three Cell (Bio-Rad). Gels were stained with 0.125% (w/v) Coomassie Blue R-250 (Sigma) in a methanol/acetic acid/water (50%/10%/40%, v/v/v) (Merck) solution and destained in methanol/acetic acid/water (20%/10%/70%, v/v/v) solution. For glycoprotein detection, gels were stained using the periodic acid-Schiff reagent (Segrest & Jackson, 1972).

2.6. Protein quantification

Soluble proteins in each fraction were quantified by the Bradford method (Bradford, 1976). Bovine serum albumin (BSA) (Sigma) was used as a standard and the measurements were performed using a Shimadzu 1620 UV–vis spectrophotometer (Shimadzu, Kyoto, Japan).

Micro-Kjeldahl technique for total nitrogen quantification was performed using a Kjeltec Auto 1030 Analyzer (Tecator, Höganäs, Sweden). Soluble fractions were obtained as described above (except for the protease inhibitor cocktail) and combining the five supernatants (LS (×2), HS, E and H) as quadruplicates. Protein content was estimated by Eq. (1):

$$P = \frac{(V_{HCl} - V_{HCl-blank}) \times N_{HCl} \times f \times a \times 100}{m} \quad (1)$$

where P is the protein content (g/100 g seeds); m is the seed sample mass (g); V_{HCl}, the volume of standard acid (ml), $V_{HCl-blank}$, the volume of standard acid needed to titrate a reagent blank (ml); N_{HCl}, the normality of the acid; f, the standard acid dilution factor; and a is the product of the factor used to transform total organic nitrogen values into protein values (6.25) and the molecular mass of nitrogen (14 g/mol).

2.7. N^{ε}-carboxymethyl lysine (CML) assay

CML was determined with a novel competition-based ELISA assay, using an N^{ε}-(carboxymethyl)lysine specific monoclonal antibody (mouse monoclonal 4G9; Alteon Inc., Ramsey, NY). The assay is CML-specific and shows no cross-reactivity. The assay was calibrated with 6-(*N*-carboxymethylamino) caproate, which refers to the epitope recognized by the mouse monoclonal antibody 4G9. Streptavidin-coated 96-well microtitre plates (Roche Diagnostics, GmbH, Penzberg, Germany) were incubated with biotin-labelled AGE–bovine serum albumin (100 ng/well in 100 μl) for 1 h. ELISA plates were washed extensively three times with washing buffer (10 mM Tris–HCl, 150 mM NaCl and 0.05% w/v Tween (ICI America Ltd., Bridgewater, NJ). Seed samples were ground and pre-incubated with proteinase K to liberate CML epitopes; protease was inactivated by addition of 1 mM of PMSF. Seed samples as well as CML standards (50 μl/well) were then simultaneously incubated with peroxidase-conjugated monoclonal antibody (50 μl/well) against CML for 1 h at room temperature. After three subsequent washing steps, colour reaction was induced by addition of 100 μl of ABTS solution/well with 0.3 g/l of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid (Roche Diagnostics, Burgess Hill, UK). Absorbance was read in a microtitre ELISA plate reader (SLT spectra, SLT Labinstruments Inc., Groedig, Austria) at 405 nm. Results show CML levels expressed as the number of single CML epitopes in ng per g of seeds. All samples were run in duplicate. The sensitivity of this competitive ELISA assay was 5 ng CML/ml with intra-assay and inter-assay precision less than 4% and 5%, respectively.

2.8. Fluorescence development during storage

In order to determine the accumulation of fluorescent products from Maillard-type reaction, fluorescence was measured in soluble protein fractions. Soluble protein fractions were diluted, in order to reach absorbance values lower than 0.1 at the excitation wavelength (380 nm) to avoid inner filter effect (Matiacevich & Buera, 2006). Fluorescence spectra were analyzed in a Shimadzu RF-540 spectrofluorometer equipped with a xenon lamp (150 W) and coupled to a Shimadzu DR-3 data recorder with a CRF-1 program cassette. Excitation and emission fluorescence spectra were registered and intensity maxima were calculated using Eq. (2) (Matiacevich & Buera, 2006):

$$UF = \frac{UF_x \cdot f \cdot d - b}{m \cdot g} \quad (2)$$

where UF_x was the fluorescence measured for each soluble fraction; f, the sensitivity factor of the instrument; d, the dilution factor; b, the y-intercept of the calibration curve of quinine sulphate of 0.1 N H_2SO_4; m, the slope of the calibration curve; and g, the seed mass (g) used for protein extraction. All reagents used were of analytical grade.

2.9. Statistical analysis

Data were analyzed and plotted using STATISTICA v. 6.0 and Prism Graphpad v. 3. ANOVA or Kruskal–Wallis tests were conducted for analysis of variance and Pearson's correlation coefficient was used to determine correlations between variables.

3. Results and discussion

3.1. Seed storage behaviour

Seed preservation of both cultivars was tested before storage (control) and after 6 months storage at 14% RH and ambient laboratory temperature. Results are shown in Table 1. Germination of cv. Ollagüe seeds diminished noticeably during storage. The changes in viability were less pronounced, but also significantly reduced. This indicated that the damage occurred in embryonic tis-

Table 1
Seed storage behaviour. Seed testing results for cv. Baer II and cv. Ollagüe before (control) and after 6 months storage at 14% RH and ambient laboratory temperature.

(%)	Cv. Baer II		Cv. Ollagüe	
Water content	4.6 ± 0.1		4.8 ± 0.1	
	Control	6 Months	Control	6 Months
Viability	100	100	95 ± 5	85 ± 5
Germination	100	95 ± 3	73 ± 10	33 ± 6

sues during storage did not cause seed death but was enough to compromise the germination process. Cv. Baer II seeds were better preserved than cv. Ollagüe seeds after 6 months storage under the same conditions. Differences in germination rate were observed among cultivars, although those differences were barely significant in viability values (Table 1). Seeds preserved for the same period (6 months) in gene bank conditions maintained invariable germination and viability values, interestingly showing the high impact of storage conditions on seed stability. This is the first study introducing differences in storage behaviour between quinoa cultivars.

3.2. Storage effects on seed protein solubility

Protein quantification by Bradford method showed that total soluble protein levels diminished after storage at 14% RH and ambient laboratory temperature (Table 2). Particularly, the saline-soluble fraction (LS) decreased and the alkaline-soluble (H) fraction was slightly increased ($p < 0.05$). Differences between cultivars were observed in seeds before storage, soluble proteins in cv. Baer II being more abundant than in cv. Ollagüe. These results suggest a correlation between protein stability and seed lifespan. A positive correlation was detected between viability and protein content of the LS fraction ($p = 0.0289$) and a negative correlation for the alkaline-soluble fraction (H) ($p = 0.0206$). The same results were obtained when germination percentage was analyzed against protein solubility ($p = 0.0411$ for the LS fraction and $p = 0.0582$ for the H fraction). Seeds of both cultivars preserved under gene bank conditions maintained germination and viability values (data not shown). In addition, their protein solubility values remained almost stable (Table 2). Moreover, detailed analysis of each solubility fraction did not show significant alterations in their protein contents, relative to control non-stored seeds ($p > 0.05$). These results suggest that the modifications in protein solubility of seeds stored at ambient laboratory conditions are related to the loss of seed viability; nevertheless, a cause-effect relationship was not established. Protein glycation may reduce the sensitivity of the Bradford assay, in particular due to the alterations of arginine and lysine residues which result in protein underestimations (Brimer, Murray-McIntosh, Neale, & Davis, 1995). Therefore, the low values obtained from aged seeds by Bradford assay can be attributed to Maillard reaction occurrence, both due to soluble protein diminishing (by protein aggregation and insolubilisation), as well as arginine and lysine glycation. Under both storage conditions, water contents were similar, which suggest that differences in deterioration rates would be mostly determined by temperature.

Table 2
Soluble protein contents (determined by Bradford method) of two quinoa cultivars stored at 14% RH and ambient laboratory temperature or at gene bank. LS: low-salinity fraction; HS: high-salinity fraction; E: ethanolic fraction; H: alkaline fraction.

Storage	Fraction	Cv. Baer II (g/100 g seeds)	Cv. Ollagüe (g/100 g seeds)
Control	LS	8.1 ± 0.9	4.1 ± 0.6
	HS	1.0 ± 0.4	2.1 ± 0.3
	E	0.2 ± 0.02	0.11 ± 0.04
	H	1.2 ± 0.2	1.12 ± 0.04
	Total	10 ± 1	7.5 ± 0.3
3 Months	LS	2.5 ± 0.08	2.46 ± 0.03
	HS	1.8 ± 0.2	2.05 ± 0.09
	E	0.16 ± 0.06	0.19 ± 0.04
	H	1.1 ± 0.2	1.0 ± 0.1
	Total	5.5 ± 0.1	5.7 ± 0.3
6 Months	LS	0.95 ± 0.09	1.05 ± 0.06
	HS	0.12 ± 0.01	0.3 ± 0.1
	E	0.00	0.00
	H	1.8 ± 0.1	2.07 ± 0.04
	Total	2.9 ± 0.2	3.5 ± 0.1
Genebank	LS	4.8 ± 0.5	4.7 ± 0.5
	HS	0.14 ± 0.01	0.050 ± 0.005
	E	0.15 ± 0.02	0.16 ± 0.01
	H	0.483 ± 0.008	0.59 ± 0.04
	Total	5.5 ± 0.5	5.4 ± 0.7

3.3. Seed protein insolubilisation

In order to test the hypothesis of protein solubility variations, total seed proteins were analyzed by micro-Kjeldahl method in whole seeds and soluble and insoluble protein fractions (supernatants and pellets, respectively), obtained from seeds stored for 3 months at 14% RH and ambient laboratory temperature (Fig. 1). No significant differences were found among cultivars. With the purpose of probing the occurrence of protein insolubilisation by protein aggregation during storage, an "accelerated" storage was conducted at 75% RH and 37 °C for the same period (3 months). Under those conditions, germination and viability values dropped to zero (data not shown). Seeds of both cultivars showed significantly higher insoluble protein contents when stored under accelerated ageing conditions than after storage under ambient conditions (Fig. 1). This comparative analysis carried out in both cultivars and under different storage conditions strongly supports the hypothesis of the correlation between protein insolubilisation and seed deterioration.

When protein fractions from 6 months stored seeds were analyzed by SDS–PAGE, the whole lane was stained by Coomassie Blue, indicating a very wide and non-specific distribution of molecular weights (Fig. 2B). Moreover, very high-molecular-weight material was observed at the boundary between stacking and resolving gels in the lanes where alkaline fractions (H) were loaded. This electrophoretic pattern was consistent with non-specific cleavage, on one side, and protein crosslinking, characteristic of non-enzymatic glycosylation, on the other. Gels were also stained with periodic acid-Schiff reagent so as to detect glycosylated proteins, particularly interesting for the alkali-soluble (H) fractions (Fig. 2D). The occurrence of glycosylation is indicative of the presence of AGEs. The H fraction showed the same electrophoretic pattern when either Coomassie Blue or periodic acid-Schiff detection methods were used, thus indicating that this fraction was mainly composed of glycosylated proteins. Moreover, the protein content of this fraction increased during storage (Table 2). Both observations strongly suggest that these glycosylated proteins were associated with ageing effects.

Brinegar and Goundan (1993) described the protein composition of quinoa seeds and reported an 11S-type globulin named chenopodin. Globulins are characterized for having two heterogeneous sets of polypeptides in the size ranges 30–40 kDa and 20–25 kDa, which are joined by disulfide bonds in the native protein (Wright, 1987). A 2S cysteine-rich globulin (8–9 kDa) was also described for quinoa seeds by Brinegar, Sine, and Nwokocha (1996). In pre-germinated seed protein fractions several well-defined electrophoretic bands were observed (Fig. 2A). Both cultivars exhibited different electrophoretic patterns when revealed with Coomassie Blue. Cv. Baer ll saline-soluble extracts showed nine different bands of 7.5, 17.5, 21.2, 32.7, 34.8, 38.1, 40.4, 56.5 and 68.7 kDa, while cv. Ollagüe showed eight bands of 8.6, 18.2, 20.5, 22.8, 33.7, 41.7, 58.3 and 71.2 kDa (one band less than Cv. Baer II in the range of 40 kDa). Ethanol-soluble extracts (E) from cv. Baer II showed four bands of 8.2, 15.1, 26.9 and 32.7 kDa, while cv. Ollagüe extracts possessed five bands of 8.2, 14.5, 26.0, 34.8 and 40.7 kDa. The electrophoretic patterns of saline-soluble fractions obtained in this study allowed us to identify several bands within those ranges previously described by Brinegar and Goundan (1993), which would correspond to different isoforms of the che-

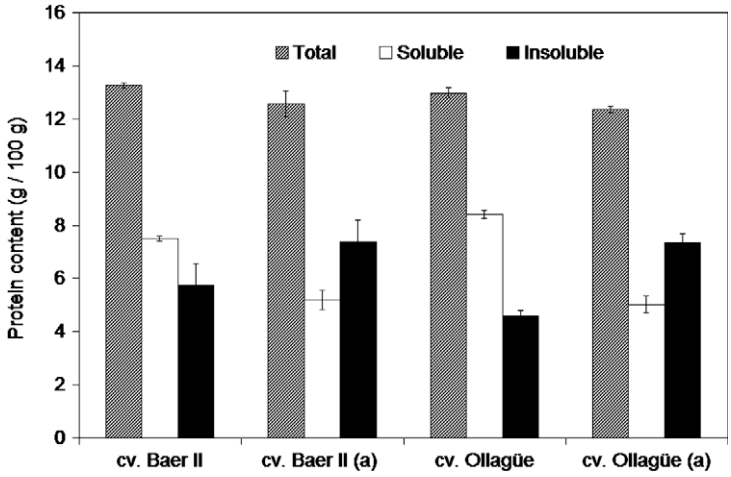

Fig. 1. Total, soluble and insoluble seed protein contents (determined by micro-Kjeldahl method) of two quinoa cultivars after 3 months storage at 14% RH and ambient laboratory temperature (control) or under accelerated ageing conditions (a) (75% RH and 37 °C).

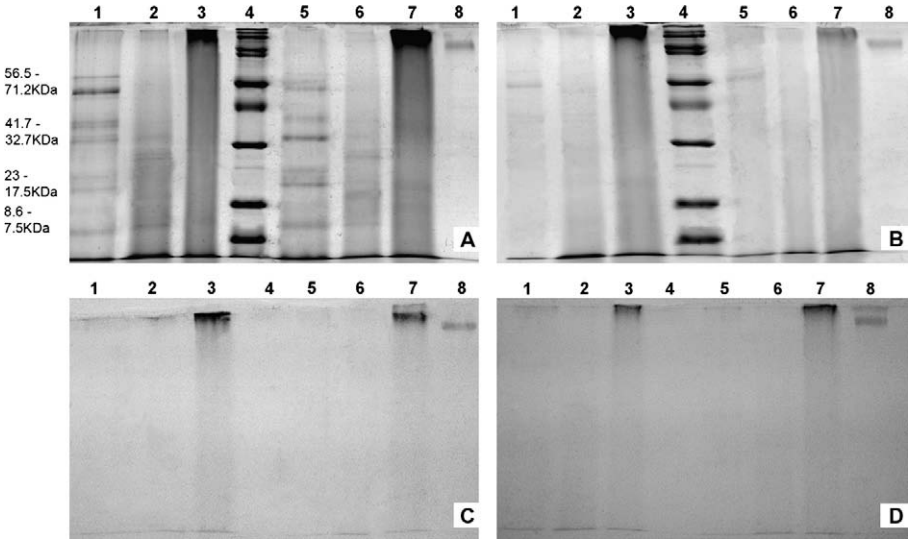

Fig. 2. SDS–PAGE of different protein fractions of two quinoa cultivars. (B, D) 6 months stored seeds; (A, C) 6 months stored seeds later humidified, so as to induce pre-germinative events. Gels were stained with Coomassie Blue for proteins (A, B) or periodic acid-Schiff for glycosylated compounds (C, D). Lanes 1–3: cv. Baer II, 1: LS + HS, 2: E, 3: H; 4: molecular weight marker; 5–7: cv. Ollagüe, 5: LS + HS, 6: E, 7: H; 8: invertase (control for glycosylated compounds). LS: low-salinity fraction, HS: high-salinity fraction, E: ethanolic fraction, H: alkaline fraction.

nopodin monomers. Likewise, the 7.5 and 8.6 kDa bands obtained in saline-soluble fractions of cv. Baer II and cv. Ollagüe, respectively, correspond to the other major storage protein, the 2S cysteine-rich globulin. In the present work, differences in seed protein composition among cultivars were found (Fig. 2A). This heterogeneity can be attributed to genetic differences, since the growing conditions set for the plants were identical for both cultivars, and can be used for the identification and classification of quinoa germplasm previously approached by Fairbanks, Burgener, Robinson, Andersen, and Ballon (1990).

The electrophoretical pattern obtained by SDS–PAGE denoted the presence of storage proteins (Fig. 2), further supporting the hypothesis of disgregation of high-molecular-weight protein aggregates created during storage. Such aggregates are most likely destroyed upon activation of repairing mechanisms during pre-germinative humidification (Bray, 1995; Leopold & Vertucci, 1989). Protein insolubilisation could be reverted during water uptake in the first stages of germination (Figs. 2A and 3). However, this ability to overcome protein insolubilisation did not necessarily lead to higher germination rates, as evidenced by the low germination percentage values exhibited by cv. Ollagüe seeds stored at 14% RH and ambient laboratory temperature (Table 1). Moreover, cv. Baer II seeds stored in the laboratory, as well as quinoa seeds stored in the gene bank, showed fair storage stability, as represented by high germination and viability values, even when protein insolubilisation occurred in both cases.

In order to further investigate seed protein damage due to the Maillard reaction, AGE accumulation was assessed by CML quantification (Table 3). CML contents were much higher in stored (aged) seeds than in control seeds of both cultivars. Moreover, humidified pre-germinated seeds showed significantly lower CML contents than untreated ones, but still higher than control seeds. Interestingly, there is a major difference in AGEs accumulation among both cultivars cv. Ollagüe being richer than cv. Baer II in CML. This is consistent with the larger loss of viability in the former cultivar due to advanced Maillard reaction.

3.4. Fluorescence development during seed ageing

Fluorescence analysis in the different protein fractions was used to study protein modification by Maillard reaction during seed storage. The excitation spectra of the different fractions of both cultivars showed several peaks at different excitation wavelengths, indicating the presence of different components with emission at approximately 450 nm. Spectra differed according to the protein fractions and were coincident at different storage conditions (data not shown). The fluorescence peak attributed to the presence of AGE in seed protein fractions is observed when excited between 320 and 380 nm (Murthy & Sun, 2000; Murthy et al., 2002, 2003), which is significantly different to the intrinsic protein fluorescence emitted by tryptophan (excitation at 280 nm/emission at 390 nm), and the basic residues of arginine and lysine are generally involved in the formation of Maillard-related fluorescent compounds (Tessier, Monnier, & Kornfield, 2002).

In this study, an excitation/emission wavelength of 380/450 nm was used for the quantitative measurement of fluorescent products accumulation due to Maillard reaction, since the fluorescence analysis revealed the presence of fluorescent compounds with these characteristics in all fractions, both cultivars and under all storage conditions.

Fluorescence intensities in extracts from 6 months stored seeds were similar or lower than those from 3 months stored seeds (Table 4), which was possibly attributable to the very low soluble protein contents in both cultivars. However, the high fluorescence values obtained from fractions with extremely low protein contents, as determined by Bradford method (Table 4, Fig. 1), suggested either the presence of non-protein fluorescent compounds or underestimation of protein content, as a consequence of impediments preventing fluorescent complexes reacting with the Bradford dye (Coomassie Blue). When pre-germinative events were induced, 6 months stored seed fractions of both cultivars showed similar fluorescence values to non-treated ones, even with the increased saline-soluble protein contents. Furthermore, this study revealed high fluorescence intensity values in seeds stored at the gene bank bearing 100% germination values.

In summary, the amount of fluorescent compounds in the 6 months stored samples were similar or lower than in the 3 months stored ones and even higher in the gene bank stored samples. This can possibly be attributed to the initial content of fluorescent compounds in the soluble protein fractions. We suggest that when the Maillard reaction proceeds, the fluorescent compounds are formed mainly linked to the proteins and such complexes become more insoluble and precipitate. Even though similar fluorescence behaviour has been attributed to Maillard products in other seeds and was related to ageing and seed deterioration (Murthy & Sun, 2000; Sun & Leopold, 1995; Wettlaufer & Leopold, 1991), our results suggest that the detected fluorophores are not solely related to the storage time and they are not eliminated during the first pre-germinative steps. The interference of certain fluorescent molecules, such as polyphenols present in the integuments and the remains of the pericarp (with excitation and

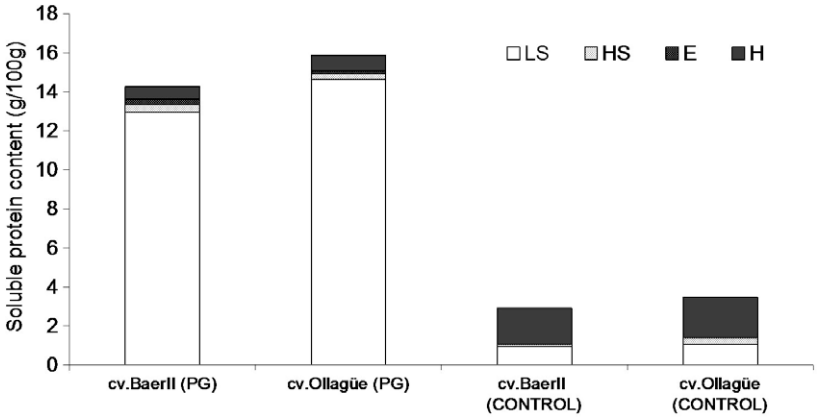

Fig. 3. Soluble protein content of seeds of two quinoa cultivars after 6 months storage at 14% RH and ambient laboratory temperature (control) and later humidified so as to induce pre-germinative events (PG). LS: low-salinity fraction (white), HS: high-salinity fraction (light grey), E: ethanolic fraction (dark grey), H: alkaline fraction (black).

Table 3
Maillard reaction assessment. CML content in seeds of two quinoa cultivars after storage at 14% RH and ambient laboratory temperature, stored and pre-germinated by humidification treatment (priming), and gene bank stored seeds (CONTROL).

Treatment	Fraction	Cv. Baer II		Cv. Ollagüe	
		Intensity (UF/g seeds)	Wavelength (nm)	Intensity (UF/g seeds)	Wavelength (nm)
3 Months stored	LS	30.1 ± 0.4	440–450	12.2 ± 0.4	440–450
	HS	12.6 ± 0.4	440–450	7.7 ± 0.4	440–445
	E	10.1 ± 0.4	437–442	7.2 ± 0.4	440–445
	H	13.1 ± 0.4	440–455	8.2 ± 0.4	445–450
6 Months stored	LS	16 ± 3	450	16.6 ± 0.2	450
	HS	3.4 ± 0.2	440–445	3.2 ± 0.2	440–445
	E	8 ± 1	440–450	4.7 ± 0.5	440–450
	H	5 ± 1	470	3.8 ± 0.4	470
6 Months stored + pre-germinative humidification	LS	25 ± 8	450	27 ± 6	450
	HS	6 ± 1	440–445	4.3 ± 0.2	440–445
	E	6.1 ± 0.7	440–450	4.1 ± 0.4	440–450
	H	2.1 ± 0.4	470	1.9 ± 0.2	470
Gene bank stored	LS	21 ± 3	450	18 ± 3	450

Table 4
Fluorescence development in seeds of two quinoa cultivars. Maximum intensity peaks and their corresponding emission wavelength ranges of each of the soluble fractions. Fluorescence values were assessed following Eq. (2). LS: low-salinity fraction, HS: high-salinity fraction, E: ethanolic fraction, H: alkaline fraction.

Treatment	Cv. Baer II mg CML/g seeds	Cv. Ollagüe mg CML/g seeds
Stored seeds	22.1 (1.92%)	55.0 (1.34%)
Stored seeds + pre-germinative humidification	20.2 (0.90%)	54.1 (0.56%)
Gene bank stored seeds	9.29 (7.58%)	3.04 (1.18%)

In brackets, % coefficient of variation.

emission maxima at 340 and 440 nm, respectively, according to Rodríguez-Delgado, Malovana, Pérez, Borges, and García Montelongo (2001)), or the formation of fluorescent, yet insoluble, high-molecular-weight aggregates may prevent AGE detection. In agreement with the results obtained by Baker and Bradford (1994) in other seeds, this study shows that the measurement of fluorescence only cannot be considered a suitable method to determine AGE accumulation in quinoa seeds.

4. Conclusions

This work establishes for the first time a direct association between seed viability and AGE accumulation due to Maillard reaction in quinoa seeds.

Quinoa seed protein insolubilisation was observed during storage. Although an association between seed conservation and protein solubility was established, protein insolubilisation would not be a determinant factor of seed viability loss, as soluble protein content values were restored by pre-germinative humidification, detected by protein quantification and electrophoretic analysis. In spite of that, our work showed high levels of AGEs in pre-germinated seeds, indicating that the repairing mechanisms were too inefficient to fully recover advanced deteriorated compounds.

Subsequently, damage appeared to be irreversible when very high levels of AGEs remained present, as in cv. Ollagüe stored seeds. In this regard, protein differential solubility may still be an appropriate indicator of protein deterioration and seed viability during storage, at the initial stages when the extent of damage is not generalized. However, fluorescence measurements cannot be considered a suitable method to determine AGEs accumulation in quinoa seeds.

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