Ageing in embryos from wheat grains stored at different temperatures: oxidative stress and antioxidant response

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Abstract

In the present work we studied oxidative stress as an important cause of seed deterioration during ageing in embryos from durum wheat grains stored at room temperature and at low temperature (10°C). The protective role of low temperature on seed viability was confirmed. The increase of hydrogen peroxide content during dry storage was strongly correlated with the decrease of germinability. Ascorbate and glutathione showed a good correlation with grain germinability and significantly increased upon imbibition, in particular in embryos from viable grains. Ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX) and catalase (CAT) were studied quantitatively (enzymatic assays). APX, GR, and GPX were also studied qualitatively by nativePAGE. The enzymes were active in dry, still viable, embryos whereas no activity was detected in non-viable embryos. With the exception of APX, all enzymatic activities decreased upon imbibition. The study of grains stored in different conditions indicated a negative correlation between the efficiency of the antioxidant enzymatic machinery and the age of the grain. The differences detected in differently stored materials confirmed that both germination parameters and the length of storage period are important in determining grain condition.

Introduction

Orthodox seeds, such as wheat grains, are tolerant to desiccation and retain viability for long periods, being able to germinate even after many years from harvest. However, during storage there is generally a gradual decline in germinability and finally a loss of the ability to germinate (Roberts 1972). Among the factors influencing the longevity of seeds, the most important ones are temperature and moisture content, thus, ageing is strictly correlated with storage conditions (Stefani *et al*. 2000; Spanò *et al*. 2004). In particular, low temperatures (10°C) play a protective role in *Triticum turgidum* L. ssp. *durum* (Desf.) ensuring the maintenance of the germination capacity for longer periods (at least 23 years) in comparison to higher temperatures, room temperature (up to 7 years) (Stefani *et al*. 2000). Seed ageing is characterised by cellular metabolic and chemical alterations including loss of membrane integrity and of enzymatic activity, impairment of RNA and protein synthesis, and DNA degradation (McDonald 1999). Free radicals, lipid peroxidation and the lack of ability of the cells to scavenge radicals are considered as the primary cause of seed damage during storage (Bailly 2004). In particular, the accumulation of reactive oxygen species (ROS) is often indicated as the major cause of seed deterioration (Lehner *et al*. 2008). To counteract the deleterious effects of ROS, plants have evolved both enzymatic and non-enzymatic defence mechanisms. High levels of the low molecular weight antioxidants, such as ascorbate (ASA) and glutathione (GSH), and enzymatic scavengers can cooperate to increase the resistance to oxidative injury (Arrigoni and De Tullio 2002). The main enzymes involved in antioxidant defence are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and the enzymes of the Halliwell-Asada pathway: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR). Many studies have been made on oxidative stress in seed ageing, however, the role of low molecular weight and enzymatic antioxidants in maintenance of seed viability has been studied mainly during accelerated ageing of orthodox seeds (Pukacka and Ratajczak 2005), with few exceptions (e.g. Goel and Sheoran 2003; Cakmak *et al*. 2010). However, previous studies have shown that lipid peroxidation and the loss of membrane phospholipids are found in accelerated ageing conditions (McDonald 1999) but not during natural ageing in prolonged storage (Petruzzelli and Taranto 1984). As conditions imposed by artificial ageing are different from constraints of natural ageing, one of the aims of the present work was to study the involvement of oxidative stress and antioxidant systems in natural ageing (room temperature and constant moisture content). With this aim, the content of hydrogen peroxide and of the low molecular weight antioxidants ASA and GSH were studied in embryos from dry and imbibed naturally ageing grains of *T. turgidum* ssp. *durum*. The activity of the main antioxidant enzymes was also studied. In particular as H_2O_2 is partially detoxified by CAT (EC 1.11.1.6), active at high peroxide concentrations in peroxisomes, and by APX (EC 1.11.1.11) in the cytosol and in chloroplast (Goel and Sheoran 2003) the activities of these enzymes were detected. DHAR (EC 1.8.5.1), GR (EC 1.6.4.2), GPX (EC 1.11.1.9) were also studied. Considering the protective role of low temperature on seed viability the antioxidative mechanisms of cold-stored grains were investigated and compared with those found in grains stored at room temperature. The comparison allowed to test if antioxidant systems are comparable in grains with the same viability but stored at different temperature. An ancillary objective was to investigate whether antioxidant systems during ageing have an effect on imbibition and/or germination performance.

Materials and methods

Plant material

Experiments were conducted in 2009. Caryopses (referred to in this paper as grains) of *Triticum turgidum* L. ssp. *durum* (Desf.) cv. Cappelli were obtained from plants cultivated in fields specifically used for experimental purposes near Pisa, Italy. Grains (dormant at harvest), which were uniform in size, were stored in the dark in sealed glass containers at room temperature (RT), (19±2°C) or at 10°C. Crops were selected after the germination energy (GE, see definition below) of a multiyear

collection of grains have been screened. The crops of grains showing the same GE were utilised: 1985 (non-viable, NV, for at least 3 years), 2004 (middle viable, MV, with an intermediate viability) and 2007 (viable, V, with full viability) for materials stored at RT; 1966 (NV), 1985 (MV) and 2000 (V) for cold stored material. Embryos (embryonic axes + scutellum) were manually isolated from dry or imbibed sterilised grains and stored in liquid nitrogen until use.

Germination energy

The seeds were sterilised for 3 min in NaOCl (1% available chlorine) and rinsed with distilled water. They were then germinated on moist filter paper (Whatman No. 2, Maidstone, England, UK) in Petri dishes and grown in the dark at 23°C for 72 h (four replicates, 50 seeds each). The percentage germination was recorded at 24, 48 and 72 h. A seed was considered to have germinated if the radicle had broken through the seed coat. Germination energy (Spanò *et al*. 2007) was calculated using the formula:

$$(n_1 \times 100 + n_2 \times 50 + n_3 \times 33.3)/ N_{tot} \quad (1)$$

where n_1 is the seeds germinated at 24 h, n_2 the seeds germinated between 24 and 48 h, n_3 the seeds germinated between 48 and 72 h, and N_{tot} is the total number of examined seeds.

Extraction and determination of hydrogen peroxide

The H_2O_2 content of isolated embryos was determined according to Jana and Choudhuri (1982). Briefly, embryos (250 mg) were ground in a mortar and homogenised with 15mL of phosphate buffer 50mMpH 6.5. The homogenate was centrifuged at 6000g for 25 min. To determine the H_2O_2 content, 3mL of extracted solution were mixed with 1mL of 0.1% titanium chloride in 20% (v/v) H_2SO_4, then the mixture was centrifuged at 6000g for 15 min and the supernatant absorbance was read at 410 nm. The amount of H_2O_2 in the extracts was calculated from a standard curve and expressed as nmol emb^{-1}. All experiments were made at least in triplicate.

Extraction and determination of ascorbate and dehydroascorbate

Ascorbate and dehydroascorbate extraction and determination were performed according to Kampfenkel *et al*. (1995) with minor modifications. Briefly, embryos (500 mg) were ground in a chilled mortar and homogenised with 3.75mL of 5% (w/v) TCA. The homogenate was centrifuged at 12 000g for 10 min at 4°C and the supernatant was used for the determination (Kampfenkel *et al*. 1995). The assay for the measurement of ASA and total ascorbate (ASA + DHA) is based on the reduction of Fe^{3+} to Fe^{2+} by ASA in an acidic solution and on the subsequent formation of complexes of Fe^{2+} with bipyridyl, giving a pink colour with maximum absorbance at 525 nm. Total ascorbate was determined after reduction of DHA to ASA by dithiothreitol and DHA level was estimated on the basis of the difference between total ascorbate and ASA value. Correction was made for colour development in the blank (absence of sample). All experiments were made at least in triplicate.

Extraction and determination of glutathione

Glutathione was extracted and determined according to Gossett *et al*. (1994). Briefly, embryos (500 mg) were homogenised in 1.5mL of ice-cold 6% (w/v) *m*-phosphoric acid (pH 2.8) containing 1mM EDTA. The homogenate was centrifuged at 20 000g for 15 min at 4°C and the supernatant was collected and stored in liquid nitrogen until use. Total glutathione (GSH +GSSG) was determined by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-glutathione reductase recycling procedure and the reaction was monitored as the rate of change in absorbance at 412 nm. GSSG was determined after removal of GSH from the sample extract by 2-vinylpyridine derivatisation. GSH was calculated by

subtracting the amount of GSSG from total glutathione. All experiments were made at least in triplicate.

Enzyme extraction and assays

Embryos were ground in liquid nitrogen in a mortar with a pestle. The extraction buffer contained 100mM potassium phosphate buffer pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA), and 1% polyvinylpyrrolidone (PVP-40). The homogenate was then centrifuged at 15 000g for 20 min. For glutathione reductase assay the supernatant was desalted on a Sephadex (Uppsala, Sweden) G-25 column. The supernatant was collected and stored in liquid nitrogen until use. Ascorbate peroxidase (APX) activity was measured according to Nakano and Asada (1981) with modification. Briefly, enzyme activity was assayed based on the decrease in absorbance at 290 nm (absorbance coefficient of 2.8mM^{-1} cm^{-1}) as ascorbate was oxidised. The reaction mixture contained 100mM potassium phosphate pH 7.5, 0.5mM ascorbate and enzyme extract (100 µg protein mL^{-1}). The reaction was started by adding 0.2mM H$_2$O$_2$. Correction was made for the low, nonenzymatic oxidation of ascorbate by hydrogen peroxide (blank). Dehydroascorbate reductase (DHAR) activity was determined as described previously by Nakano and Asada (1981). The activity of DHAR was determined by monitoring the glutathione-dependent reduction of dehydroascorbate. The enzymatic extract contained 50 µg protein mL^{-1}. The activity was determined by measuring the increase in absorbance at 265 nm for 3 min. Specific activity was calculated from the 14mM^{-1} cm^{-1} extinction coefficient. A correction for the nonenzymatic reduction of DHA by GSH was conducted in the absence of the enzyme sample (blank). Glutathione reductase (GR) activity was determined as described by Rao *et al*. (1995) following the oxidation of NADPH at 340 nm (extinction coefficient 6.2mM^{-1} cm^{-1}). Enzymatic extract contained 100 µg protein mL^{-1}. A correction for the nonenzymatic reduction of GSSG was conducted in the absence of enzyme sample (blank). Glutathione peroxidase activity was determined according to Navari-Izzo *et al*. (1997) by coupling its reaction with that of GR, using as substrate 0.45mM H$_2$O$_2$ (GPX) or 0.96mM *t*-butyl hydroperoxide (PHGPX). The activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2mM^{-1} cm^{-1}). The enzymatic extract contained 100 µg protein mL^{-1}. Catalase (CAT) activity was determined at 240 nm, as described by Aebi (1984), using as substrate 20mM H$_2$O$_2$. Enzymatic extract contained 100 µg protein mL^{-1}. A blank containing only the enzymatic solution was made. Specific activity was calculated from the 23.5mM^{-1} cm^{-1} extinction coefficient. All experiments were made at least in triplicate. All enzymatic activities were determined at 25°C and expressed as Ug^{-1} protein min^{-1}. Protein measurement was performed according to Bradford (1976), using BSA as standard.

Native PAGE and activity staining

Samples were subjected to discontinuous 10% PAGE under nondenaturating, nonreducing conditions, as described by Laemmli (1970) except that SDS was omitted. Electrophoretic separation was performed at 4°C with a constant current of 35mA per gel (Rao *et al*. 1995). After completion of electrophoresis the gels were stained for the detection of the activities of APX, GR and GPX. For APX activity detection, the running buffer contained 2mM ascorbate and the gels were prerun for 30 min to allow the ascorbate, present in the running buffer, to enter the gel before sample application (Mittler and Zilinskas 1993), then 100 µg of protein per lane were loaded on the gel. Following electrophoresis, gels were equilibrated, incubated and washed according to Rao *et al*. (1995). Activity bands were negatively stained by submerging the gels in a solution of 50mM potassium phosphate buffer (pH 7.8) containing 28mM tetramethyl ethylene diamine and 2.45mM nitroblue tetrazolium for 10–15 min. The reaction was stopped by a brief wash in water. To verify the H$_2$O$_2$-dependence of APX bands, a gel was also incubated in a reaction solution without H$_2$O$_2$ (De Gara *et al*. 2000).

For GR activity detection, the gels were washed and incubated according to Hou *et al*. (2004). One hundred µg of protein per lane were loaded on the gel. GR activity was negatively stained in darkness with a solution containing 1.2mM MTT and 1.6mM PMS for 5–10 min at room temperature with gentle shaking. Bands of GR activity were visible as clear zones against a purple background. Gels for the GPX activity detection were washed and incubated as in Lin *et al*. (2002). Two hundred µg of protein per lane were loaded on the gel. The incubation was made in the presence of 0.45mM H_2O_2 (GPX) or 0.96mM *t*-butyl hydroperoxide (PHGPX). The GPX activity was developed as the GR activity.

Statistical analysis
Data were subjected to one-way ANOVA and differences among treatments were determined by Bonferroni *post-hoc* test. Data are expressed as the mean values ± s.e. of at least three replicates.

Results

Viability and H_2O_2
The wheat grain viability was assessed on the basis of the germination energy. Grains viability clearly depended on storage conditions and grains stored for 24 years (1985) at 10°C were still viable, while the same grains stored at RT were NV (Table 1). This confirms that low temperatures slow down seed ageing and extend seed viability. Storage of in a dry state was characterised in wheat embryos by an increase in H_2O_2 content (Fig. 1) that was gradual in grains stored at 10°C. In embryos with intermediate viability (MV), peroxide content was significantly higher in grains stored at low temperature than in grains stored at room temperature; NV embryos showed a considerable increase in H_2O_2 content, which was ~2 times higher than in the dry V embryos. Imbibition for 24 h caused little variation in the peroxide content with the exception of embryos from NV grains, which showed a significant decrease of the presence of this signalling molecule.

Ascorbate and glutathione
Embryos from dry grains contained a negligible quantity of ASA (Fig. 2*a*) that decreased with decreasing viability. Imbibition caused a strong increase in pool of total ascorbate in embryos from V and MV grains with a maximum content in embryos from V grains stored at room temperature (the youngest grains). This increase was due to the increase in both the reduced (Fig. 2*a*) and the oxidised (Fig. 2*b*) form. Total ascorbate pool decreased in parallel to the loss of seed viability. Dry embryos were characterised by a low level of glutathione, which was not detectable in NV embryos (Fig. 3). Upon imbibition the glutathione level strongly increased of ~4 times, in both the ageing treatments; this increase in the glutathione amount was almost exclusively due to the reduced form (Fig. 3*a*), while GSSG (Fig. 3*b*) maintained quite low levels. Upon imbibition the differences between materials with different viability became negligible. Glutathione was not detectable in imbided NV embryos.

Antioxidant enzymes
Different antioxidant enzymatic systems were analysed and results are reported in Figs 4–7. No enzymatic activity was detected in embryos from dry or imbibed NV grains. As shown by the enzymatic assays, APX (Fig. 4*a*) was already present in dry embryos and its activity significantly increased after imbibition. APX activity remained almost constant in dry embryos from V and MV grains stored at RT. In contrast, a remarkable decrease in this activity was observed in dry embryos from MV cold-stored grains. The presence of APX isoenzymes (Fig. 4*b*) in wheat embryos and their pattern were analysed by native PAGE. Several proteins with ascorbate oxidising capability were present both in dry and in imbibed embryos. Upon imbibition two new bands appeared in all of the materials whereas some other bands decreased particularly in cold-stored grains. GR (Fig. 5*a*) and

DHAR (Fig. 5c) were already present in dry embryos but their activity generally decreased after imbibition. As for APX, DHAR and GR activities were almost constant in embryos from RT V and MV grains, but they significantly decreased during ageing in cold-stored materials. Activity gels (Fig. 5b) of extracts from viable grain embryos showed the appearance of new electrophoretic bands of GR upon imbibition. GPX (Fig. 6a) and PH-GPX (Fig. 6b), specific for lipid hydroperoxides, were already present in dry embryos, having comparable activities in V and MV embryos from grains stored at room temperature. However, the activity was significantly higher in V than in MV embryos isolated from material stored at 10°C. Imbibition stressed the differences between the different storage conditions. Activity gels (Fig. 6c, d) showed two different bands which could be detected in all materials; however, a third activity band, with lower mobility, was detectable in embryos from grains stored at room temperature. This band was not present in imbibed embryos. CAT (Fig. 7) showed similar activity in dry embryos, with the exception of cold-stored MV embryos, where the activity was significantly higher. Imbibition was characterised by a general decrease in the activity of this H_2O_2 scavenging enzyme.

Discussion

Ageing determines a marked decrease in wheat seed viability. In agreement with previous studies (Stefani *et al.* 2000; Spanò *et al.* 2007) this loss of viability was slowed by storage at low temperature (10°C). The loss of germinability has been attributed to the accumulation of ROS (Wojtyla *et al.* 2006) and the subsequent oxidative stress, which has been indicated as an important cause of seed deterioration associated with ageing (McDonald 1999). Ageing induced in dry embryos an increase in the content of H_2O_2, an indicator of incipient tissue damage; NV materials had the highest content of this stress indicator. These results are consistent with previous studies showing a progressive impairment of membrane integrity during storage (Spanò *et al.* 2007). The results of H_2O_2 content stress the importance of the storage conditions and of the age of the grain. In fact, embryos from grains with the same germination energy had a higher content of hydrogen peroxide in cold-stored material. This might be due to the length of the storage period which was significantly longer in cold-stored grains (24 years) than in grains stored at RT (5 years). H_2O_2 content can decrease by the action of protective mechanisms such as the non-enzymatic antioxidants ASA and GSH (Arrigoni and De Tullio 2002), present with negligible values both in RT and 10°C stored material. Noteworthy, glutathione was not detectable in embryos from NV grains and this is an indirect confirm of the possible implication of GSH in the regulation of plant cell growth and division (Potters *et al.* 2004). The activities of the enzymes involved in the ascorbate/glutathione cycle, APX, DHAR and GR remained almost unchanged during ageing of embryos from V and MV dry grains stored at RT. In contrast, these activities decreased when the grains were stored at 10°C, in accordance with studies on artificial seed ageing of wheat (Lehner *et al.* 2008). Data of hydrogen peroxide and of enzyme activities further stress that artificial ageing and natural ageing impose different constraints on seeds. The different results obtained in embryos from grains stored under different conditions show that the efficiency of the antioxidant enzymatic machinery is strictly correlated with the age of the grain. Moreover, differently stored grains with the same viability did not have the same enzymatic activity. The loss of viability in embryos of MV wheat grains stored at 10°C was accompanied by a progressive impairment of the efficiency of the antioxidant defence system which determined an increase in H_2O_2 content and even the high CAT activity could not counteract the significant increase in this ROS. Thus, a correlation was generally observed between enzymatic activity and age of grains rather then between enzymatic activity and GE. This is in accordance with previous studies where nucleolytic activity was strictly correlated with the age of wheat grains (Spanò *et al.* 2007). In NV embryos there was no activity of APX, DHAR, GR and CAT; hence, the antioxidant machinery was unable to remove the ROS produced during ageing and peroxide content reached the highest value. Imbibition did not cause significant variations in peroxide content, but there was a strong decrease in the content

of this molecule in embryos from imbibed NV grains. Obviously, the strong decrease in H_2O_2 content could not be due to enzymes as no antioxidant activity has been detected in NV embryos. To assess whether hydrogen peroxide could be transferred during imbibition to the endosperm, the content of this ROS was also measured in this seed compartment. Results (data not shown) were consistent with a partial redistribution of this molecule between the two parts of the grain, as a significant increase of H_2O_2 content was detected in endosperms from NV grains. A progressive impairment of the membrane was demonstrated in ageing wheat caryopses (Spanò *et al.* 2007) and the leakage of H2O2 through the deteriorated membranes of NV grains upon imbibition must be considered too (data not shown), as this ROS was detected in NV grain imbibition water. The restoration of the oxidative metabolism induced by imbibition is accompanied by changes in the redox state and in the metabolism of ascorbate and glutathione, molecules playing a key role in ROS scavenging. Upon imbibition, the increase in the ascorbate pool is correlated with germination ability, as both cell division and cell expansion require ascorbate (Tommasi *et al.* 2001). In accordance the total ascorbate pool decreased in parallel with the loss of seed viability. We note that the increase in GSH detected upon imbibition was not accompanied by changes in GSSG content and, as already reported in literature (Torres *et al.* 1997), in imbibed embryos the glutathione redox status shifted to the reduced form both in unaged and in aged seeds. With the exception of APX, which increased in imbibed embryos, the activity of the other antioxidant enzymes generally decreased. Therefore the enzymes of the Halliwell-Asada cycle seem to play only a marginal role in ascorbate and glutathione supply in the 24 h-imbibed embryos. The activity of PHGPX might indicate the need of membrane protection against ROS-induced lipid peroxidation (Yang *et al.* 2005). In conclusion, our results demonstrate that ageing in embryos of wheat grains is associated with an increase in the levels of peroxide and with an impairment in the antioxidant defence system with a good correlation between the content of low molecular weight antioxidants and grain germinability. As shown for ribonucleases (Spanò *et al.* 2007), differently stored seeds having the same germination energy, have important metabolic differences and metabolism is subjected to regulation depending mostly on the length of the storage period. Cold slows down the loss of germination but long storage progressively alters metabolism. So the loss of seed viability induced by ageing can derive from different mechanisms active in different ageing conditions (i.e. temperature, water content) and caution should be used when conclusion on ageing are inferred from studies made on artificially aged seeds.

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Legends of figures

Fig. 1. Concentration of H_2O_2 in wheat embryos isolated from dry or 24 h imbibed viable (V), middle viable (MV), non-viable (NV) grains stored at room temperature (RT) or 10°C. Data points represent means±s.e. of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$.

Fig. 2. Concentration of (*a*) ascorbate (ASA) and (*b*) dehydroascorbate (DHA) in wheat embryos isolated from dry or 24 h imbibed viable (V), middle viable (MV), non-viable (NV) grains stored at room temperature(RT) or 10°C. Data points represent means±s.e. of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$.

Fig. 3. Concentration of (*a*) glutathione (GSH) and (*b*) oxidised glutathione (GSSG) in wheat embryos isolated from dry or 24 h imbibed viable (V), middle viable (MV), non-viable (NV) grains stored at room temperature(RT) or 10°C. Data points represent means±s.e. of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$.

Fig. 4. (*a*) Enzymatic activity and (*b*) activity gel of ascorbate peroxidase (APX) in wheat embryos isolated from dry or 24 h imbibed viable (V), middle viable (MV), non-viable (NV) grains stored at room temperature(RT) or 10°C. Activities are expressed in Ug^{-1} protein min–1. New activity bands, appearing upon imbibition are indicated by arrows. Data points represent means±s.e. of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$. In each lane, 100 µg protein was loaded.

Fig. 5. (*a*) Enzymatic activity and (*b*) activity gel of glutathione reductase (GR) and (*c*) enzymatic activity of dehydroascorbate reductase (DHAR) in wheat embryos isolated from dry or 24 h imbibed viable (V), middle viable (MV) grains stored at room temperature (RT) or 10°C. Activities are expressed in Ug^{-1} protein min^{-1}. Data points represent means±s.e of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$. In each lane, 100 µg protein was loaded.

Fig. 6. (*a*) Enzymatic activity and (*c*) activity gel of glutathione peroxidase (GPX) and (*b*) enzymatic activity and (*d*) activity gel of PHGPX in wheat embryos isolated from dry or 24 h imbibed viable (V), middle viable (MV) grains stored at room temperature (RT) or 10°C. Activities are expressed in Ug^{-1} protein min^{-1}. Data points represent means±s.e. of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$. In each lane, 200 µg protein was loaded.

Fig. 7. Enzymatic activity of catalase (CAT) in wheat embryos isolated from dry or 24 h imbibed V, MV grains stored at room temperature (RT) or 10°C. Activities are expressed in Ug^{-1} protein min^{-1}. Data points represent means±s.e. of three replicates. Different letters shown above the bars indicate means that are significantly different at $P < 0.05$.

Table 1. **Germination percentage and germination energy (GE) of grains of *Triticum turgidum* ssp. *durum* cv. Cappelli stored at room temperature or 10°C**

Grains were imbibed for 24, 48 and 72 h. Grains with a 0 GE had remained non-viable for at least 3 years. RT, room temperature

Viability	Harvest year	Germination (%)			GE
		24 h	48 h	72 h	
NV	1985 RT	0	0	0	0
MV	2004 RT	12	66	68	40
V	2007 RT	100	100	100	100
NV	1966 10°C	0	0	0	0
MV	1985 10°C	26	69	69	47
V	2000 10°C	100	100	100	100

Fig. 1
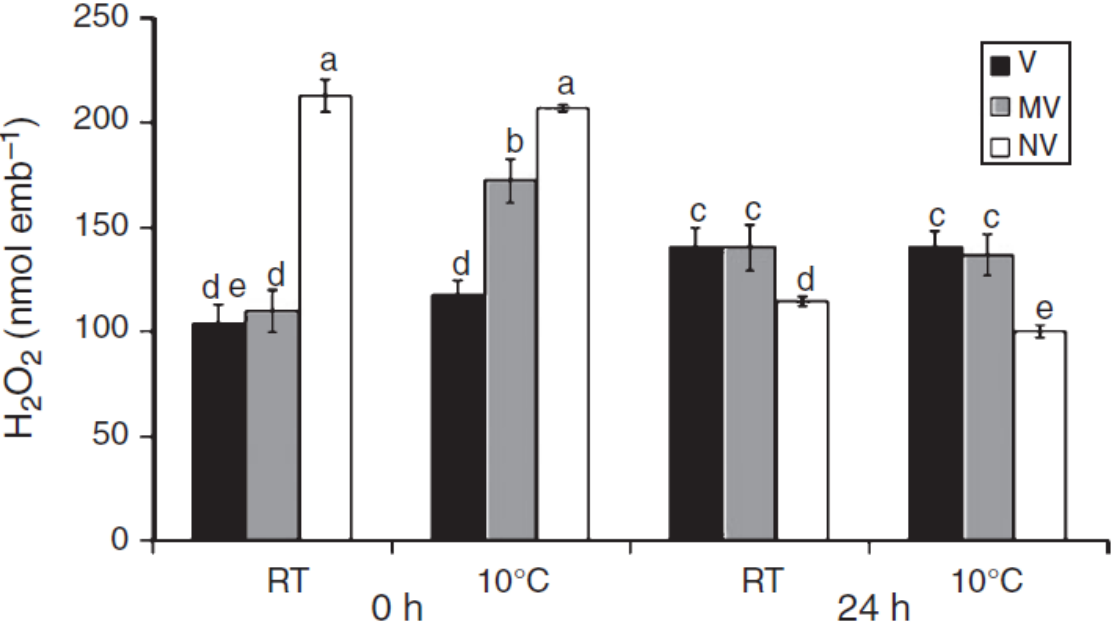
Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Fig.7