Responses to desiccation injury in developing wheat embryos from naturally- and artificially-dried grains

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Abstract

Grains of wheat (Triticum durum Desf. cv. Cappelli) were collected at different stages of maturation. To test whether the response to desiccation injury is correlated with physiological stage and/or water content, one lot of grains at each stage was artificially dried until its water content was comparable to that of the subsequent stage. Several stress parameters were analysed in embryos isolated from naturally- and artificially-dried grains. In particular, the content of dehydrins, hydrogen peroxide and the activity of ribonucleases and of antioxidant enzyme were studied. Neutral ribonucleases, unlike acidic ones, seemed to be correlated with grain water content. In parallel with these analyses the presence of dehydrins was also examined. Dehydrin accumulation was found to vary with drying rate, and more isoforms of dehydrin were present in artificially dried than in naturally-dried grains. Artificial drying resulted in accumulation of hydrogen peroxide, while the content of this molecule was much lower in embryos from naturally fully-ripened grains. While the activities of ascorbate peroxidase (APX, EC 1.11.1.11), dehydroascorbate reductase (DHAR, EC 1.8.5.1), glutathione reductase (GR, EC 1.6.4.2), and glutathione peroxidase (GPX, EC 1.11.1.9), were generally higher early in maturation, the activity of catalase (CAT, EC 1.11.1.6) increased in mid-maturation and decreased in fully-ripe embryos. Different protective mechanisms seem to act in combination but their relative importance changes as maturation progresses and in response to the drying method.

Keywords:

Antioxidant activity – Dehydrin - Hydrogen peroxide – Ribonucleases - *Triticum durum*

Abbreviations: APX, Ascorbate peroxidase; CAT, Catalase; DHAR, Dehydroascorbate reductase; GPX, Glutathione peroxidase; GR, Glutathione reductase; LEA proteins, Late embryogenesis abundant proteins; ROS, Reactive oxygen species.

1. Introduction

Desiccation is a natural stage of seed development that enables seeds to maintain germinability for long periods under adverse conditions [1]. Maturation drying, necessary for the completion of the life cycle of orthodox seeds, ensures the switch from a developmental mode to a germinative mode [2]. On the other hand, natural drying induces stress (water deficit), which results in the formation of reactive oxygen species (ROS) that cause denaturation of proteins, DNA damage and lipid peroxidation, with negative effects on overall metabolism [3]. Seeds are well endowed with antioxidant molecules, and scavenging systems are likely involved in the early neutralisation of potentially toxic ROS formed during water loss [1]. Protection against ROS may be mediated by hydrogen peroxide scavenging enzymes, such as catalase (CAT), glutathione peroxidase (GPX) and enzymes of the ascorbate-glutathione cycle [4]. Ascorbate peroxidase, for example, catalyses the removal of H2O2, and its possible involvement in protection against oxidative stress occurring during seed desiccation has been considered [5]. Fluctuations in enzymatic activities have been detected during seed ripening of a number of different species. CAT increases during seed development and is closely related to moisture content in sunflower seeds [6]. In lupin embryos CAT activity increases until physiological maturity and decreases during seed dehydration to levels higher than in young embryos [7]. A decline in CAT activity has however been detected in ripening kernels of *Triticum durum* [5]. Specific RNases are associated with wheat embryo water content: its isozymes decrease during maturation drying [8] and reappear with the progressive increase in water content during imbibition [9]. The results of these studies also suggest the importance of physiological state, as these ribonucleases appear more precociously in dormant- than non-dormant-embryos during germination. In orthodox seeds the different mechanisms of protection associated with maturation drying include the accumulation of di-and oligosaccharides [10] and the synthesis of late embryogenesis abundant proteins, LEA [11,12]. In particular, LEA proteins accumulate in seeds during maturation drying [13] and this accumulation in seeds suggests their involvement in protective reactions promoting maintenance of embryo structures under conditions of water deficit [14,15]. Dehydrins seem to protect cells by maintaining protein structures and water binding under conditions of cell dehydration [11,12,14,16]. In GM-tobacco it has been demonstrated that this class of proteins could act as free-radical scavengers under conditions of cold stress [17]. In the present study, developing grains of *T. durum* cv. Cappelli were analysed. To test whether the capacity to respond to desiccation injury is correlated with physiological stage and/or with water content, a comparison was made between embryos from seeds dried naturally during maturation and embryos from seeds dried artificially to reach the water content typical of the subsequent stage. The content of hydrogen peroxide, an important signalling molecule of stress, and the activity of antioxidant enzymes were studied. Responses to natural and artificial drying were examined by studying the activities of RNases and the presence of protective molecules, in particular the presence of dehydrins and the activities of the antioxidant enzymes APX, DHAR, GR, GPX and CAT. The damage related to desiccation was quantified as H_2O_2 production.

2. Results

2.1. Grain development and germination

Grain development was studied from milk stage to fully-ripe stage. Grain moisture content is expressed as a percentage on a fresh weight basis (Table 1). Fresh mass increased from milk to dough stage (data not shown). Further maturation of grains corresponded to a sharp increase in water loss and during this desiccation stage the water content decreased to 12% of fresh weight. Seeds with water content above 26% did not germinate until five days of imbibition (Table 1). Seeds collected at later stages of development germinated with germination percentages that increased as water content decreased. Artificial drying led to germination in earlier stages (dough stage, Table 1). Similar results were obtained from isolated embryos (data not shown).

2.2. RNase activity gels

Different forms of RNase were present in developing wheat embryos (Fig. 1): acidic, neutral salt-stimulated and neutral salt-inhibited [8]. The continuous presence of a single band of acidic RNases was typical of developing grain embryos. Artificial drying had no significant effect on the pattern of acid RNases (Fig. 1). Neutral RNases, on the contrary, seemed to be related to the hydration state of grains (Fig. 1 B and C). There was a decrease of about 50% in the intensity of band activity from dough to wax stage, until its disappearance in the last stage in both naturally- and artificially-dried grains.

2.3. Accumulation of dehydrins

LEA proteins were present with bands of MW from 25 kDa to 15 kDa (Fig. 2). Fewer and fainter bands were detected in the first stage considered (milk stage), while a similar pattern with a higher number of broader bands was typical of the mid-development stages (dough and wax). In fully-ripe grain embryos a higher number of bands was present. In embryos from all the developmental stages, artificial drying induced patterns of dehydrins similar to those detected in embryos from fully-ripe grains.

2.4. H_2O_2 content

After a slight increase between the first and the second stages, H_2O_2 content remained nearly constant and then fell to 32 mmol g^{-1} DW at full maturity (Fig. 3). Artificial drying of grains resulted in a general decrease in peroxide content, though the last step in artificial desiccation (12% water content) produced H_2O_2 levels higher than those in naturally-dried seeds (12% water content).

2.5. Antioxidant enzyme activities

APX, DHAR, GR, GPX and CAT activities are expressed as U g^{-1} protein (Figs. 4 and 5). APX activity (Fig. 4) was about 377 U g^{-1} protein at the milk stage and decreased during later stages of development. After artificial drying APX activity remained almost constant at a value similar to those measured in naturally dehydrated grain embryos with comparable water content. Artificial drying reduced APX activity to values lower than those recorded in embryos from milk stage grains. DHAR and GR activities were high in the early stage of maturation. GR activities (Fig. 4) stabilised at a lower level until the end of seed maturation. DHAR activity (Fig. 4) increased in fully-ripe embryos, though it never reached the high value found in young ones. With the exception of DHAR activity in embryos at milk stage, which remained high, artificial drying reduced all enzyme activity to values similar to those in embryos from grains of comparable water content. GPX activity (Fig. 5) decreased until the dough and wax stages and then increased, though never reaching the values found in milk

stage embryos. Although artificial drying induced a decrease in the activity of this H_2O_2 scavenging enzyme in young embryos, it had no significant effect on its values in later stages of development. CAT activity (Fig. 5) increased up to the dough and wax stages and then decreased, though it remained higher in embryos from fully-ripe seeds than in young embryos. Artificial drying lead to a marked increase in CAT activity, reaching the value of 47 U g^{-1} protein found in embryos from fully dried grains.

3. Discussion

Desiccation is a natural stage of orthodox seed development and is generally accepted as important for cereals to acquire germinability of the whole seed [18], switching seeds from a developmental mode to a germinative one [2]. In the present study, grains and isolated embryos germinated only when the grain water content fell below 42%. Premature desiccation induced more precocious germination, indicating that germinability is correlated with the hydration state of grains. However artificially-dried seeds were characterised by lower germinability than naturally-dried seeds with comparable water content, suggesting that the physiological state of the developing grain also plays an important role. Drying seems to mark a transition point for neutral ribonucleases, which undergo qualitative and quantitative changes in enzymatic activity. The shift from a development to a germination mode caused by drying is associated with a progressive decrease in this RNase activity. The presence of neutral ribonucleases seems to be correlated with grain water content. In fact, this enzymatic activity decreases progressively during maturation drying [8] and then rises again with the progressive increase in water content during imbibition [9]. This is further confirmed by the results of artificial drying, during which the pattern of neutral ribonucleases was mirrored by water content. Surviving dehydration requires a complex set of mechanisms working in close coordination. LEA proteins play an important role in response to various stress factors, including water stress [16] and several LEAs, such as dehydrins, accumulate in seeds during maturation drying [19,20]. Different patterns of dehydrin bands were detected depending on developmental stage: in the early stage of maturation (milk stage) there were fewer and fainter bands than in later stages (dough and wax). Artificial drying brought about an increase in bands, whose pattern was similar to that of fully-ripe embryos. Therefore, it seems that dehydrin accumulation is not regulated by the degree of water loss, but rather by the rate of drying [13]. Removal of water in plant tissues is accompanied by an increased production of ROS [13,21]. Orthodox seeds possess several mechanisms to scavenge ROS in order to protect themselves against their destructive activity [22]. The present study has addressed some of the enzymatic detoxifying mechanisms that enable ROS elimination. H_2O_2 can be directly decomposed by CAT, or by the enzymes of the ascorbate-glutathione cycle. This latter cycle involves APX, DHAR, GR, amongst others, and GPX may also catalyse the reduction of H2O2 [6]. According to Lehner et al. [4] the antioxidant systems sufficiently protect seeds against oxidative damage during dehydration. In early development (milk stage, 56% water content), when both mitochondria and chloroplasts are active, antioxidant enzymatic activities were generally higher than in subsequent stages. For water content below about 25-26%, peroxide content rapidly decreased, which might be due to a decrease in the source of ROS.

In mid-development (dough and wax stages) the loss of APX activity might be counterbalanced by the increase in CAT activity, another important ROS-scavenging enzyme. The increase in CAT activity in mid-development could be due to the involvement of this enzyme in fatty acid metabolism, which is high during this stage of grain ripening [5,23]. Maintaining viability in seeds with low water content requires different protective mechanisms acting at different stages: in early development (milk stage) antioxidant enzymes

play the major role, in mid-development dehydrins and CAT carry out the main protective mechanisms, while in fully-ripe embryos, as mentioned above, ROS decrease and antioxidant mechanisms are no longer necessary. The activities of enzymes responsible for H_2O_2 removal fall remarkably in dehydrated grain. Artificial drying causes more severe stress than natural drying. The grain defence systems seem sufficient to protect embryos effectively, except for the last stage, when even the relatively high antioxidant enzyme activity is unable to lower peroxide levels. While water content is clearly important in the grain's response to desiccation injury, its developmental state and the rate of drying, which are different in naturally- and artificially-dried grains, seem to be important factors as well.

3. Materials and methods

4.1. Plant material

Grains of *T. durum* Desf. cv. Cappelli were obtained from plants cultivated in fields specifically used for experimental purposes near Pisa, Italy. Grains were harvested at four different stages of ripening as shown in Table 1. Artificial drying was performed in a glass desiccator, under vacuum, at room temperature and in the presence of drierite. Every 18 h lots of grains were collected, embryos were isolated and water content was determined, as indicated below. Embryos from grains with water content typical of the subsequent ripening stage were stored in liquid nitrogen until use. Calculations of seed fresh weight, dry weight and moisture content were based on weights determined before and after oven drying of grain samples at 100 °C for 24 h. Grains of all ripening stages, naturally and artificially dehydrated, were surface sterilised for 3 min in NaOCl (1%, v/v, available chlorine) and rinsed before use. Wheat grainswere germinated in Petri dishes on water-moistened Whatman No. 2 filter paper at 23 ° ± 1 °C in the dark. The percentage germination was recorded after 1, 2, 3, 4 and 5 days. Three replications with 50 grains each were made. Embryos (axes + scutellum) were hand-isolated, immediately after material collection by mean of a gouge, from sterilised grains and transferred to Petri dishes onto a layer of Whatman No. 2 filter paper imbibed with sterile germination medium: 10 mM Tris-HCl pH 7, 20 mM KCl, 10 mg mL^{-1} sucrose and 50 mg mL^{-1} chloramphenicol [24]. Germination was evaluated and embryos were considered to have germinated when the roots were at least 2 mm long. Five replications with 10 embryos each were made. Embryos utilised for RNase, dehydrin, H_2O^2 and antioxidant enzyme determination were stored at -20 °C until use.

4.2. RNase extraction and electrophoresis

Extraction of ribonucleases was made according to Spanò et al. [25]. Enzymatic extract was stored in liquid nitrogen until use. Electrophoresis was carried out according to Blank and McKeon [26] with minor modifications [25] using 12.5% SDS-PAGE gels containing 2.4 mg mL^{-1} Torula yeast RNA. Following electrophoresis, gels were washed, specifically incubated and stained as in Spanò et al. [25]. In particular gels were incubated for 90 min at 37 °C in: 100 mM imidazole buffer pH 8.0 for the salt-inhibited neutral RNases; 100 mM imidazole buffer pH 8.0 containing 200 mMKCl for the salt-stimulated neutral RNases; 50 mMsodium acetate buffer pH 5.7 for the acidic RNases. Gels were scanned with a GS-690 imaging densitometer (Bio-Rad, Hercules, CA, USA).

4.3. Protein extraction, electrophoresis and western blotting

Embryos were frozen and ground to a fine powder in liquid nitrogen. The proteins were precipitated with 10% (w/v) TCA and 0.07% b-mercaptoethanol in acetone, washed and

completely dried. Dried powder was solubilised in loading buffer [25] and the samples were boiled for 5 min and centrifuged for 5 min at 10,000xg. The supernatant was subjected to discontinuous electrophoresis (12.5% SDS-PAGE). The separated polypeptides were transferred onto a nitrocellulose membrane for 2 h at 300 mA. After transfer, the membrane was blocked over night with 5% (w/v) dry non-fat milk in 20 mM TriseHCl pH 7.5 with 500 mM NaCl (TBS) containing 0.1% (w/v) Tween 20 (TBST). Then the membrane was incubated for 1 h with a polyclonal rabbit anti-dehydrin antibody (Stressgen) at a dilution of 1/500 in TBS. After three washes of 20 min each in TBST, the membrane was incubated for 1 h with the secondary antibody, a goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad) at a dilution of 1/1000 in TBS. After three washes of 20 min each in TBST, the membrane was developed by incubating for 5 min in BCIP/NBT colour development solution prepared as in instructions of Bio-Rad.

4.4. Extraction and determination of hydrogen peroxide

H_2O_2 contents of isolated embryos were determined according to Jana and Choudhuri [27]. Embryos (250 mg) were ground in a mortar and homogenised with 15 mL of phosphate buffer 50 mM pH 6.5. The homogenate was centrifuged at 6000xg for 25 min. To determine the H_2O_2 content, extracted solution (3 mL) was mixed with 1 mL of 0.1% titanium chloride in 20% (v/v) H_2S0_4, then the mixture was centrifuged at 6000xg for 15 min and the supernatant absorbance at 410 nm was read. The amount of H_2O_2 in the extracts was calculated from standard curve. Results were expressed as mmol g^{-1} DW. 4.5. Enzyme extraction and assays
Embryos were ground in liquid nitrogen with a mortar and pestle. Extraction buffer contained 100 mM potassium phosphate buffer pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1% polyvinylpyrrolidone (PVP-40). The homogenate was then centrifuged at 15,000xg for 20 min. For GR the supernatant was desalted on a Sephadex G-25 column. The supernatant was collected and stored in liquid nitrogen until use. Ascorbate peroxidase activity was measured according to Nakano and Asada [28] with modification. Enzyme activity was assayed from the decrease in absorbance at 290 nm (absorbance coefficient of 2.8 mM^{-1} cm^{-1}) as ascorbate was oxidised. The reaction mixture contained 100 mM potassium phosphate pH 7.5, 0.5 mM ascorbate and enzyme extract (100 mg protein mL^{-1}). The reaction was started by adding 0.2 mM H_2O_2. Correction was made for the low, non-enzymatic oxidation of ascorbate by hydrogen peroxide. Dehydroascorbate reductase activity was determined as described previously by Nakano and Asada [28]. The activity of DHAR was determined by monitoring the glutathione-dependent reduction of dehydroascorbate. Enzymatic extract contained 50 mg 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 14 mM^{-1} cm^{-1} extinction coefficient. A correction for the non-enzymatic reduction of DHA by GSH was carried out in the absence of the enzyme sample (blank). Glutathione reductase activity was determined as described by Rao et al. [29] following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}). Enzymatic extract contained 100 mg protein mL^{-1}. A correction for the non-enzymatic reduction of GSSG was carried out in the absence of NADPH (blank). Glutathione peroxidase activity was determined according to Navari-Izzo et al. [30] by coupling its reaction with that of GR, using 0.45 mM H_2O_2 as substrate. Enzymatic extract contained 100 mg protein mL^{-1}. Specific activity was calculated from the 6.2 mM^{-1} cm^{-1} extinction coefficient. Catalase activity was determined as described by Aebi [31]. Enzymatic extract contained 100 mg protein mL^{-1}. A blank containing only the enzymatic solution was made. Specific activity was calculated from the 23.5 mM^{-1} cm^{-1} extinction coefficient. All enzymatic activities were determined at 25 °C and expressed as U

g^{-1} protein. Three replications for each enzyme extract were made. Protein measurement was performed according to Bradford [32], using BSA as standard.

References

[1] O. Leprince, G.A.F. Hendry, B.D. McKersie, The mechanism of desiccation tolerance in developing seeds, Seed Sci. Res. 3 (1993) 231-246.
[2] A.R. Kermode, Regulatory mechanisms in the transition from seed development to germination: interactions between the embryo and the seed environment. in: J. Kigel, G. Galili (Eds.), Seed Development and Germination. Marcel Dekker, New York, 1995, pp. 273-332.
[3] J.M. Hansen, Y.M. Go, D.P. Jones, Nuclear and mitochondrial compartmentation of oxidative stress and redox signalling, Annu. Rev. Pharmacol. Toxicol. 46
(2006) 215-234.
[4] A. Lenher, C. Bailly, B. Flechel, P. Poels, D. Côme, F. Corbineau, Changes in wheat seed germination ability, soluble carbohydrate and antioxidant enzymes activities in the embryo during the desiccation phase of maturation, J. Cereal Sci. 43 (2006) 175-182.
[5] L. De Gara, M.C. de Pinto, V.M.C. Moliterni, M.G. D'Egidio, Redox regulation and storage processes during maturation in kernels of *Triticum durum*, J. Exp. Bot. 54 (2003) 249-258
[6] C. Bailly, J. Leymarie, A. Lehner, S. Rousseau, D. Côme, F. Corbineau, Catalase activity and expression in developing sunflower seeds as related to drying, J. Exp. Bot. 55 (2004) 475-483.
[7] M. Garnczarska, W. Bednarski, M. Jancelewicz, Ability of lupine seeds to germinate and to tolerate desiccation as related to changes in free radical level and antioxidants in freshly harvested seeds, Plant. Physiol. Biochem. 47 (2009) 56-62.
[8] I. Grilli, P. Meletti, C. Spanò, Ribonucleases during ripening and after-ripening in *Triticum durum* embryos, J. Plant. Physiol. 159 (2002) 935-937.
[9] C. Spanò, R. Buselli, I. Grilli, Dormancy and germination in wheat embryos: ribonucleases and hormonal control, Biol. Plant. 52 (2008) 660-667.
[10] C. Bailly, C. Audigier, F. Ladonne, M.H. Wagner, F. Coste, F. Corbineau, D. Côme, Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality, J. Exp. Bot. 52 (2001) 701-708.
[11] T.J. Close, Dehydrins: a commonality in the response of plants to dehydration and low temperature, Physiol. Plant. 1544 (1997) 196-206.
[12] J.M. Farrant, C. Bailly, J. Leymarie, B. Hamman, D. Côme, F. Corbineau, Wheat seedlings as a model to understand desiccation tolerance and sensitivity, Physiol. Plant. 120 (2004) 563-574.
[13] C. Vertucci, J.M. Farrant, Acquisition and loss of desiccation tolerance. in: J. Kigel, G. Galili (Eds.), Seed Development and Germination. Marcel Dekker, New York, 1995, pp. 237-272.
[14] C.R. Allagulova, F.R. Gimalov, F.M. Shakirova, V.A. Vakhitow, The plant dehydrins: structure and putative functions, Biochemistry 68 (2003) 1157e1165.
[15] K. Goyal, L.J. Walton, A. Tunnacliffe, LEA proteins prevent protein aggregation due to water stress, Biochem. J. 388 (2005) 151-157.
[16] T.J. Close, Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins, Physiol. Plant. 97 (1996) 795-803.
[17] M. Hara, S. Tershima, T. Fukaya, T. Kuboi, Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco, Planta 217 (2003) 290-298.

[18] L. Jiang, A.R. Kermode, Role of desiccation in the termination of expression of genes for storage proteins, Seed Sci. Res. 4 (1994) 149-173.
[19] M. Black, F. Corbineau, H. Gee, D. Côme, Water content, raffinose and dehydrins in the induction of desiccation tolerance in immature wheat embryos, Plant. Physiol. 120 (1999) 463-471.
[20] J. Boudet, J. Buitink, F.A. Hoekstra, H. Rognaux, C. Larre, P. Satour, O. Leprince, Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance, Plant. Physiol. 140 (2006) 1418-1436.
[21] C. Bailly, Active oxygen species and antioxidants in seed biology, Seed Sci. Res. 14 (2004) 93-107.
[22] S. Pukacka, E. Ratajczak, Ascorbate and glutathione metabolism during development and desiccation of orthodox and recalcitrant seeds of the genus *Acer*, Funct. Plant Biol. 34 (2007) 601-613.
[23] D.N. Stokes, T. Galliard, J.L. Harwood, Changes in the lipid composition of developing wheat seeds, Phytochemistry 25 (1986) 811-815.
[24] L.I. Caers, W.J. Peumans, A.R. Carlier, Preformed and newly synthesized messenger RNA in germinating wheat embryos, Planta 144 (1979) 491-496.
[25] C. Spanò, C. Crosatti, R. Pacchini, P. Meletti, I. Grilli, Ribonucleases during cold acclimation in winter and spring wheats, Plant. Sci. 162 (2002) 809-815.
[26] A. Blank, T.A. McKeon, Single-strand-preferring nuclease activity in wheat leaves is increased in senescence and is photoregulated, Proc. Natl. Acad. Sci. USA 86 (1989) 3169-3173.
[27] S. Jana, M.A. Choudhuri, Glycolate metabolism of three submerged aquatic angiosperm during aging, Aquat. Bot. 12 (1982) 345-354.
[28] Y. Nakano, K. Asada, Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts, Plant. Cell. Physiol. 22 (1981) 867-880.
[29] M.V. Rao, A.H. Beverley, D.P. Ormrod, Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Role of antioxidant enzymes, Plant. Physiol. 109 (1995) 421-432.
[30] F. Navari-Izzo, S. Meneguzzo, B. Loggini, C. Vazzana, C.L.M. Sgherri, The role of the glutathione system during dehydration of *Boea hygroscopica*, Physiol. Plant. 99 (1997) 23-30.
[31] H. Aebi, Catalase in Vitro, Meth. Enzymol. 105 (1984) 121-125.
[32] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248-254.

Legends of figures

Fig. 1. Activity gels of acidic (A, 20 kDa in size), neutral salt-stimulated (B, 24 and 26 kDa in size), neutral salt-inhibited (C, 27 kDa in size) RNases in wheat embryos isolated from grains at different stages of ripening, as indicated in Table 1. The approximate molecular weights, estimated by comparison with protein standards run in the same gel, are indicated on the right.

Fig. 2. Changes in dehydrin-related proteins in wheat embryos isolated from grains at different stages of ripening, as indicated in Table 1. 10 mg of protein per lane were loaded on the gel. Dehydrins were detected by western blotting. Independent protein extractions and immunoblots were performed in triplicate. The approximate molecular weights, estimated by comparison with protein standards run in the same gel, are indicated on the right.

Fig. 3. Concentration of hydrogen peroxide in wheat embryos isolated from grains at different stages of ripening, as indicated in Table 1. Data points represent means ± SE of three replicates. Different letters shown above the bars show means that are significantly different at $P < 0.05$.

Fig. 4. Activities of ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) in wheat embryos isolated from grains at different stages of ripening, as indicated in Table 1. Activities are expressed in U g^{-1} protein. Data points represent means ± SE of three replicates. Different letters shown above the bars show means that are significantly different at $P < 0.05$. Similar results were obtained in at least three independent experiments.

Fig. 5. Activities of glutathione peroxidase (GPX) and catalase (CAT) in wheat embryos isolated from grains at different stages of ripening, as indicated in Table 1. Activities are expressed in U g^{-1} protein. Data points represent means ± SE of three replicates. Different letters shown above the bars show means that are significantly different at $P < 0.05$. Similar results were obtained in at least three independent experiments.

Table 1
Water content and germination after 5 days of grains of *T. durum* Desf. naturally-and artificially-dried. Measurements were carried out in triplicate. Means and standard errors are indicated. Values followed by the same letter were not significantly different ($P < 0.05$).

Material		Water FW^{-1} %	Germination (5 days) %
1	Milk-ripe	56 ± 2 a	0 d
2	Dough-ripe	48 ± 5 ab	0 d
3	Wax-ripe	26 ± 4 c	21 ± 2 b
4	Fully-ripe	12 ± 1 d	40 ± 2 a
1*	Milk-ripe +2**	42 ± 3 b	0 d
2*	Dough-ripe +2**	33 ± 4 c	4 c
3*	Wax-ripe +1**	12 ± 1 d	19 ± 1 b

*Desiccated material.
**Days of desiccation treatment.

Fig. 1

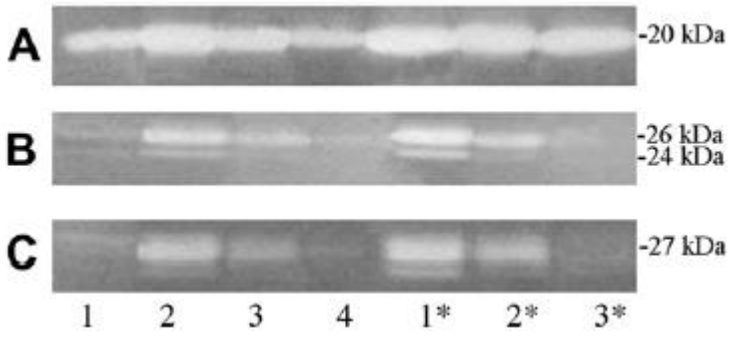

Fig. 2

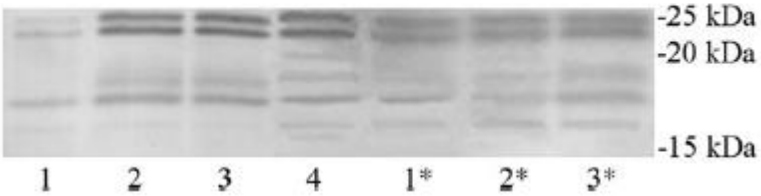

Fig. 3

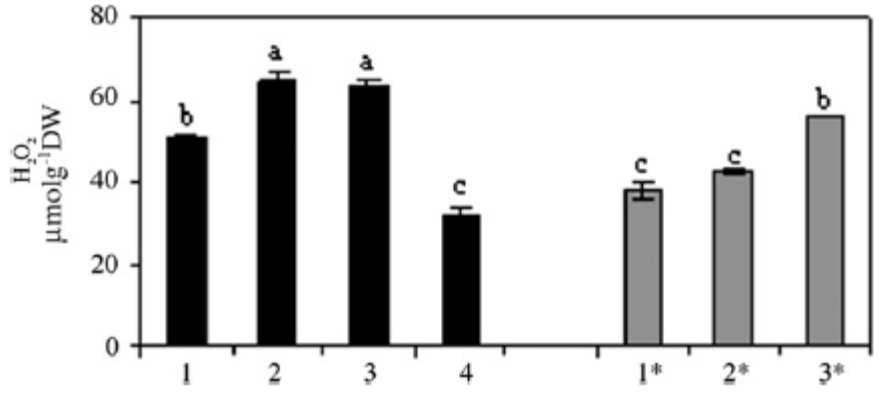

Fig. 4

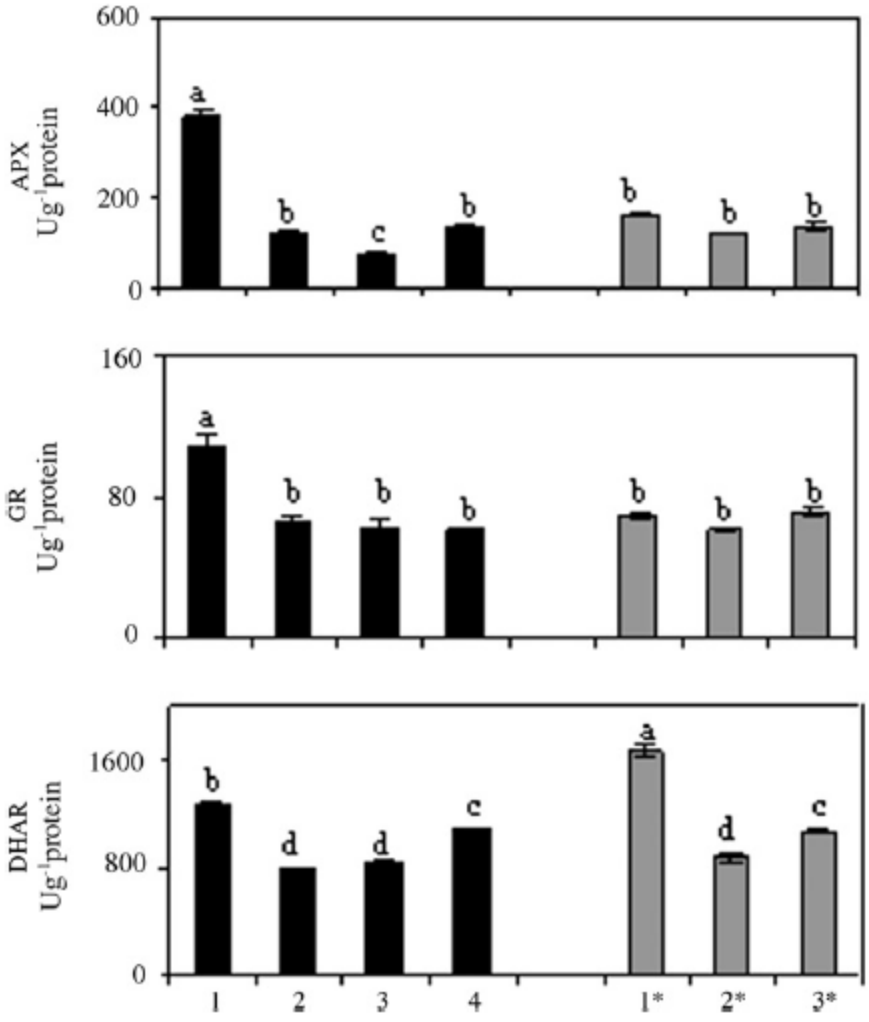
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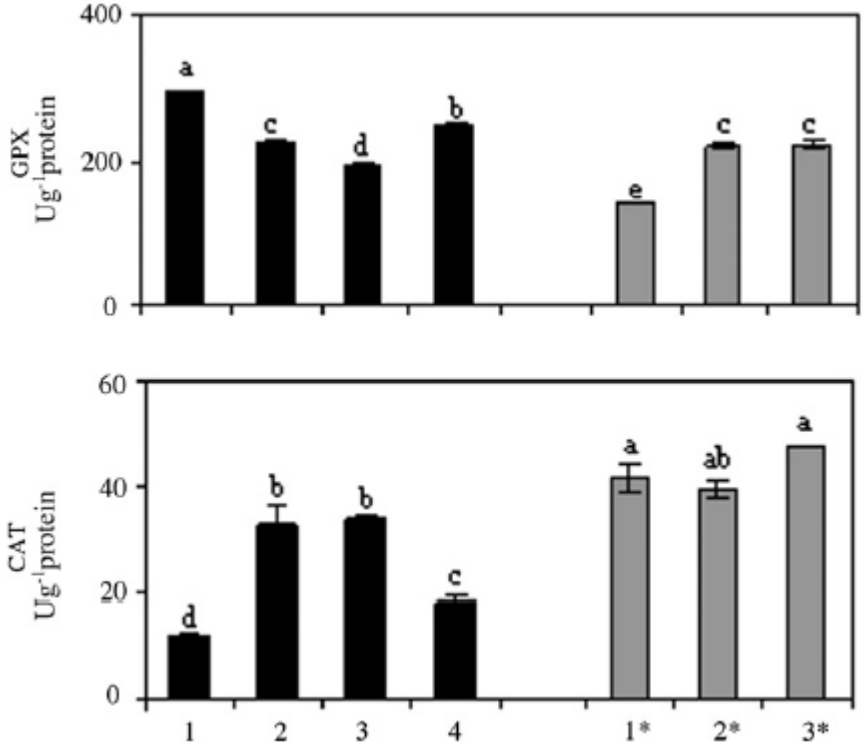
Fig. 5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CAT
Ug-1protein
60
40
20
0
d
b
b
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a
ab
a
1
2
3
4
1*
2*
3*