Antioxidant systems in sunflower (*Helianthus annuus L.*) seeds as affected by priming

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Abstract

Priming treatment of sunflower (*Helianthus annuus* L., cv Briosol) seeds for 7 days at 15°C with a polyethylene glycol solution at −2.0 MPa strongly improved their subsequent germination at 15°C on water. This stimulatory effect of priming remained after drying back the seeds at 20°C for 3 days. Malondialdehyde (MDA) and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were measured in control unprimed seeds, primed seeds, seeds primed then dried, and after 3 and 6 hours of imbibition of control and dried primed seeds in order to determine whether the cell antioxidant systems were involved in the beneficial effect of priming. The osmotreatment resulted in a strong increase in SOD and CAT activities but did not markedly affect MDA and GR activity. Following the 3 days of drying, MDA increased and the enzyme activities became similar to those measured in dry unprimed seeds, although the stimulatory effect of priming on germination remained. Imbibition of control dry seeds was associated with an increase in MDA and a decrease in CAT and GR activities, whereas reimbibition of dried primed seeds resulted in a decrease in MDA and an increase in SOD, CAT and GR activities. Isoform patterns on native gels showed no difference between treated (priming with or without subsequent drying) and control seeds for SOD (7 isoforms) and GR (5 isoforms), but the osmotreatment did induce a second isoform of CAT. The results obtained indicate that the CAT isoform pattern might be used as an indicator of the priming treatment that promotes germination. Involvement of the antioxidant systems in seed vigour is discussed.

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Abbreviations: AOS = active oxygen species; CAT = catalase; GR = glutathione reductase; MDA = malondialdehyde; MTG = mean time to germination; PEG = polyethylene glycol; SOD = superoxide dismutase

Keywords: catalase, germination, glutathione reductase, *Helianthus annuus*, lipid peroxidation, osmopriming, sunflower seed, superoxide dismutase

Introduction

Osmopriming consists of the incubation of seeds in an osmoticum, usually a salt or polyethylene glycol (PEG) solution, in order to control imbibition and prevent radicle protrusion. It is a well known technique used to improve subsequent germination in numerous species, especially under suboptimal conditions (Bradford, 1986; Karssen *et al.*, 1989). In practice, priming is followed by dehydration of seeds to their initial moisture content while retaining the benefit of the treatment. Germination of primed seeds is less sensitive to temperature and oxygen deprivation than that of unprimed seeds (Brocklehurst and Dearman, 1983; Bradford, 1986; Corbineau *et al.*, 1994; Özbingöl *et al.*, 1998). In the case of sunflower, for example, osmopriming with a PEG solution at −2 MPa for 3–5 days at 15°C strongly increased subsequent germination at suboptimal temperatures (5 to 15°C) and in hypoxia (1 to 3% oxygen) (Smok *et al.*, 1993; Chojnowski *et al.*, 1997).

Metabolic events, such as protein, RNA and DNA syntheses (Bray, 1995), DNA replication (Lanteri *et al.*, 1994) or β-tubulin accumulation (De Castro *et al.*, 1995), are associated with priming. In sunflower seeds, both respiratory activity and ability to convert 1-aminocyclopropane 1-carboxylic acid (ACC) to ethylene increased after priming (Chojnowski *et al.*, 1997). It would be of a considerable practical value to have biochemical or molecular markers for evaluating the efficiency of priming. With pepper and tomato seeds, a positive correlation was found between the DNA replication, measured as the increase in nuclei arrested in the G2 phase of the mitotic cell division cycle, and the beneficial effect of the osmotic treatment (Lanteri *et al.*, 1994; Özbingöl, 1998). Job *et al.* (1997) also demonstrated that an 11 S globulin

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β-chain was a good marker of sugarbeet seed priming.

Priming can improve the germination of aged seeds (Fujikura and Karssen, 1992; Van Pijlen *et al.*, 1995; Bailly *et al.*, 1998). In sunflower seeds, recovery of germinability of aged seeds by priming was associated with a lower rate of lipid peroxidation (Bailly *et al.*, 1998). This apparently was caused by the restoration of antioxidant mechanisms, in particular of the activities of catalase (CAT) and glutathione reductase (GR) (Bailly *et al.*, 1998). These enzymes control, in part, the rate of lipid peroxidation by scavenging H_2O_2 and by producing the antioxidant glutathione, respectively. These data suggested that the rate of germination of aged sunflower seeds was related to scavenging of toxic active oxygen species (AOS) and lipid peroxidation.

The aims of the present work were to determine (i) whether the beneficial effect of priming on the germination of non-aged sunflower seeds can also be related to increased scavenging of AOS, and (ii) whether activities of enzymes related to these processes, such as CAT, superoxide dismutase (SOD) and GR, can be used as markers of the treatment. After specifying the effects of osmopriming on germination, the rate of lipid peroxidation was determined by measuring malondialdehyde (MDA) levels, and the activities of the main AOS detoxifying enzymes and expression of their isoforms were measured.

Materials and methods

Plant material

Sunflower (*Helianthus annuus* L., cv Briosol) seeds harvested in 1995 were obtained from Cargill (Peyrehorade, South-West of France). They were stored at 20°C (70 % RH) immediately after harvest and were non-dormant when used in the experiments (Corbineau *et al.*, 1990). Whole seeds (achenes) were used for germination experiments and priming treatments, but all biochemical measurements were made with naked seeds (i.e. true seeds without pericarp).

Germination experiments

Germination assays were performed in darkness at 15°C in 9-cm Petri dishes (25 seeds per dish, 8 replicates) on a layer of cotton wool moistened with distilled water. A temperature of 15°C (a suboptimal temperature for the germination of sunflower seeds) was chosen instead of 25°C (the thermal optimum) because it allows a better expression of the effects of various treatments on germination (Corbineau *et al.*, 1990; Chojnowski *et al.*, 1997). A seed was considered as germinated when the radicle protruded the envelopes (seed coat + pericarp). Germination counts were made daily for 5 days. The results presented are the means of the germination percentages obtained in 8 replicates ± standard deviation (SD). Germination rate is expressed as the mean time to germination (MTG, in days) which was calculated as follows:

$$MTG = [n_1 \times 1 + (n_2 - n_1) \times 2 + (n_3 - n_2) \times 3 + (n_4 - n_3) \times 4 + (n_5 - n_4) \times 5] / n_5$$

where n_1, n_2, n_3, n_4 and n_5 are the mean percentages of germinated seeds obtained at days 1, 2, 3, 4 and 5 in 8 replicates.

Priming treatment

Priming was carried out for 7 days at 15°C by incubating seeds on cotton wool moistened with a solution of PEG 8000 (Sigma) at −2.0 MPa, which has been shown to be the best conditions for osmopriming of sunflower seeds (Smok *et al.*, 1993). Primed seeds were subsequently rinsed with distilled water and then dried at room temperature for 3 days on filter paper. After priming, seed moisture content (dry weight basis) was around 31.5% and decreased to 28, 16 and 10 % after 1, 4 and 8 h of drying, respectively. The moisture content of primed seeds redried for 3 days was similar to that measured in untreated seeds (5.7 %, dry weight basis). Dry weight was obtained by oven drying the seeds at 105°C for 3 days.

In some experiments, dried unprimed seeds and redried primed seeds were imbibed at 15°C on distilled water (as in germination assays) for 3 and 6 h. These durations of imbibition were not sufficient to allow radicle protrusion.

Evaluation of lipid peroxidation

Lipid peroxidation was evaluated by spectrophotometric measurements of malondialdehyde (MDA) levels, according to Heath and Parker (1968) as described elsewhere (Bailly *et al.*, 1996). This method has previously been shown to be an accurate one for evaluation of lipid peroxidation in sunflower seeds (Bailly *et al.*, 1998).

Enzyme extraction and assays

Superoxide dismutase (SOD, EC 1.15.11), catalase (CAT, EC 1.11.1.6) and glutathione reductase (GR, EC 1.6.4.2) were extracted and assayed as previously described (Bailly *et al.*, 1996). The SOD, CAT and GR activities of each extract were measured 3 times, and the results presented correspond to the means of the values obtained with 3 different extracts ± SD. Values were calculated per mg of protein (specific activity)

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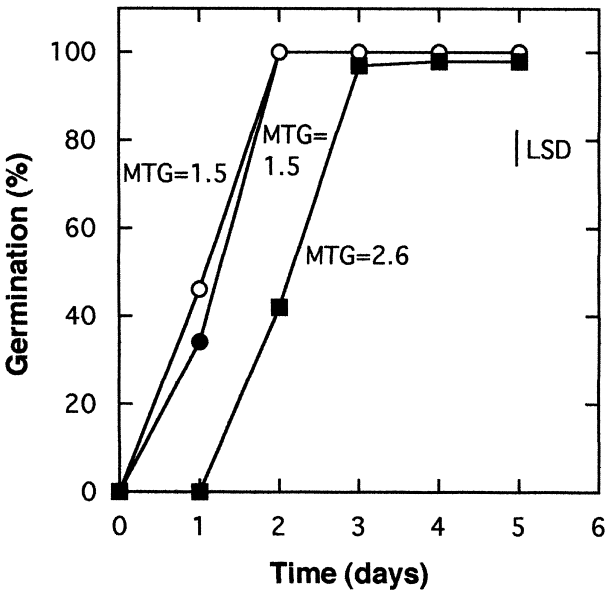

Figure 1. Effects of 7 days of osmopriming (−2.0 MPa) on sunflower seed germination. Time course of germination at 15°C of control unprimed seeds (■), non-redried primed seeds (○) and redried primed seeds (●). Vertical bar denotes the largest SD (LSD). The values indicated on the curves correspond to the mean time to germination (MTG) for each assay. Results are means of 8 measurements.

and are expressed as % of those obtained with dry, unprimed seed controls. Protein content of the extracts was determined according to the method of Bradford (1976) using the BioRad protein assay kit with bovine serum albumin as a standard.

Gel electrophoresis and enzyme activity staining

All gels (precast minigels, 5 × 4 cm) were run on a PhastSystem (Pharmacia).

SOD isozymes were separated by isoelectric focusing on polyacrylamide gels (PhastGel IEF 4–6.5, Pharmacia), and SOD activity was detected according to Beauchamp and Fridovich (1971) by incubating gels under light in a solution of 40 mM Tris-HCl (pH 8.0) containing 5 mM $MgCl_2$, 0.65 mM phenazine methosulfate, 0.36 mM nitroblue tetrazolium and 0.4 mM β-NADP.

CAT isoforms were separated on native 7.5% polyacrylamide gels (PhastGel homogenous 7.5, Pharmacia). Activity staining was carried out by immersing the gels in 0.003% H_2O_2 (v/v), then in a mixture of 1.5% $FeCl_3$ (w/v) and 1.5% potassium ferricyanide (w/v) as described by Woodbury *et al.* (1971).

GR isozymes were electrophoresed on native 12.5% polyacrylamide gels (PhastGel homogenous 12.5, Pharmacia). Activity staining was adapted from Anderson *et al.* (1990) and was carried out in 0.4 mM NADPH, 3.4 mM oxidized glutathione, 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.3 mM 2,6-dichlorophenol indophenol and 50 mM Tris-HCl (pH 7.75).

SOD, CAT and GR isozyme activities were analysed by determining photon density of the gels with a LKB Ultroscan XL laser densitometer using a Gel Scan XL software program.

Results

Effects of priming on seed germination

Untreated sunflower seeds fully germinated after 3 days at 15°C (Fig. 1). Osmopriming in PEG markedly shortened the subsequent lag-time of germination on water, irrespective of redrying the seeds. Mean time for germination of primed seeds was 1.5 day without redrying or after redrying, i.e. about 60% of that of unprimed seeds (2.6 days).

Effects of priming on MDA content and on SOD, CAT and GR activities

Figure 2 shows the effects of 7 days of osmopriming, followed or not by drying, on lipid peroxidation

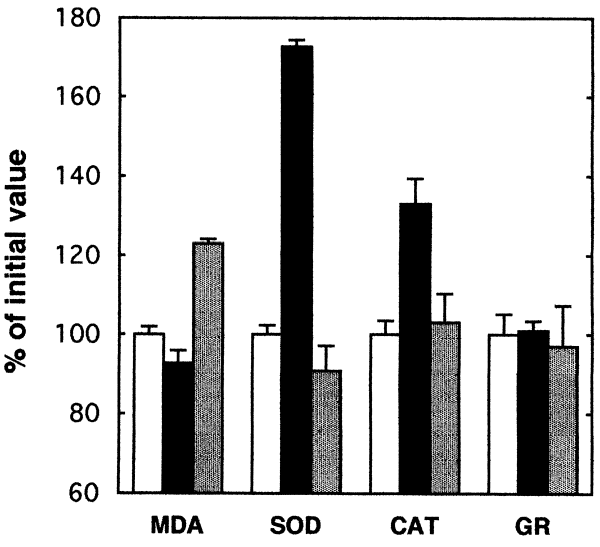

Figure 2. Effects of 7 days of osmopriming (−2.0 MPa) on level of MDA and activities of SOD, CAT and GR. White bars, control unprimed dry seeds; black bars, non-redried primed seeds; grey bars, redried primed seeds. Results are percentages of values obtained with control unprimed dry seeds and are means of 9 measurements ± SD.

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Table 1. Water content of control unprimed seeds and redried primed seeds before imbibition and after 3 and 6 h of imbibition at 15°C. Means of 4 replicates ± SD.

Duration of imbibition (h)	Water content (% dry weight)	
	control unprimed seeds	redried primed seeds
0	5.7 ± 0.1	5.6 ± 0.1
3	33.5 ± 0.1	32.7 ± 0.5
6	40.6 ± 0.3	40.1 ± 0.2

(estimated by malondialdehyde content) and on the activities of SOD, CAT and GR. MDA content decreased slightly after osmopriming but increased markedly after the subsequent drying of primed seeds. SOD activity was strongly enhanced by 7 days of priming, whereas subsequent drying led to an activity slightly lower than that found in control unprimed seeds. Osmopriming increased CAT activity by approx. 30%, but drying back the seeds restored the initial activity. No significant differences in GR activity were observed after priming or priming and drying the seeds.

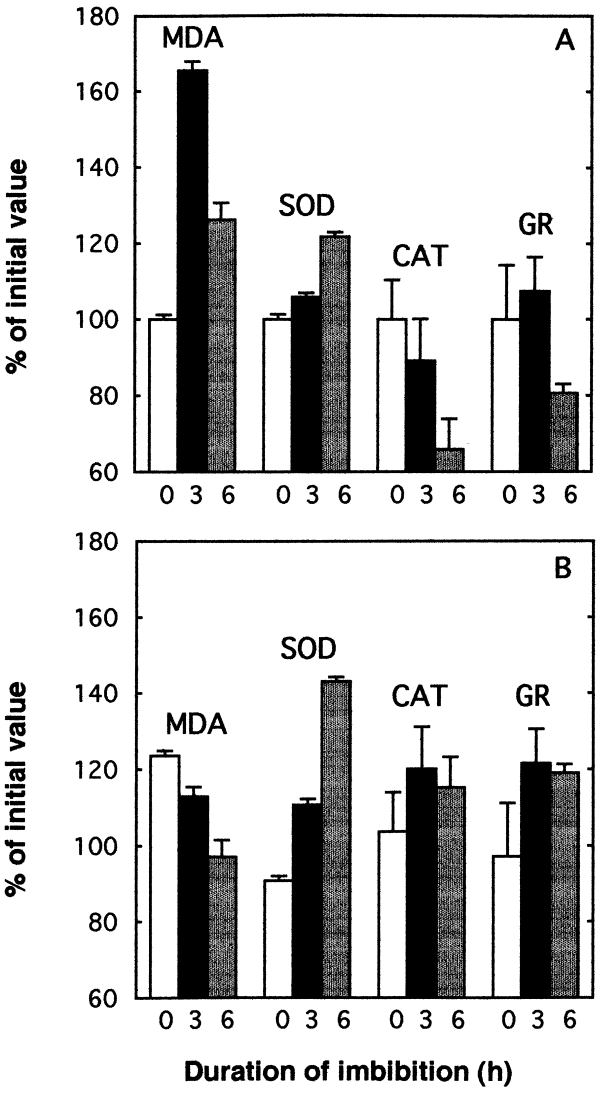

Figure 3. Effects of the duration of imbibition at 15°C of control unprimed seeds (A) and redried primed seeds (B) on level of MDA and activities of SOD, CAT and GR. Results are expressed as percentages of initial values (i.e. values obtained with dry unprimed seeds) and are means of 9 measurements ± SD.

Changes in MDA content and in SOD, CAT and GR activities during subsequent imbibition on water

To more precisely study the effects of priming on lipid peroxidation and activities of the main antioxidant enzymes during the first stage of germination, control unprimed seeds and redried primed seeds were placed on water at 15°C for 3 and 6 h before measuring MDA content and SOD, CAT and GR activities.

Table 1 shows that the water uptake was similar in both types of seeds. After 6 h of imbibition, moisture contents reached approx. 40% (dry weight basis).

In control seeds, MDA levels increased sharply during the first 3 h of imbibition, then decreased after 6 h but remained higher than in dry seeds (Fig. 3A). In redried primed seeds, MDA levels slightly decreased during the 6 first hours of imbibition and reached a value similar to that measured in dry unprimed seeds (Fig. 3B).

SOD activity increased during imbibition of control unprimed seeds (Fig. 3A) and much more in primed seeds (Fig. 3B). Imbibition induced a continuous decrease in CAT activity in control seeds (Fig. 3A), whereas it did not significantly change this activity in primed seeds (Fig. 3B). GR activity decreased markedly after 6 hours of imbibition in control seeds (Fig. 3A), but it was maintained at a high level in primed seeds (Fig. 3B).

Effects of priming on isoform profiles of the enzymes

The isoform profiles of the three enzymes were the same for undried primed seeds and primed seeds subsequently redried (data not shown). Sunflower seeds contained 7 isoforms of SOD (Fig. 4), the ones between pI 5.2 and 6.5 being the most abundant. No

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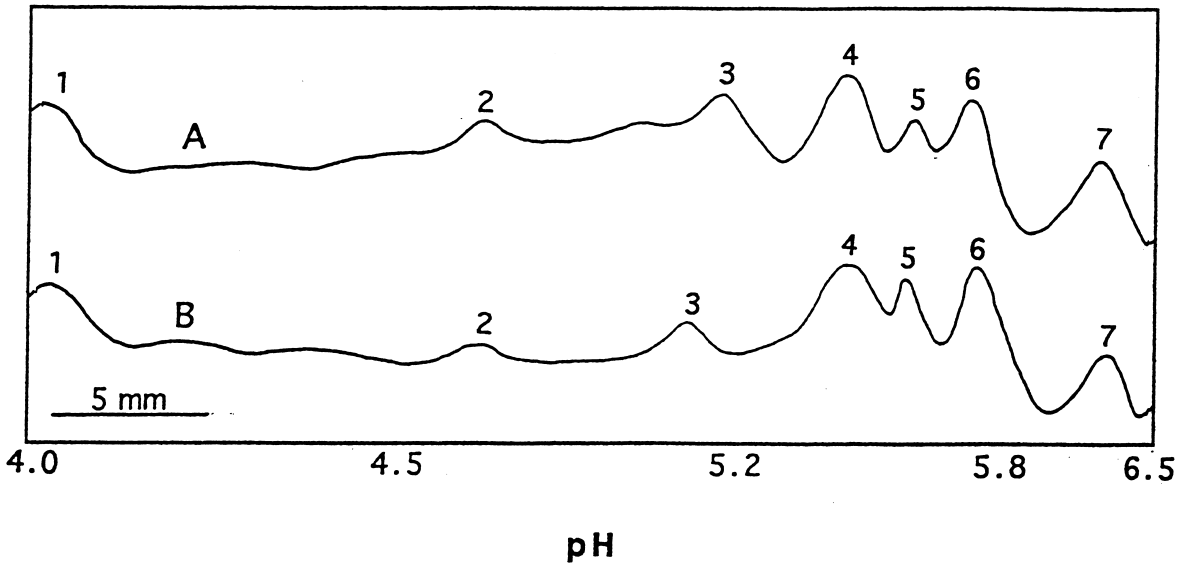

Figure 4. Densitometer readings of isoelectric focusing gels showing SOD isoforms in control unprimed seeds (A) and in primed seeds (B). Peaks are numbered according to increasing pI.

change in the isoform pattern was detected after 7 days of osmopriming (Fig. 4). Figure 5 displays the isoform profiles of CAT. Control unprimed seeds contained a single CAT isoform. Osmopriming led to the appearance of a clearly visible additional band of a lower molecular weight, whereas the previously observed band emerged as a wider peak. Protein extracts obtained from control unprimed seeds or osmoprimed seeds displayed the same five distinct isoforms of GR on native gels (Fig. 6).

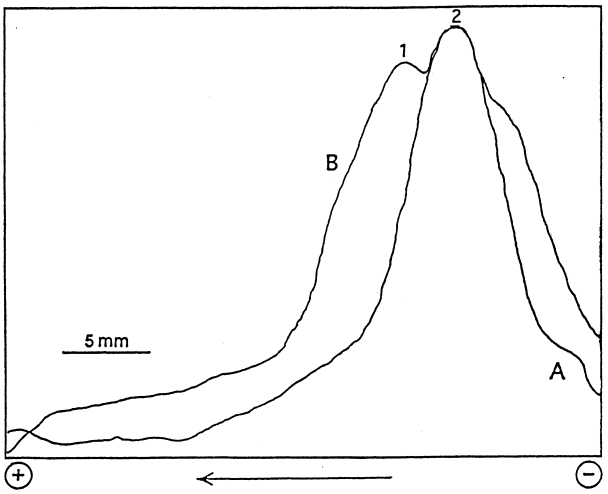

Figure 5. Densitometer readings of native gels showing CAT isoforms in control unprimed seeds (A) and in primed seeds (B). Peaks are numbered according to increasing molecular weight.

Discussion and conclusion

As previously shown by Smok *et al.* (1993) and Chojnowski *et al.* (1997), priming of sunflower seeds markedly increased their germination rate at 15°C by shortening the lag-time (Fig. 1). In this work, we were interested in finding a parameter to evaluate whether or not seeds had been primed. The results indicate that such a parameter may be sought in enzymatic AOS scavenging.

Lipid peroxidation was evaluated by determination of seed malondialdehyde content. It only slightly decreased after 7 days of osmopriming (Fig. 2), which suggests that seed water uptake during priming is probably not associated with lipid peroxidation. Redried primed seeds had a significantly higher level of MDA than control dry seeds (Fig. 2). It seems, therefore, that loss of water during dehydration results in deleterious oxidative processes and lipid peroxidation. Such a phenomenon has been observed in plants subjected to drought (Price and Hendry, 1989; Smirnoff, 1993; Inzé and Van Montagu, 1995) or during dehydration of radicles of germinating maize seeds (Leprince *et al.*, 1990), and seeds and pollen (Priestley *et al.*, 1985). The activities of SOD and CAT were strongly increased after priming (Fig. 2), thus enhancing the antioxidant defence of the cells. This can be related to the increased metabolic activity occuring during priming, which may cause AOS production as secondary products of respiration by mitochondria or lipid degradation by glyoxysomes. Although chilling is known to generate an antioxidant response in

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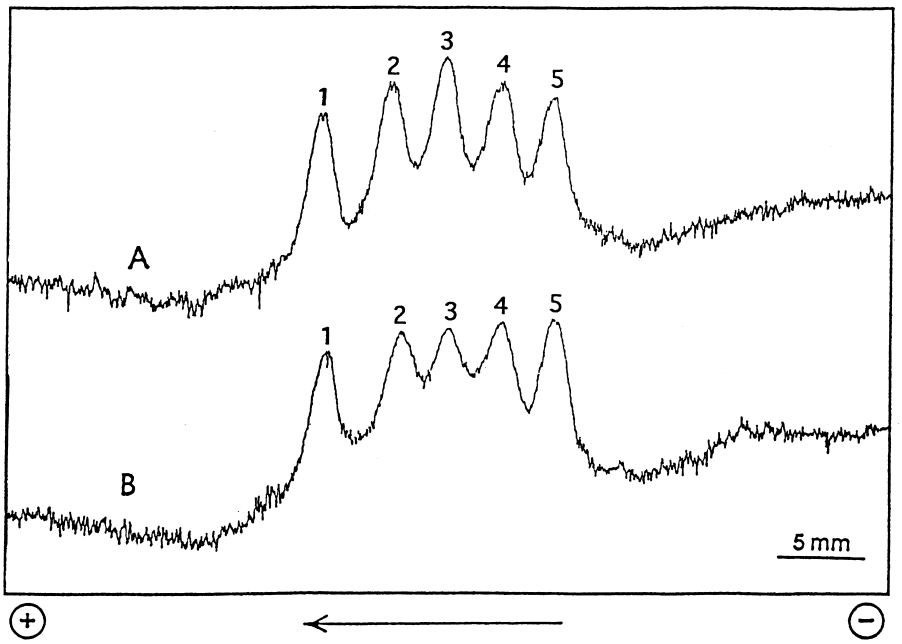

Figure 6. Densitometer readings of native gels showing GR isoforms in control unprimed seeds (A) and in primed seeds (B). Peaks are numbered according to increasing molecular weight.

numerous plants (Bowler *et al.*, 1992; Walker and McKersie, 1993; Hodges *et al.*, 1997), increased SOD and CAT activities during priming at 15°C are probably not due to a combination of osmopriming and cold stress, since this temperature does not result in chilling injury in sunflower seeds or seedlings (Gay *et al.*, 1991). Scavenging of superoxide radicals and H_2O_2 through SOD and CAT activities (Bowler *et al.*, 1992) may also explain why there was no increase in lipid peroxidation in imbibed primed seeds. Results obtained with redried primed seeds, which displayed a high MDA content, are not contradictory to this hypothesis since dehydration decreased both SOD and CAT activities (Fig. 2).

Although the activities of SOD, CAT and GR were similar in dry unprimed seeds and redried primed seeds (Fig. 2) and the water uptake was also similar in both types of seeds (Table 1), priming markedly improved the AOS scavenging during the first hours of imbibition, as MDA content decreased in primed seeds even when it increased in control untreated ones (Fig. 3). This lower rate of lipid peroxidation may be due to a higher efficiency of the antioxidative system in primed seeds. Indeed, activities of SOD, CAT and GR were higher during imbibition in primed seeds than in control ones (Fig. 3), which could, therefore, prevent deleterious oxidative processes such as lipid peroxidation. CAT and GR activities decreased during the first 6 hours of imbibition of untreated seeds (Fig. 3A), while they slightly increased in primed seeds (Fig. 3B). The shorter lag-time of germination of redried primed seeds may therefore be related to higher AOS scavenging during the first stages of imbibition. This phenomenon may result from mRNA synthesis during priming, as for other proteins (Bray, 1995), and/or synthesis of AOS scavenging enzymes during subsequent imbibition.

Seven isozymes of SOD were observed by isoelectric focusing separation (Fig. 4), which is consistent with the results obtained in vegetative tissues of other plant species (Giannopolitis and Ries, 1977; Asins *et al.*, 1995; Acevedo and Scandalios, 1996). As osmopriming did not lead to the synthesis of new isoforms (Fig. 4), the increase in total SOD activity in hydrated primed seeds must be due to a synthesis or an increase in activity of the preexisting isozymes.

CAT activity was enhanced by a 7-day osmopriming treatment whereas subsequent drying restored the initial activity (Fig. 2). CAT has been demonstrated to play a key role in germination of aged sunflower seeds, and its activity has been closely correlated with the recovery of germination by priming (Bailly *et al.*, 1998). Only one CAT isoform was observed in control unprimed seeds, but priming induced the appearance of an additional isozyme (Fig. 5). The synthesis of this second CAT isoform may be related to a particular role of CAT which is to remove H_2O_2 produced by β-oxidation of fatty acids

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during germination (Ni and Trelease, 1991; Willekens *et al.*, 1995) and then probably also during priming. In dicotyledonous oil-rich seeds such as sunflower, β-oxidation takes place in glyoxysomes during mobilization of oil bodies during germination (Tchang *et al.*, 1980; Huang *et al.*, 1983). However, glyoxysomes have been also identified in cotyledons of dry seeds (Bewley and Black, 1994), and they have been shown to proliferate concomitantly with a decrease in the number of storage lipid bodies in carrot embryos during priming (Dawidowicz-Grzegorzewska, 1997). Besides these ultrastructural observations, increased activities of glyoxysomal enzymes, such as isocitrate lyase, during priming (Fu *et al.*, 1988; Smith and Cobb, 1991) suggest that β-oxidation occurs during priming. Protection against environmental stresses and involvement in glyoxysome metabolism are carried out by distinct CAT isoforms belonging, respectively, to what has been called class II and class III (Willekens *et al.*, 1995). The presence of both classes might be a characteristic of primed oil seeds. However, our results do not allow us to identify which of the two isoforms observed in Fig. 5 correspond to these two classes.

Previous experiments have demonstrated changes in GR activity during seed ageing and during seed reinvigoration of aged seeds by priming (De Paula *et al.*, 1996; Bailly *et al.*, 1998). However, no changes in global GR activity (Fig. 2) nor variation of the isoform pattern (Fig. 6) have been observed during priming of unaged seeds and their subsequent drying.

Our results indicate that osmopriming in sunflower seeds is associated with higher antioxidant enzyme activities which are maintained during subsequent imbibition of dried primed seeds. The data support our previous results which suggest that sunflower seed germinability and vigour are related to AOS scavenging (Bailly *et al.*, 1998). Moreover, the present experiments indicate that the CAT isoform pattern may be a good marker of priming.

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