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Effect of UV-B radiation on the activity and isoforms of enzymes with peroxidase activity in sunflower cotyledons

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Abstract

Ultraviolet-B (UV-B) radiation produces oxidative stress by increasing active oxygen species (AOS) such as singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radicals. Recent studies confirm that hydrogen peroxide is a signaling molecule that mediates responses to abiotic and biotic stresses in plants. Peroxidases are a variety of enzymes that catalyze the breakdown of H_2O_2 with the concomitant dependent oxidation of a wide variety of substrates. The behavior of activities and isoforms of catalase (CAT), ascorbate peroxidase (APX) and peroxidases (POD) was investigated in the cotyledons of *Helianthus annuus* L. subjected to UV-B radiation. Under UV-B treatments (15 and 30 kJ m^{-2}) and later recovery in darkness or in white light, APX activity remains unaltered. Compared to control, CAT and guaiacol peroxidase (GPX) activities were increased at the two UV-B doses and after dark recuperation, but both enzymes returned to controls values when plants were treated with 30 kJ m^{-2} UV-B radiation and recovered under white light. Control sunflower cotyledons had one CAT and four APX isoforms which were altered by UV-B irradiation and recuperation treatments. In control cotyledons were observed two isoforms of POD (POD1 and POD2). Only POD2 activity was increased by 15 kJ m^{-2} and decreased by 30 kJ m^{-2} UV-B radiation, but both the POD activities increased when plants were recovered in darkness or in white light. We observed a new POD isoform (POD3) after dark recuperation when plants were treated with 30 kJ m^{-2} UV-B dose. Chromatography in a Mono Q column showed three α-naphthol activity peaks corresponding to the three isoforms observed in stained gels, but only POD3 had activity when guaiacol was used as substrate. On the other hand, treatments with different hydrogen peroxide concentrations increased the activity of the two POD isoforms (POD1 and POD2) observed in control cotyledons, but POD3 was absent. Sunflower plants acclimatize themselves to UV-B radiation by induction of different isoforms of POD. Catalase, APX, POD1 and POD2 work directly as oxygen species scavenger and POD3 could play a role in polyphenols metabolism, increasing the antioxidant capacity or cross-linking UV-absorbing phenolics.

Keywords: Ascorbate peroxidase; Catalase; *Helianthus annuus* L.; Hydrogen peroxide; Peroxidase; UV-B radiation

1. Introduction

Plants are affected by different kind of stresses which are often species or location specific. They include drought, high salinity, extremes temperatures, water logging, mineral nutrient deficiency, metal toxicity, pollutants and ultraviolet-B (UV-B) radiation (Smirnoff, 1998).

Depletion of the stratospheric ozone layer is leading to an increase in solar UV-B radiation (280–320 nm) reaching the earth's surface (Smirnoff, 1998). UV-B damages living organisms because their cellular components such as proteins and nucleic acids absorb this energy-rich radiation. Many studies have shown deleterious UV-B effects on plants including reduced photosynthesis, biomass reduction, decreased protein synthesis, impaired chloroplast function, damage to DNA, etc. which are extensively reviewed by Jordan (1996). UV-B radiation also produces oxidative stress (Rao et al., 1996; Dai et al., 1997; A.-H.-Mackerness et al., 2001; Costa et al., 2002), increasing active oxygen species (AOS) generation such as singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radicals (A.-H.-Mackerness et al., 1998). Besides, it has been demonstrated that white light ameliorates UV-B-induced responses, including gene expression.

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These effects were the consequence of photosynthetic electron transport and photophosphorylation (Jordan, 2002 and references therein).

Recent studies confirm that hydrogen peroxide is a signaling molecule that mediates responses to abiotic and biotic stresses in plants (Levine et al., 1994; Vanlerberghe and McIntosh, 1996; Van Breusegem et al., 2001; Neill et al., 2002). High AOS levels initiate signaling responses that include enzyme activation, gene expression, programmed cell death and cellular damage (A.-H.-Mackerness et al., 2001). In plant cells, hydrogen peroxide can be generated by specific enzymes such as superoxide dismutase (SOD), NADPH oxidase and peroxidases (POD) (Neill et al., 2002) and under most conditions, cellular antioxidant systems efficiently remove H_2O_2. Thus, the oxidative effects of various stimuli could be mediated via reductions in the activities of antioxidant enzymes, rather than by increased H_2O_2 generation.

An efficient antioxidant defense system is present in plants to counteract oxidative stress. It is composed by enzymatic and non-enzymatic mechanisms. The former includes SOD, catalase (CAT), POD, ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDAR) and glutathione reductase (GR). The non-enzymatic mechanisms consist of antioxidants such as ascorbate, glutathione, α-tocopherol and carotenoids (Ahmad, 1995).

CAT, APX and a variety of general peroxidases catalyze the breakdown of H_2O_2. In plants, CAT (EC 1.11.1.6) is one of the main H_2O_2-scavenging enzymes that dismutates H_2O_2 into water and O_2, and it is a constitutive component of peroxisomes (Corpas et al., 1999). APX (EC 1.11.1.11) is a specific peroxidase that catalyzes the elimination of the toxic product H_2O_2 at the expense of oxidizing ascorbate to monodehydroascorbate. APX isozymes are distributed in at least four cells compartments: the stroma, the thylakoid membrane, the microbody and the cytosol (Asada, 1992). Peroxidases (EC 1.11.1.7) are enzymes that catalyze the H_2O_2-dependent oxidation of a wide variety of substrates, mainly phenolics (Dunford, 1986), and they are often found in multiple molecular forms. Peroxidases are heme proteins involved in numerous physiological roles in plant tissues, including lignin biosynthesis, indole-3-acetic acid degradation, wound healing and pathogen defense (Sato et al., 1993; Bestwick et al., 1998; Bernards et al., 1999; Kawano, 2003).

There is much evidence showing that the amounts and activities of enzymes involved in AOS scavenging are altered by environmental stresses such as chilling, drought and high salinity (Smirnoff, 1998). In a previous study carried out in our laboratory, we demonstrated the deleterious effects of UV-B radiation on the antioxidant defense system in sunflower (Costa et al., 2002).

In the present work, we investigated the behavior of enzymes that catabolize hydrogen peroxide. CAT, APX and POD activities and isoforms were analyzed in sunflower cotyledons subjected to UV-B radiation after dark and white light recuperation.

2. Material and methods

2.1. Plant material and treatments

Sunflower (*Helianthus annuus* L., cv. Paraiso 5, supplied by Nidera Inc.) seeds were surface sterilized with NaClO (8% active Cl_2) at 40% for 10 min and then thoroughly rinsed with distilled water. They were germinated in plastic pots filled with vermiculite and irrigated with Hoagland's nutrient solution (Hoagland and Arnon, 1957) for 5 days. Plants were grown at 25/20 °C with a 16 h photoperiod under fluorescent white light (175 μmol m^{-2} s^{-1}) in a controlled environmental growth chamber. Five days after germination, cotyledons were subjected only to UV-B radiation using a UV-B lamp (UVM-57 chromato-Vue, UVP, San Gabriel, CA, USA) (290–320 nm) at an irradiance of 5.2 W m^{-2} ultraviolet light at plant level. UV-B was filtered through 0.13 mm thick cellulose acetate filter (to avoid transmission below 290 nm) for UV-B treatments or through 0.13 mm thick Mylar Type S filter (absorbing radiation below 320 nm) for control treatments. The two doses used were adjusted by exposure of plants during 50 and 100 min to the illumination source at time zero. Using the generalized plant response function and normalizing the spectral irradiance at 300 nm, according to Caldwell (1971), the biologically effective UV-B doses employed were 15 and 30 kJ m^{-2}. After irradiation, some plants were left to recover during 20 h in the dark or in white light in order to evaluate the development of effects under these conditions. UV-B radiation doses employed in this study are similar to those recorded in a wide range of South America areas. For hydrogen peroxide treatment, plants were irrigated during 20 h in the dark with nutrient solution devoid of H_2O_2 (control) or containing 0.1, 1, 10 and 100 μM of H_2O_2 and then cotyledons were isolated and used for determinations.

2.2. Enzyme preparation and assay

Extracts for the determination of CAT, APX and guaiacol peroxidase (GPX) activities were prepared from 1.0 g of cotyledons homogenized under ice-cold conditions in 3 ml of extraction buffer, containing 50 mM phosphate buffer (pH 7.4), 1 mM EDTA, 1 g PVP and 0.5% (v/v) Triton X-100. The homogenates were centrifuged at 10,000 × g for 30 min and the supernatant fraction was used for the assays. Catalase (EC 1.11.1.6) activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first-order reaction constant ($k' = k$[CAT]) of the decrease in H_2O_2 absorption was determined and the catalase content in picomole per milligram protein was calculated using $k = 4.7 \times 10^7$ M^{-1} s^{-1}

(Chance et al., 1979). Ascorbate peroxidase (EC 1.11.1.11) activity was measured in fresh extracts and was assayed as described by Nakano and Asada (1981) using a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.5 mM ascorbate and 0.1 mM EDTA. The hydrogen peroxide-dependent oxidation of ascorbate was followed monitoring the absorbance decrease at 290 nm (ε: 2.8 mM^{-1} cm^{-1}). Guaiacol peroxidase activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2.

2.3. Native PAGE and activity staining

Equal amounts of protein (50 μg) from cotyledon extracts were subjected to discontinuous PAGE under non-denaturing and non-reducing conditions as described by Laemmli (1970), except that SDS was omitted. Electrophoretic separation was performed at 4 °C for 3 h with a constant current of 30 mA per gel using Mini-Protean 3 Electrophoresis System (BIORAD). Staining of POD isozymes was achieved as following: the gels were equilibrated with sodium phosphate buffer (50 mM, pH 7) for 30 min, then they were incubated in 50 mM sodium phosphate buffer containing 5 mM α-naphthol and 0.33 mM H_2O_2 during 15 min (Ferrer et al., 1990). The ferricyanide staining for CAT isozymes was carried out using the method described by Woodbury et al. (1971). The gels were washed three times for 15 min each in distilled water to remove interfering buffer salts, and then they were suspended in a 3.27 mM H_2O_2 solution and were gently rocked for 25 min. The gels were rinsed quickly in H_2O and stained in a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride (equal volumes of 2% solutions of each component added sequentially). As soon as a green color began to appear, they were washed three times with water. For detection of APX activity, samples were subjected to native PAGE as described above, except that the carrier buffer contained 2 mM ascorbate. The gels were prerun for 30 min to allow ascorbate to enter in the gel prior to the application of the samples (Mittler and Zilinskas, 1993). Following electrophoretic separation, gels were equilibrated with 50 mM sodium phosphate buffer (pH 7) and 2 mM ascorbate for 30 min. Then, the gels were incubated with 50 mM sodium phosphate buffer (pH 7) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. Finally, they were washed with buffer during 1 min and placed in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and 2.45 mM nitroblue tetrazolium (NBT) with gentle agitation. The reaction was continued for 10–15 min and stopped by a brief wash in deionized water. The APX activity was observed as a clear band on a purple-blue background. All gels were photographed, scanned and analyzed using Gel-Pro Analyzer 3.1 software (Media Cybernetics).

2.4. Chromatography

Extract of cotyledons subjected to 30 kJ m^{-2} UV-B radiation and recovered in darkness was prepared in 50 mM Tris–HCl buffer (pH 8) containing 1 mM EDTA, 1% (w/v) PVP, 1% (v/v) Triton X-100 and 5 mM ascorbate. After centrifugation at 10,000 × *g* for 30 min, the sample was applied to a Sephadex G-25 column in order to eliminate low weight molecules. Then, the eluate was filtered through a membrane filter (Millipore, pore size 0.22 μm). One millilitre of the extract was loaded onto an anion-exchange column (Mono Q 5/5), attached to a fast protein liquid chromatography system (FPLC; Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with buffer A (20 mM Tris–HCl, pH 8). A NaCl gradient was used (0–0.2 M). Fractions of 0.4 ml were collected at a rate of 1 ml min^{-1} and assayed for peroxidase activity using guaiacol (by measuring the increase in absorption at 470 nm) or α-naphthol (by measuring the increase in absorption at 605 nm) as substrates.

2.5. pI determination

For p*I* determination, the three peroxidase fractions from the above chromatography assay were concentrated by ultracentrifugation. IEF were performed in a Phast System unit (Pharmacia) using a broad (3–9) pH range in the pre-cast gels as well as protein p*I* markers (broad p*I* range). The gels were revealed with Coomassie Blue R 350 for p*I* markers and with α-naphthol for POD samples.

2.6. Protein determination

Protein concentration was evaluated by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.7. Statistics

Values in the text and tables indicate mean values ± S.D. Levels of significance in the differences were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

3. Results

3.1. Enzyme activities

Different behavior of the enzymes with peroxidase activity was observed under UV-B treatments. While APX activity remain unaltered after the two UV-B radiation doses and after recuperation, CAT and GPX showed a similar response, increasing at the two UV-B irradiation doses (by about 20% for CAT and 37% for GPX, respect to control values). After 20 h of recovery in the dark, CAT and GPX increased in cotyledons exposed to 15 and 30 kJ m^{-2} UV-B radiation doses by about 77 and 58% compared to controls, respec-

Table 1
Effect of UV-B treatments on cotyledons peroxidase activities

Treatment (kJ m^{-2})	CAT (pmol mg^{-1} protein)	APX (U mg^{-1} protein)	GPX (U mg^{-1} protein)
Control	0.30 ± 0.01^{a}	11.4 ± 1.2^{a}	13.8 ± 1.2^{a}
15.0	0.36 ± 0.01^{b}	9.3 ± 1.0^{a}	18.7 ± 1.1^{b}
30.0	0.36 ± 0.01^{b}	10.1 ± 1.0^{a}	19.1 ± 1.1^{b}
Recovered in darkness			
15.0	0.56 ± 0.03^{c}	13.2 ± 1.1^{a}	21.7 ± 1.1^{c}
30.0	0.50 ± 0.03^{c}	12.9 ± 1.0^{a}	22.0 ± 1.0^{c}
Recovered in light			
30.0	0.28 ± 0.02^{a}	9.3 ± 1.2^{a}	14.4 ± 1.2^{a}

Enzymatic activities were assayed as described in Section 2. Data are the means ± S.D. of three different experiments with two replicated measurements ($n = 6$). Different letters within columns (a–c) indicate significant differences ($P < 0.05$) according to Tukey's multiple range test. One unit of APX forms 1 nmol of ascorbate oxidized/min under the assay conditions. One unit of GPX forms 1 nmol of guaiacol oxidized/min under the assay conditions.

tively (Table 1). After recuperation under white light, both the enzyme activities showed similar values to controls (Table 1).

3.2. Isoforms of enzymes with peroxidase activity in sunflower cotyledons subjected to UV-B radiation

As it can be seen in Fig. 1A and B, control as well as treated plants showed one CAT isoform (CAT1); meanwhile, APX presented four bands (APX1, APX2, APX3 and APX4). CAT activity slightly increased at the two UV-B doses radiation and in recovery in darkness (Fig. 1A, Table 2). Despite the fact that total APX activity was similar in control and treated plants (Table 1), APX isoform activities responded in a different way (Fig. 1B, Table 2). While APX4 activity significantly decreased with both UV-B doses respect to control, APX2 significantly increased with 15 and 30 kJ m^{-2} UV-B radiation, with respect to control values. Recovery in darkness or under white light also decreased APX4 activity (Fig. 1B, Table 2). Only POD2 activity were increased by 15 kJ m^{-2} and decreased by 30 kJ m^{-2} UV-B radiation, but both peroxidase activities increased when plants were recovered in darkness or in white light (Fig. 1C, Table 2).

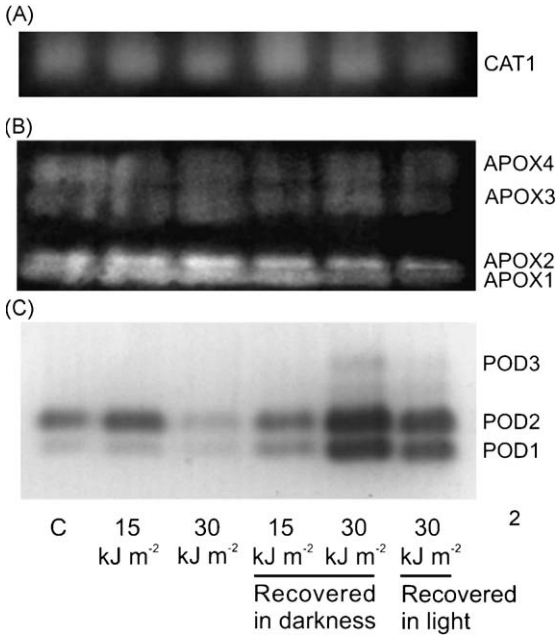

Fig. 1. Effect of UV-B radiation on CAT, APX and POD isoforms. Proteins (50 μg per well) were separated by native PAGE (10% polyacrylamide gels) and stained for (A) catalase, (B) ascorbate peroxidase and (C) peroxidase activities as described in Section 2. Gels presented are representative of three independent experiments with two replicated measurements ($n = 6$).

Surprisingly, a new band (POD3) was observed when plants were left to recover during 20 h in the dark after 30 kJ m^{-2} UV-B irradiation (Fig. 1C).

3.3. Chromatography analysis of cotyledons peroxidase isozymes

In order to characterize POD isoforms, a chromatography analysis of extracts from plants irradiated with 30 kJ m^{-2} UV-B and recovered in darkness was performed. Elution profile of extracts from cotyledons in an anion-exchange column showed three peaks (p1, p2 and p3) using α-naphthol as a substrate (Fig. 2). These peaks correspond to the three isoforms observed in POD stained gels (p1, p2 and p3 correspond

Table 2
Quantification of enzymes like peroxidase isoform activities in gels by densitometric scanning

	C	15 (kJ m^{-2})	30 (kJ m^{-2})	Recovered in darkness		Recovered in light
				15 (kJ m^{-2})	30 (kJ m^{-2})	30 (kJ m^{-2})
CAT1	1.00 ± 0.00^{a}	1.20 ± 0.04^{b}	1.14 ± 0.07^{b}	1.64 ± 0.14^{c}	1.44 ± 0.09^{c}	0.96 ± 0.04^{a}
APX4	1.00 ± 0.00^{a}	0.50 ± 0.05^{b}	0.58 ± 0.06^{b}	0.53 ± 0.05^{b}	0.59 ± 0.04^{b}	0.56 ± 0.01^{b}
APX3	1.00 ± 0.00^{a}	0.97 ± 0.03^{a}	1.03 ± 0.07^{a}	1.03 ± 0.07^{a}	1.04 ± 0.09^{a}	1.07 ± 0.08^{a}
APX2	1.00 ± 0.00^{a}	1.56 ± 0.03^{b}	1.80 ± 0.09^{b}	1.09 ± 0.09^{a}	0.91 ± 0.09^{a}	0.92 ± 0.08^{a}
APX1	1.00 ± 0.00^{a}	1.05 ± 0.06^{a}	1.07 ± 0.06^{a}	1.09 ± 0.09^{a0}	0.96 ± 0.09^{a}	0.91 ± 0.09^{a}
POD2	1.00 ± 0.00^{a}	1.25 ± 0.07^{b}	0.54 ± 0.07^{c}	1.16 ± 0.02^{b}	2.40 ± 0.30^{d}	1.29 ± 0.17^{b}
POD1	1.00 ± 0.00^{a}	1.09 ± 0.05^{a}	0.98 ± 0.08^{a}	1.19 ± 0.09^{b}	2.24 ± 0.53^{c}	2.17 ± 0.24^{c}

Peroxidase activities in gels of the Fig. 1 were quantified by densitometric scanning and expressed as arbitrary units taken control as 1 unit. Data are the means ± S.D. of three independent experiments with two replicated measurements ($n = 6$). Different letters within row (a–c) indicate significant differences ($P < 0.05$) according to Tukey's multiple range test.

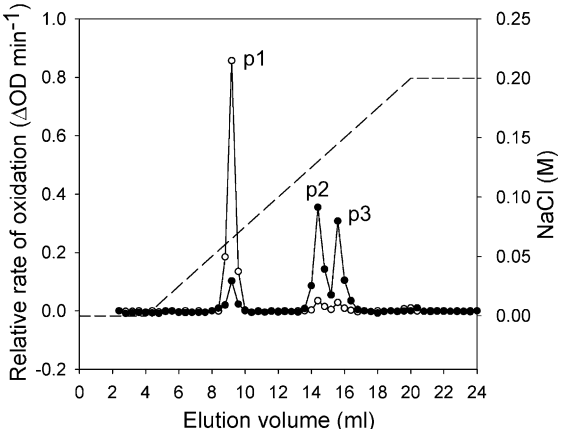

Fig. 2. FPLC fractionation of cotyledons peroxidases. Sunflower plants were irradiated with 30 kJ m^{-2} UV-B and left to recover for 20 h in the dark. Proteins were loaded in a Mono Q column and eluted by a NaCl gradient (−). Elution profile of peroxidase activity using guaiacol (open symbols) or α-naphthol (closed symbols) as substrates.

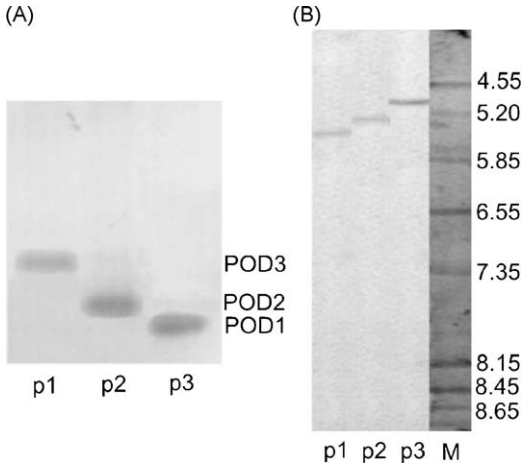

Fig. 3. (A) Native PAGE and (B) isoelectric focusing of cotyledon anionic isoperoxidases separated by FPLC. p1–p3 represent POD activity peaks (FPLC) and M represents p*I* marker. POD samples were stained using α-naphthol, and p*I* marker was revealed with Coomassie Blue R.

to POD3, POD2 and POD1, respectively), as it was determined performing native PAGE of the peaks (Fig. 3A). To test substrate specificity, peaks were analyzed using guaiacol or α-naphthol. Protein from p1 (corresponding to POD3 isoform) showed to be more specific for guaiacol, whereas protein from p2 and p3 used mainly α-naphthol as substrate (Table 3).

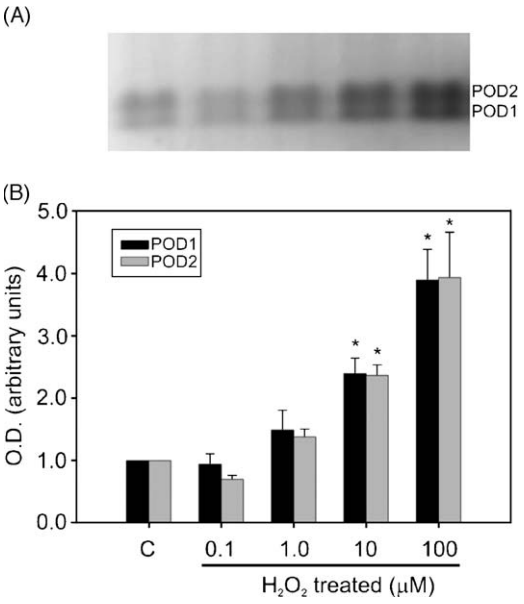

Fig. 4. Effect of hydrogen peroxide treatments on peroxidase isoforms. Proteins (50 μg per well) were separated by native PAGE (10% polyacrylamide gels) and stained for peroxidase activity as described in Section 2. Peroxidase activities in gels were quantified by densitometric scanning and expressed as arbitrary units taken control as 1 unit. Values are the means of three different experiments with two replicated measurements, and bars indicate S.D. ($n = 6$). *Significant differences ($P < 0.05$) according to Tukey's multiple range test.

3.4. IEF determination

Isoelectric point determination of each isoform showed that POD1, POD2 and POD3 have a p*I* of 5.0, 5.2 and 5.4, respectively (Fig. 3B), corroborating the anionic nature of these cotyledon peroxidases.

3.5. Cotyledon peroxidase isoforms in H_2O_2-treated plants

In order to elucidate the isoforms pattern of peroxidases subjected to a reactive oxygen species, sunflower cotyledons were treated with different hydrogen peroxide concentrations. As shown in Fig. 4, H_2O_2 enhanced the activities of the two peroxidase isoforms present in control cotyledons (POD1 and POD2) in a dose dependent manner, except for the lower H_2O_2 concentration which had no effect on POD activities. Nevertheless, H_2O_2 did not contribute to the appearance of POD3 isoform that was present when plants were subjected

Table 3
Substrate specificity of peroxidases from sunflower cotyledons separated by FPLC

Peak	Guaiacol activity (ΔOD min^{-1} mg^{-1} protein)	α-Naphtol activity (ΔOD min^{-1} mg^{-1} protein)	Guaiacol/α-naphtol
p1 (POD3)	0.490 ± 0.021	0.061 ± 0.010	8.03
p2 (POD2)	0.079 ± 0.008	0.867 ± 0.017	0.09
p3 (POD1)	0.064 ± 0.008	0.694 ± 0.016	0.09

Enzymatic activities were assayed as described in Section 2. Data are the means ± S.D. of three different experiments with two replicated measurements ($n = 6$).

to 30 kJ m^{-2} UV-B radiation and further recuperation in darkness.

4. Discussion

Repair and acclimation responses are readily induced in response to UV exposure in many species (Frohnmeyer and Staiger, 2003). A typical repair mechanism is the light-dependent photoreactivation by photolyases resulting in the restoration of UV-damaged DNA to its native form (Britt, 1999). Acclimation responses include increased oxygen radical scavenging activity (Strid et al., 1994; Rao et al., 1996) and the accumulation of soluble UV-screening flavonoids (Olsson et al., 1999; Warren et al., 2002).

The effect of UV-B radiation on free radical production and scavenging, as well as on cell membranes in plants, have been well documented (Takeuchi et al., 1995; Rao et al., 1996; Costa et al., 2002). Various environmental stresses cause H_2O_2 accumulation in leaves and the regulation of these enhanced H_2O_2 levels is of utmost importance in plant cell metabolism. Hydrogen peroxide, an active oxygen species, is known to diffuse across biological membranes and cause cellular damage. The accumulation of H_2O_2 produced an increase in CAT and APX activities in potato tubers during low-temperature storage (Mizuno et al., 1998). Studies carried out in rice (*Oryza sativa*) leaves demonstrated that after supplemental UV-B radiation, CAT and SOD activities were enhanced; meanwhile, no differences were observed in APX (Dai et al., 1997). In sunflower cotyledons, the induction of antioxidant enzymes with peroxidase activity (CAT and GPX) indicated that hydrogen peroxide participates actively on UV-B plant response. It has been demonstrated that GPX activity increases under UV-B irradiadion (Rao et al., 1996), UV-C treatment (Zacchini and de Agazio, 2004) and salinity (Parida et al., 2004), and this increase has been used as an indicator for different abiotic stresses. The fact that GPX was induced with both the UV-B treatments and even more in darkness recovery let us to assume that the photosynthetic electron transport and photophosphorylation allowed the major plant recuperation.

Despite the fact that total APX activity had no modification, their isozymes were clearly affected demonstrating a selective response of each isoform, presumable due to its location. These results are in agreement with those reported by Lee et al. (2001), who found induction of only cytosolic isoforms under salt stress in leaves of rice plants. Also, other biotic and abiotic stresses lead to selective increase of different APX isoforms (Smirnoff, 2000 and references therein).

POD usually occurs as multiple molecular forms (isozymes); the function of these isozymes and its regulation remain largely unknown. POD requires H_2O_2 as an essential substrate and, therefore, POD metabolizes H_2O_2 to water. Anionic PODs are believed to utilize phenolic compounds to initiate the chain reaction that leads to lignification. In *Nicotiana sylvestris*, an increased activity of the anionic phenol-oxidizing peroxidase correlated with increased tolerance to UV radiation (Jansen et al., 2001). In *Arabidopsis thaliana*, the enhancement of POD activity specific to coniferyl alcohol and the synthesis of several new isoforms suggested that UV-B radiation might have enhanced the synthesis of secondary metabolites (Rao et al., 1996). Our results clearly demonstrate that in sunflower cotyledons, subjected to UV-B radiation, differences in anionic peroxidase isozymes expression patterns occurred (Fig. 1C). In the present study, under 30 kJ m^{-2} UV-B radiation and further recovery in the dark or in white light, a general induction of enzymes involved in hydrogen peroxide breakdown took place. This fact indicates that AOS (such as H_2O_2) scavenging mechanisms were still operative. Taking into account that H_2O_2 increased POD1 and POD2 activities in sunflower cotyledons (Fig. 4), these isoforms could be related to abiotic stresses involved in AOS formation. Fig. 4 also demonstrated that POD3 is not induced in cotyledons treated with H_2O_2. This result let us to speculate that POD3 could not contribute to UV tolerance by removing UV-B induced H_2O_2. This interpretation is in agreement to that described by Jansen et al. (2001), who demonstrated that phenol-oxidizing peroxidases unlikely contribute to UV tolerance as a result of their oxygen radical scavenging activity.

Peroxidases are capable of oxidizing a wide range of substrates. Separation of cotyledons peroxidase isozymes from plants subjected to 30 kJ m^{-2} UV-B radiation and left to recover in the dark, revealed that guaiacol has major affinity to POD3 than α-naphthol, although Ferrer et al. (1990) had demonstrated that α-naphthol is more sensitive to isoperoxidase activities. Since guaiacol is a natural substrate, is more probably that POD3 has a role in UV-B acclimation by acting in phenolic metabolism. UV-B-absorbing pigments, like flavonoids, are an important line of defense against UV-B radiation and in addition can enhance antioxidant capacity (Jordan, 2002). Yamasaki et al. (1997) proposed a flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2 under conditions of high stress or rapid growth or when ascorbate availability is limited.

In conclusion, this work demonstrated that UV-B radiation induced selective adaptive responses in affected plants. We propose that *Helianthus annuus* L. metabolizes UV-B-induced AOS through peroxidase-related antioxidant enzymes. CAT increased its activity but the pattern of isoforms remained similar to those found in controls, and APX did not alter either its total activity or its number of isoforms under UV-B radiation. Sunflower plants could acclimate themselves by induction of other types of peroxidases; POD1 and POD2 could work directly as oxygen species scavenger. POD3 could play a role either in polyphenols metabolism, increasing the antioxidant capacity or in the cross-linking of various UV-absorbing phenolics and, therefore, could be involved in plant cell wall architecture. Such complexity in POD responses could be explained, considering the multi-

functional role that isoforms of peroxidase might play in plant stress response.

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