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Oxidative Damage in Pea Plants Exposed to Water Deficit or Paraquat[1]

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The application of a moderate water deficit (water potential of −1.3 MPa) to pea (*Pisum sativum* L. cv Lincoln) leaves led to a 75% inhibition of photosynthesis and to increases in zeaxanthin, malondialdehyde, oxidized proteins, and mitochondrial, cytosolic, and chloroplastic superoxide dismutase activities. Severe water deficit (−1.9 MPa) almost completely inhibited photosynthesis, decreased chlorophylls, β-carotene, neoxanthin, and lutein, and caused further conversion of violaxanthin to zeaxanthin, suggesting damage to the photosynthetic apparatus. There were consistent decreases in antioxidants and pyridine nucleotides, and accumulation of catalytic Fe, malondialdehyde, and oxidized proteins. Paraquat (PQ) treatment led to similar major decreases in photosynthesis, water content, proteins, and most antioxidants, and induced the accumulation of zeaxanthin and damaged proteins. PQ decreased markedly ascorbate, NADPH, ascorbate peroxidase, and chloroplastic Fe-superoxide dismutase activity, and caused major increases in oxidized glutathione, NAD^+, NADH, and catalytic Fe. It is concluded that, in cv Lincoln, the increase in catalytic Fe and the lowering of antioxidant protection may be involved in the oxidative damage caused by severe water deficit and PQ, but not necessarily in the incipient stress induced by moderate water deficit. Results also indicate that the tolerance to water deficit in terms of oxidative damage largely depends on the legume cultivar.

In natural conditions crops are often exposed to various environmental stresses that decrease production. At the whole-plant level, the effect of stress is usually perceived as a decrease in photosynthesis and growth, and is associated with alterations in C and N metabolism. At the molecular level, the negative effect of stress on leaves may be in part a consequence of the oxidative damage to important molecules, as a result of the imbalance between production of activated O_2 and antioxidant defenses (Foyer et al., 1994).

This hypothesis is very plausible because chloroplasts are a major source of activated O_2 in plants (Asada and Takahashi, 1987; Foyer et al., 1994), and because antioxidants, which may play a critical role in preventing oxidative damage, are greatly affected by environmental stress (Bowler et al., 1994). In chloroplasts the superoxide radical (O_2^-) is produced by photoreduction of O_2 at PSI and PSII, and singlet O_2 is formed by energy transfer to O_2 from triplet excited state chlorophyll (Asada and Takahashi, 1987). H_2O_2 can originate, in turn, from the spontaneous or enzyme-catalyzed dismutation of O_2^-. Other subcellular compartments of leaves, such as peroxisomes and mitochondria, are also potential generators of O_2^- and H_2O_2, mainly as a consequence of electron transport and enzymic reactions (Del Rio et al., 1992). Fortunately, in optimal conditions leaves are rich in antioxidant enzymes and metabolites and can cope with activated O_2, thus minimizing oxidative damage. Antioxidants include ASC, GSH, carotenoids, α-tocopherol, SOD, catalase, and the enzymes of the ASC-GSH cycle (Foyer et al., 1994). Antioxidant metabolites are present in chloroplasts at very high concentrations (10–20 mM ASC and 1–4 mM GSH), and, apart from their obvious role as enzyme substrates, they can react chemically with almost all forms of activated O_2 (Halliwell and Gutteridge, 1989). The importance of ASC and GSH as antioxidants is underlined by the results of a recent study showing that overexpression of GR in chloroplasts doubles the concentrations of ASC and GSH in leaves and confers increased resistance to oxidative stress (Foyer et al., 1995).

An aspect frequently overlooked in studies on free radicals and antioxidants is the pro-oxidant properties of Fe. The so-called "catalytic Fe" catalyzes the decomposition of H_2O_2 and lipid hydroperoxides to hydroxyl and alkoxyl radicals, respectively. Both free radicals are extremely cytotoxic and promote lipid peroxidation (Halliwell and Gutteridge, 1989). Lipid peroxidation is commonly taken as an indicator of oxidative stress. Lipid peroxides are quantified by the TBA test, which is easy to perform and allows the results to be conveniently expressed as TBARS. However, results from the TBA test need to be compared with more specific assays. Because the hydroxyl radical and other highly reactive species can oxidize proteins in addition to lipids, the suitability of lipid peroxidation for diagnosing oxidative stress can be tested by measuring protein oxidation in leaves exposed to adverse conditions.

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Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate; CuZn-, Fe-, or Mn-SOD, SODs containing Cu and Zn, Fe, or Mn as metal cofactors; DR, dehydroascorbate reductase; GR, glutathione reductase; GSSG, oxidized glutathione; MDA, malondialdehyde; MR, monodehydroascorbate reductase; PQ, paraquat; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TBARS, 2-thiobarbituric acid-reactive substances; Ψ_w, water potential.

In this paper we report the results of detailed measurements of the physiological status, oxidant damage, catalytic Fe, and antioxidant enzymes and metabolites of pea (*Pisum sativum* L.) leaves exposed to two types of stress of agronomic interest, water deprivation and PQ treatment. Two intensities of water stress were applied for comparison with our previous work using a different pea cultivar (Moran et al., 1994), and PQ, a potent herbicide that exacerbates O_2^- radical production, was used as a model to study oxidative stress (Asada and Takahashi, 1987; Bowler et al., 1994), especially those aspects for which little or no information is currently available.

MATERIALS AND METHODS

Plant Material and Stress Treatments

Pea (*Pisum sativum* L. cv Lincoln) plants were grown in controlled environment chambers as described by Gogorcena et al. (1997) using a PPFD of 350 μmol m^{-2} s^{-1} and one-half-strength Hoagland nutrient solution containing 45 μM Fe as Sequestrene 330 (Ciba-Geigy, Basilea, Switzerland). Approximately 30 d after sowing, plants were divided at random into five groups, which received the treatments described below.

Physiological measurements and harvest of leaves for all treatments were made when plants were at the late-vegetative growth stage. Leaves to be used for biochemical determinations were frozen in liquid N_2 immediately after harvest and stored at −80°C until required for analyses.

Water Deficit

This was induced by withholding irrigation until leaf Ψ_w values of −1.30 ± 0.04 MPa (D_1) and −1.93 ± 0.05 MPa (D_2) were reached, which usually occurred after approximately 7 and 9 d, respectively.

PQ

Plants were sprayed with 100 μM methyl viologen (Sigma) in 0.25% Tween 20 and exposed to light (500 μmol $m^{-2}s^{-1}$) for 6 h. Immediately after this period, physiological parameters were measured and leaves were harvested and stored as indicated below. Plants showed some signs of wilting but no apparent necrotic lesions.

Control (C_1) for Water Deficit

Plants were kept in optimal water conditions (leaf Ψ_w values of −0.50 ± 0.02 MPa) over the treatment period.

Control (C_2) for PQ

Plants were sprayed with 0.25% Tween 20 and exposed to light (500 μmol m^{-2} s^{-1}) for 6 h. Leaf Ψ_w values were similar to those of C_1 plants.

Physiological Parameters

Leaf Ψ_w was measured 2 h after the commencement of the photoperiod with a pressure chamber (Soil Moisture Equipment, Santa Barbara, CA). After determination of Ψ_w, rates of photosynthesis, transpiration, and stomatal conductance were measured with a LI-6200 portable photosynthesis system equipped with a LI-6250 CO_2 analyzer (Li-Cor, Lincoln, NE). Chlorophylls *a* and *b*, β-carotene, and xanthophylls were extracted with acetone from flash-frozen leaf discs and quantified by an HPLC method that permitted the complete separation of all photosynthetic pigments (Val et al., 1994). Chlorophylls were also quantified spectrophotometrically (Lichtenthaler, 1987) to confirm the HPLC data. Water contents were determined from leaf samples (0.5 g) after drying at 80°C for 3 d. The epoxidation index was calculated as (% violoxanthin × 2 + % antheraxanthin)/200 and is equivalent to the "epoxidation state" used by others (Demmig-Adams and Adams, 1993).

Catalase, Enzymes of the ASC-GSH Cycle, and Related Metabolites

Antioxidant enzymes were extracted from 0.25 g (catalase and APX) or 0.5 g (DR, MR, and GR) of leaves with optimized media (Moran et al., 1994). The homogenate was strained through one layer of Miracloth (Calbiochem) and centrifuged at 15,000*g* for 20 min. All operations were performed at 0 to 4°C. Catalase activity was determined by following the decomposition of H_2O_2 at 240 nm (Aebi, 1984). Total and cytosolic APX activities were measured following the oxidation of ASC at 290 nm (Asada, 1984). Estimates of the cytosolic enzyme activity were made by omitting ASC from the extraction medium. In the absence of ASC, chloroplastic APX, but not the cytosolic isoform, is inactivated within 1 min (Nakano and Asada, 1987). DR activity was assayed by measuring the reduction of ASC at 265 nm (Nakano and Asada, 1981). MR (Dalton et al., 1992) and GR (Dalton et al., 1986) activities were determined by following the oxidation of NADH and NADPH at 340 nm, respectively. Where appropriate, controls were run to correct for nonenzymatic rates, and buffers and reagents were treated with Chelex resin to avoid contamination by trace amounts of transition metal ions. Soluble protein in leaf extracts was quantified using a commercial dye (Bio-Rad) and BSA as the standard.

ASC was extracted from 0.5 g of leaves with 5 mL of 5% (w/v) metaphosphoric acid and quantified by its ability to reduce Fe^{3+} at acidic pH (Law et al., 1983). GSH and GSSG were extracted from 0.5 g of leaves with 5 mL of 5% (w/v) sulfosalicylic acid. The homogenate was filtered and centrifuged, and the concentrations of GSH plus GSSG and GSSG were determined in two aliquots of the supernatant essentially by the method of Law et al. (1983). Pyridine nucleotides were extracted from 30 mg of leaves with 1 mL of 0.1 M NaOH (reduced forms) or 5% (w/v) TCA (oxidized forms) (Gogorcena et al., 1995) and were quantified by an enzymatic-cycling method (Matsumura and Miyachi, 1980).

SOD: Activity, Isoforms, and Subcellular Location

For determination of total SOD activity, 0.5 g of leaves were homogenized with 5 mL of 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA, 1% (w/v) PVP-10, and 0.1% (v/v) Triton X-100. After centrifugation at 15,000*g* for 20 min, extracts were depleted of small molecules by exhaustive dialysis against 5 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA. Total SOD activity was assayed by its ability to inhibit the reduction of ferric Cyt *c* by the O_2^- generated with a xanthine-xanthine oxidase system. The reaction mixture contained 10 μM KCN to inhibit Cyt *c* oxidase without affecting CuZn-SOD activity. One unit of activity was defined as the amount of enzyme required to inhibit ferric Cyt *c* reduction by 50% (McCord and Fridovich, 1969). Isoforms of SOD were resolved by nondenaturing 15% PAGE and stained for activity essentially as described by Donahue et al. (1997). Assignment of CuZn-, Fe-, and Mn-isoforms was performed by selective inhibition with KCN or H_2O_2 (Salin and Lyon, 1983), and their relative abundances were calculated by densitometry.

Leaf organelles were purified by differential and Percoll density-gradient centrifugation, according to published protocols (Palma et al., 1986; Sandalio et al., 1987; Struglics et al., 1993). The pellet obtained after the initial centrifugation at 2,200*g* for 5 min was resuspended in wash medium and layered on top of a discontinuous Percoll gradient (Sandalio et al., 1987). The gradient was centrifuged at 13,000*g* for 35 min, and the two higher-density bands containing chloroplasts were recovered (Palma et al., 1986). The supernatant obtained after the initial centrifugation was centrifuged again at 2,200*g* for 5 min. The pellet was discarded and the supernatant was centrifuged at 12,000*g* for 15 min. The final pellet was resuspended in wash medium, applied to a Percoll gradient, and centrifuged as before (Sandalio et al., 1987). The bands corresponding to mitochondria and peroxisomes were recovered, and the mitochondria were repurified on a second Percoll gradient as described by Struglics et al. (1993).

All organelles banded in the gradients at their expected equilibrium densities (Palma et al., 1986; Sandalio et al., 1987; Struglics et al., 1993). Isolated organelles were washed twice and broken by resuspension in SOD extraction medium. After clearing the samples by centrifugation, the composition of SOD isoforms was determined by native PAGE as indicated above. Purity of organelles was assessed using the following markers: chlorophyll for chloroplasts (Lichtenthaler, 1987), Cyt *c* oxidase for mitochondria (Schnarrenberger et al., 1971), and catalase for peroxisomes (Aebi, 1984).

Catalytic Fe

Leaves (0.5 g) to be used to quantify catalytic Fe were extracted with 6 mL of Chelex-treated 25 mM potassium phosphate buffer (pH 7.0) and were fractionated by ultrafiltration on Centricon-3 membranes (Amicon). The concentration of catalytic Fe in the low-molecular-mass fraction of leaves was measured as the amount of TBARS produced from DNA in the presence of the sample (containing Fe^{3+}), the antibiotic bleomycin, and ASC as the reductant of Fe^{3+} (Evans and Halliwell, 1994).

Oxidative Damage of Lipids and Proteins

Lipid peroxides were extracted by grinding in an ice-cold mortar 0.5 g of leaves with 5 mL of 5% (w/v) metaphosphoric acid and 100 μL of 2% (w/v) butyl hydroxytoluene (in ethanol). Homogenates were filtered through one layer of Miracloth and centrifuged at 15,000*g* for 20 min. The chromogen was formed by mixing 0.5 mL of supernatant, 50 μL of 2% (w/v) butyl hydroxytoluene, 0.25 mL of 1% (w/v) TBA (in 50 mM NaOH), and 0.25 mL of 25% (v/v) HCl, and by incubating the reaction mixtures at 95°C for 30 min (Minotti and Aust, 1987). A blank for all samples was prepared by replacing the sample with extraction medium, and controls for each sample were prepared by replacing TBA with 50 mM NaOH.

After the reaction was stopped by cooling the samples in an ice bath, two protocols were followed. For determination of TBARS, the chromogen formed was extracted by adding 1.5 mL of 1-butanol, the tubes were vigorously shaken, the organic (upper) phase was separated by low-speed centrifugation, and the absorbance of TBARS was read at 532 nm. For MDA determination, the chromogen was extracted by adding 0.4 mL of 1-butanol. The organic phase was obtained as before, the process was repeated, and the pooled organic phase was evaporated under N_2 and kept at −80°C until use. The samples were resuspended in 100 μL of HPLC solvent, and an aliquot of 20 μL was immediately injected. The HPLC equipment (Waters) included a photodiode-array detector (model 996) controlled by Millennium software. The $(TBA)_2$-MDA adduct was resolved on an Ultrasphere C_{18} column (5 μm, 25 cm × 4.6 mm; Beckman) and was eluted with 5 mM potassium phosphate buffer (pH 7.0) containing 15% acetonitrile and 0.6% tetrahydrofuran (Draper et al., 1993). The flow rate was 1 mL min^{-1} and detection was at 532 nm.

Calibration curves were made using 1,1,3,3-tetraethoxypropane (Sigma) in the range of 0 to 2 nmol (TBARS) and 0 to 1 nmol (MDA). Tetraethoxypropane is stoichiometrically converted into MDA during the acid-heating step of the assay. Recovery experiments were performed by adding 0.25 to 2 nmol (for TBARS) or 0.2 to 1 nmol (for MDA) of tetraethoxypropane at the moment of extraction and by taking into account the amounts of endogenous TBARS.

Proteins were extracted from 0.5 g of leaves as described in detail (Moran et al., 1994), and oxidative damage was quantified as total protein carbonyl content by reaction with 2,4-dinitrophenylhydrazine after the removal of possible contaminating nucleic acids with 1% (w/v) of streptomycin sulfate (Levine et al., 1990).

RESULTS

Photosynthesis, Water Status, and Pigment Composition

Pea plants were subjected to two intensities of water stress, as indicated by their leaf Ψ_w and water content relative to controls (C_1 plants) that had the same age but

Table I. *Physiological parameters of pea leaves subjected to water deficit or treated with PQ*

Plant treatments were: C_1, control for water deficit treatment; D_1, moderate water deficit; D_2, severe water deficit; C_2, control for PQ treatment; and PQ treatment. Values are means of 5 to 14 independent plant samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ based on Duncan's multiple range test.

Parameter	Treatment				
	C_1	D_1	D_2	C_2	PQ
Photosynthesis (μmol CO_2 m^{-2} s^{-1})	8.82a	2.20b	0.18c	5.94d	0c
Stomatal conductance (cm s^{-1})	0.35a	0.05b	0.03c	0.13d	ND[a]
Transpiration (mmol H_2O m^{-2} s^{-1})	2.71a	0.56b	0.43b	1.44c	ND
Dry weight (mg $plant^{-1}$)	479a	309b	360bc	444ac	427ac
Water content, (% water of leaves)	85.7a	84.1b	78.0c	85.4a	78.0c
Soluble protein (mg g^{-1} dry wt)	113.5a	105.2a	80.5b	104.5a	75.3b

[a] ND, Not determined.

had been kept in optimal water conditions throughout the experiment. At a leaf Ψ_w of −1.3 MPa (D_1 plants) there was a reduction in leaf water content from 85.7 to 84.1% and decreases of 75% in photosynthesis, 79% in transpiration, and 87% in stomatal conductance (Table I). At this level of stress there was only a minor effect on the contents of chlorophylls *a* and *b*, β-carotene, neoxanthin, and lutein, whereas the xanthophyll cycle pigment pool was significantly affected, with a decrease in the epoxidation index from 0.92 in C_1 plants to 0.71 in D_1 plants (Table II).

A further decrease of Ψ_w to −1.9 MPa (D_2 plants) led to a reduction in leaf water content to only 78% and to the almost complete suppression of net photosynthesis. This was accompanied by decreases of 92% in stomatal conductance and of 84% in transpiration. Other general indicators of leaf activity, such as dry weight, soluble protein, chlorophylls *a* and *b*, β-carotene, neoxanthin, and lutein, were reduced by 21 to 38% (Tables I and II). The effect of severe water deficit was more pronounced on the xanthophyll cycle components. In leaves of D_2 plants the contents of antheraxanthin and zeaxanthin increased 2.1- and 5.7-fold, respectively, at the expense of violaxanthin, which decreased by 61%. Hence, the epoxidation index declined to 0.57 in D_2 plants.

The same parameters were also measured in plants of the same age sprayed with PQ 6 h before sampling to assess the damage inflicted on the photosynthetic machinery. Leaves of PQ plants had no measurable photosynthesis and contained only 78% water, exactly the same as for D_2 plants (Table I). Treatment with PQ had no effect on leaf dry weight but lowered the contents of soluble protein, chlorophylls *a* and *b*, β-carotene, neoxanthin, and lutein by 20 to 30%, and that of violaxanthin by 72%. In contrast, the content of antheraxanthin was doubled and that of zeaxanthin reached 0.46 μg cm^{-2} (Table II). Consequently, the epoxidation index declined from 0.97 to 0.56 in PQ-treated plants.

Antioxidant Enzymes

In D_1 plants the activities of the enzymes involved in the ASC-GSH cycle were similar to or slightly lower than those of C_1 plants (Table III). This level of stress also had a moderate effect on catalase activity (21% decrease) and total SOD (27% increase). Severe water deficit (D_2 plants), however, caused decreases ranging from 40 to 55% in all antioxidant activities, except MR and total SOD, which remained nearly constant (Table III). Likewise, estimates of cytosolic APX activity using an extraction medium devoid of ASC indicated that D_1 and D_2 plants retained most if not all of this activity (data not shown). In contrast, exposure of plants to PQ had a drastic effect on most antioxidant activities. Catalase and GR decreased by 48%, DR by 64%, total APX by 83%, and cytosolic APX by 92%. As with water deficit, MR activity remained nearly constant following PQ treatment (Table III).

Table II. *Photosynthetic pigments in pea leaves subjected to water deficit or treated with PQ*

Plant treatments and statistical analysis are as described in Table I. Values are means of three to four independent plant samples.

Pigment	Treatment				
	C_1	D_1	D_2	C_2	PQ
	$\mu g\ cm^{-2}$				
Chlorophyll *a*	25.93a	21.46b	18.14b	35.51c	28.55a
Chlorophyll *b*	9.82ab	8.93bc	7.78c	13.53d	11.16a
β-Carotene	3.19a	2.40bc	1.98c	4.21d	2.83ab
Neoxanthin	1.65a	1.37b	1.18b	2.22c	1.70a
Lutein	5.78a	5.23ab	4.45b	7.99c	5.90a
Violaxanthin	1.51a	0.89b	0.59c	2.17d	0.60c
Antheraxanthin	0.16a	0.26ab	0.35b	0.17a	0.35b
Zeaxanthin	0.07a	0.28b	0.40c	0a	0.46c

Table III. *Antioxidant enzymes in pea leaves subjected to water deficit or treated with PQ*

Plant treatments and statistical analysis are as described in Table I. Values are means of 5 to 20 independent plant samples.

Enzyme[a]	Treatment				
	C_1	D_1	D_2	C_2	PQ
APX	61.4a	60.1a	36.8b	59.4a	10.2c
DR	3.21a	3.03a	1.43b	3.86a	1.40b
MR	27.4a	26.1ab	25.3b	24.9b	22.8c
GR	6.67a	5.80b	3.14c	7.62a	3.65c
Catalase	7.03a	5.54b	4.01c	7.92a	4.14c
SOD[b]	778a	987b	845a	1161c	643d

[a] Activities are expressed in units min^{-1} g^{-1} dry weight. Units are: APX and DR, μmol ASC; MR, μmol NADH; GR, μmol NADPH; and catalase, mmol H_2O_2. [b] SOD activity is expressed in units g^{-1} dry weight. SOD units were calculated as described in "Materials and Methods."

Five SOD isoforms were separated in extracts of pea leaves by nondenaturing PAGE (Fig. 1). These isoforms were identified and labeled by increasing mobility as Mn (no inhibition by KCN or H_2O_2), Fe-1, and Fe-2 (no inhibition by KCN but inhibited by H_2O_2), and CuZn-1 and CuZn-2 (inhibited by both KCN and H_2O_2). Densitometric analysis of activity gels showed that the Mn, Fe-1, Fe-2, CuZn-1, and CuZn-2 isoforms accounted for approximately 60, 6, 2, 7, and 25% of total SOD activity, respectively. In an attempt to localize the Fe-SOD isoforms at the subcellular level, leaves were fractionated using Percoll-density gradients. Cross-contamination between organelles was evaluated using marker molecules. Chloroplasts were contaminated by <2% with mitochondria and peroxisomes, and mitochondria were contaminated by <15% with chloroplasts and peroxisomes. Comparison of SOD composition in the leaf extract, the mitochondria, and the two fractions of chloroplasts indicated that the Mn and CuZn-2 isoforms are located in the mitochondria and chloroplasts, respectively, and strongly suggested that the CuZn-1 isoform, which is most abundant in the unfractionated soluble extract, is located in the cytosol but also in the mitochondria (Fig. 2). All of these results are consistent with earlier studies on SOD subcellular localization in pea leaves (Palma et al., 1986; Sandalio et al., 1987; Sen Gupta et al., 1993).

Very recently, Donahue et al. (1997) found two Fe-SODs in pea leaves, although these activities were only unmasked when gels were preincubated with KCN to inhibit the CuZn-SODs. In our case, the Fe-SODs exhibited the expected relative mobilities between those of Mn- and CuZn-SODs (Figs. 1 and 2). At least one Fe-SOD is located in the chloroplasts (Fig. 2), which is also in agreement with previous work on Fe-SOD localization in other higher plants (Bowler et al., 1994). We could not detect Fe-SOD in mitochondria (Fig. 2) or in peroxisomes (data not shown).

According to the isoform patterns observed for each treatment, the 27% increase of total SOD activity in D_1 plants can be ascribed to the increase in the Mn- and CuZn-SODs. In D_2 plants total SOD activity, as well as the activities of the CuZn-isoforms, declined back to control or lower levels, but Mn-SOD activity further increased (Fig. 1; Table III). The overall decrease of total SOD activity in PQ plants may result from the inhibition of Fe-SODs (Fig. 1) and CuZn-SODs. The inhibition of both CuZn-SODs was observed in some leaf samples (data not shown) but not in others (Fig. 1).

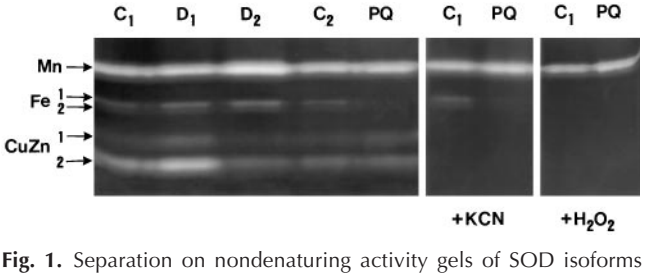

Fig. 1. Separation on nondenaturing activity gels of SOD isoforms from pea leaves subjected to water deficit or treated with PQ. In each case 45 μg of protein per lane was loaded. Identification of SOD isoforms was performed by pre-incubation of gels with 3 mM KCN or 5 mM H_2O_2 for 60 min prior to activity staining. Plant treatments are as described in Table I.

Antioxidant Metabolites and Nucleotides

Leaves of D_1 plants had 31% less ASC and GSH than C_1 plants but had a similar GSSG content (Table IV). Levels of NAD^+ and NADH were also similar in D_1 and C_1 plants, but leaves of the former contained 14% less $NADP^+$ and 25% less NADPH. Further intensification of water stress resulted in a 40% decrease in ASC and a 69% decrease in GSH relative to C_1 plants, but GSSG content was unchanged. Accordingly, the GSH/GSSG ratio decreased from 22.5 in C_1 plants to 13.9 in D_1 plants and 7.8 in D_2 plants. Overall, most of the GSH plus GSSG pool remained

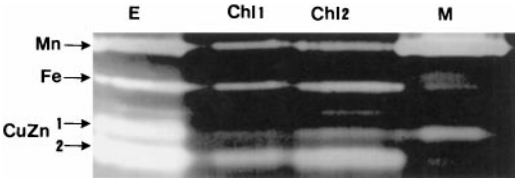

Figure 2. Subcellular localization of SOD isoforms in pea leaves. E, Whole-leaf extract; Chl1 and Chl2, fractions 1 and 2 of chloroplasts; and M, mitochondria. Lanes were loaded with 40 to 60 μg of protein.

Table IV. *Nonenzymic antioxidants and pyridine nucleotides in pea leaves subjected to water deficit or treated with PQ*

Plant treatments and statistical analysis are as described in Table I. Values are means of 6 to 14 independent plant samples.

Metabolite	Treatment				
	C_1	D_1	D_2	C_2	PQ
	nmol g^{-1} dry wt				
ASC	18,900a	13,100b	11,400b	21,400a	2,700c
GSH	2,250a	1,530b	700c	1,990ab	530c
GSSG	100a	110a	90a	140b	200c
NAD^+	194.7ab	177.4ac	157.4c	142.9c	228.0b
NADH	17.2ab	21.0ac	20.2ac	15.0bd	23.4c
$NADP^+$	56.9a	48.7bc	43.2b	55.8ac	30.0d
NADPH	51.2a	38.4b	21.4c	87.2d	8.8e

in the GSH form: 96 and 89% in C_1 and D_2 plants, respectively (Table IV).

Leaves of PQ plants had 87% less ASC, 73% less GSH, and 43% more GSSG than C_2 plants (Table IV). Consequently, the GSH/GSSG ratio declined from 14.2 to 2.65, and the GSH/(GSH plus GSSG) ratio from 93 to 73%. The effect of PQ on the pyridine nucleotide content was surprisingly variable. Leaves exposed to the herbicide had 46% less $NADP^+$ and 90% less NADPH than untreated leaves, whereas the levels of NAD^+ and NADH were 60% greater (Table IV).

Catalytic Fe and Oxidative Damage of Biomolecules

Catalytic Fe was not detectable in leaves of C_1, C_2, and D_1 plants, but was found in D_2 plants and, at a much larger concentration, in PQ plants (Fig. 3A). When desferrioxamine (200 μM), which binds strongly to Fe and converts it in a catalytically inactive form, was included in the reaction mixture, bleomycin-dependent DNA damage was suppressed. This confirmed that the DNA damage, which is the basis for the bioassay of catalytic Fe, was caused by the Fe present in the samples. Additional controls including PQ (25–100 μM) in the reaction mixtures were used to verify that endogenous PQ in leaves was not interfering with the assay.

Lipid peroxidation in leaves was measured spectrophotometrically as the content of TBARS and by HPLC as the content of MDA (Fig. 3B). For a closer comparison, we used in both cases identical extraction medium containing butyl hydroxytoluene to avoid artifactual formation of peroxides during tissue grinding. The same extracts were also used in the two assays. Recovery of standard MDA added to plant tissue prior to extraction was 88% for the TBARS method and 99% for the HPLC method. According to the TBA test, leaves of C_1 and D_1 plants had approximately 150 nmol of MDA equivalents per gram dry weight, but this value was 23% greater in D_2 plants. Using the same units, levels of TBARS in PQ plants were not found to differ significantly from those of C_2 plants (Fig. 3B).

The specific quantification of MDA by HPLC allowed us to ascertain that MDA is a major product of lipid peroxidation in pea leaves and to eliminate possible interferences with other TBARS. Identification of the peak of the $(TBA)_2MDA$ adduct was based on the retention time and visible spectrum, which were obtained on-line with the photodiode-array detector (Fig. 4). Both parameters were identical to those of the adduct made from authentic MDA. Levels of MDA were substantially lower than those of TBARS for all treatments. The content of MDA per gram dry weight of leaves in D_1 and D_2 plants was 24 and 45% greater than in C_1 plants, but there was no difference between that of PQ and C_2 plants (Fig. 3B).

The amount of oxidatively modified proteins was estimated by the carbonyl assay and expressed on a dry weight (Fig. 3C) or a protein basis, showing a similar trend in both cases for all plant treatments. The leaf content of oxidized proteins per gram dry weight increased by 36, 67, and 30% in D_1, D_2, and PQ plants compared with the controls. The corresponding increases per milligram of protein were 47, 135 and 80%. These observations show that the damage to leaf proteins increases with stress because there is a reduction in the soluble protein content of leaves upon exposure to water deficit or PQ (Table I).

DISCUSSION

The application of a moderate water stress to pea plants inhibited photosynthesis by 75%, which was probably attributable at least in part to CO_2 limitation by stomatal closure because there were large decreases in stomatal conductance and transpiration (Table I), but no changes or only small changes in pigments other than those of the xanthophyll cycle (Table II). In contrast, the application of a severe water stress caused the virtual disappearance of photosynthesis, a marked increase in antheraxanthin and zeaxanthin, and a substantial reduction in all of the other pigments. In this case, the inhibition of photosynthesis was probably due to both CO_2 limitation and damage of the photosynthetic apparatus. This damage may arise from the lowering of antioxidant protection (Tables III and IV) and from an insufficient capacity of D_2 plants to dissipate excess excitation energy through the xanthophyll cycle. The sustained increase of zeaxanthin in D_1 and D_2 plants is consistent with previous reports on *Nerium oleander* and cotton plants, and is associated with a decrease in photochemical efficiency; the increase in zeaxanthin also confirms that this pigment is a sensitive indicator of plant

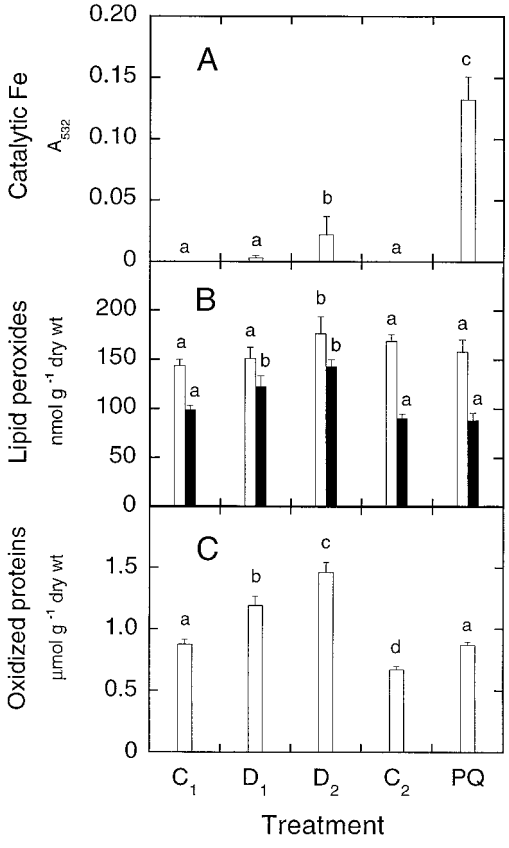

Figure 3. Catalytic Fe (A), oxidized lipids (B), and oxidized proteins (C) in pea leaves subjected to water deficit or treated with PQ. Catalytic Fe is expressed as A_{532} because actual concentrations are dependent on the assay conditions and on the form in which Fe is present (free or bound to chelates). In our conditions (25-μL samples, incubation at 37°C for 120 min), 0.2 absorbance units correspond to approximately 1 μM of catalytic Fe. The content of lipid peroxides is expressed as nanomoles of TBARS (□) or MDA (■) per gram dry weight, and that of oxidized proteins as micromoles of total carbonyl groups per gram dry weight. Values are means ± SE of six to nine independent plant samples. Plant treatments and statistical analysis for each parameter are as described in Table I.

stress (Demmig-Adams and Adams, 1993; Smirnoff, 1993). Likewise, the treatment with PQ caused almost identical decreases in photosynthesis, water content, and soluble protein to those of D_2 plants, as well as the accumulation of similarly high levels of antheraxanthin and zeaxanthin (Tables I and II).

In D_1 plants there were already symptoms of moderate oxidative stress, such as an increase in total and CuZn-SOD activity and in the contents of oxidized proteins and MDA (Table III; Figs. 1 and 3C). Leaves of D_1 plants retained most of their antioxidant capacity and had virtually no catalytic Fe, which may explain why oxidative damage in D_1 plants was incipient compared with D_2 plants. Thus, in D_2 and PQ plants there was a substantial decrease in antioxidant defenses along with an increase in catalytic Fe and protein damage (Tables III and IV; Fig. 3, A and C).

There were several indications that the functioning of the ASC-GSH cycle was limited or even impaired in D_2 and PQ plants. In both cases there was a shortage of photosynthetic NADPH, which is required for GR activity (Fig. 5). Total APX activity and ASC content showed an identical moderate reduction (40%) in D_2 plants; both parameters decreased in parallel, but to a much larger extent (83–87%), in PQ plants (Tables III and IV). The identical decline in APX activity and ASC induced by severe water deficit or PQ strongly suggests that the loss in enzyme activity can be ascribed at least in part to substrate depletion (Fig. 5). The decrease of ASC in D_2 and PQ plants may be due to its direct destruction by O_2^- and derived species, but also to the consumption of ASC for zeaxanthin synthesis and tocopherol regeneration (Smirnoff, 1993). Another factor indicating a limited operation of the ASC-GSH cycle in D_2 and PQ plants is the large decreases in GSH (70%) and GR activity (52%). The decrease of GSH in PQ plants may be explained in part by oxidation of GSH to GSSG by activated O_2, whereas in D_2 plants the decrease is probably a result of the inhibition of GSH synthesis, a phenomenon consistent with the general inhibition of protein synthesis and high turnover rates that occur following the gradual imposition of water stress (Fig. 5). The lack of GSSG accumulation in D_2 plants is also consistent with the observation that GSH oxidation does not occur in plants until the loss of water content is very near the limit of survival (Smirnoff, 1993).

Water deficit and PQ are known to enhance O_2^- production in the chloroplasts (Asada and Takahashi, 1987; Price et al., 1989). In this respect, the induction of chloroplastic and cytosolic CuZn-SODs in D_1 plants (Fig. 1) may be interpreted as a response to augmented O_2^- generation in both cellular compartments. On the other hand, the inhibition of chloroplast Fe-SOD but not of the corresponding CuZn-SOD in PQ plants (Fig. 1) suggests a greater lability of the former to PQ-imposed stress. In this scenario Fe-SOD would be inhibited by PQ but CuZn-SOD activity would be maintained or inhibited, depending on the level of PQ

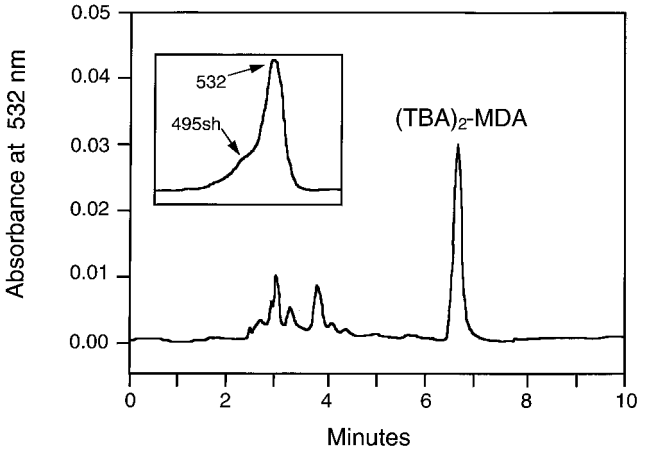

Figure 4. Analysis of MDA in pea leaves by HPLC with diode-array detection. The chomatogram was obtained at 532 nm and visible spectra (400–600 nm) were recorded on-line during the HPLC analysis. Inset, Visible spectrum of the $(TBA)_2$-MDA complex at the retention time (approximately 6.7 min) of the peak, showing a peak at 532 nm and a shoulder (sh) at 495 nm.

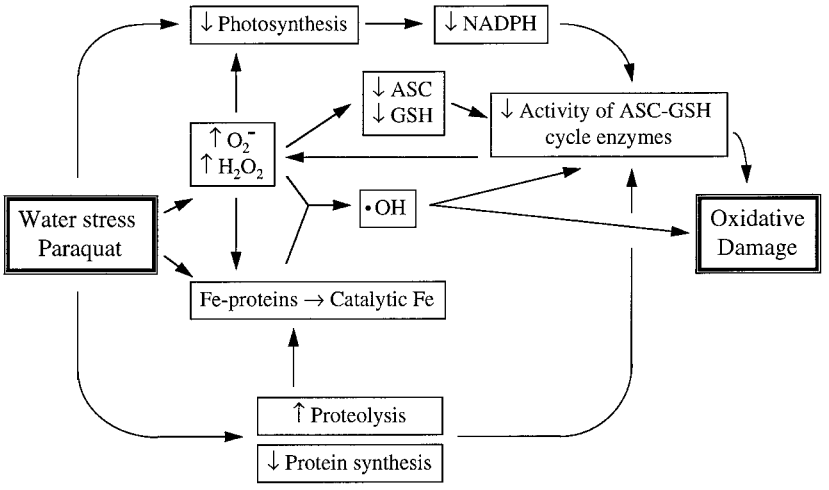

Figure 5. Scheme representing the possible sequence of events leading to oxidative damage in pea leaves subjected to severe water stress or treated with PQ.

reaching the chloroplasts. On the other hand, Mn-SOD activity was not inhibited in PQ plants (Fig. 1), and this could reflect sustained O_2^- production in mitochondria. The pool of NAD^+ plus NADH increased by 60% in PQ plants, but the NAD^+/NADH ratio remained constant (Table IV). This suggests that PQ does not affect mitochondrial respiration, in agreement with Tsang et al. (1991).

The toxicity caused by the increased generation of O_2^- and H_2O_2 may be further aggravated by the accumulation of catalytic Fe at moderate levels in D_2 plants and at high levels in PQ plants (Fig. 3A). This catalytic Fe may be released from Fe-proteins such as phytoferritin, which occurs in the chloroplasts (Briat et al., 1995) following their oxidative attack by O_2^- and/or H_2O_2. Catalytic Fe may interact in turn with O_2^- and H_2O_2 through a Haber-Weiss reaction to yield hydroxyl radicals (Fig. 5). The large increase of catalytic Fe in PQ plants further strengthens the hypothesis that the toxicity of PQ is mediated by Fe. Indirect support for this hypothesis comes from the observations that there is generation of hydroxyl radicals in PQ plants (Babbs et al., 1989) and that pretreatment of pea plants with the Fe chelator desferrioxamine lessens PQ-induced damage (Zer et al., 1994).

Finally, it is clear that the response of antioxidants to a water deficit depends on the severity of stress and on the species and age of plants (for example, see Smirnoff and Colombé, 1988; Tanaka et al., 1990; Mittler and Zilinskas, 1992, 1994). But there is also a differential sensitivity of cultivars to water deficit with respect to the induction of oxidative stress. These differences can be illustrated by comparison with our previous work (Moran et al., 1994). At the age and intensity of stress equivalent to D_1 plants, pea cv Frilene showed a reduction of 72 to 85% in catalase, DR, and GR activities, and increases of 180 to 240% in oxidized lipids and proteins (Moran et al., 1994). In cv Lincoln, however, the same activities decreased by <13% (Table III) and the increases in oxidative damage ranged from 0 to 36% (Fig. 3, B and C). In cv Frilene low-molecular-mass Fe (used as an estimate of catalytic Fe) increased 2.4-fold with water deficit, whereas in cv Lincoln catalytic Fe was not detectable. These observations indicate that, at the biochemical level, cv Lincoln is more tolerant to water deficit than is cv Frilene; the decrease in antioxidant protection and the increase in oxidative damage are closely related; and the accumulation of catalytic Fe is not the only factor likely to be involved in the initiation of oxidative damage, despite the requirement of a catalytic metal for protein oxidation (Stadtman, 1992). The last contention is substantiated by the fact that lipid peroxidation increases in leaves or other plant tissues subjected to water deficit (Moran et al., 1994; Gogorcena et al., 1995; this work) but not to other types of stress, despite the increase in catalytic Fe (Escuredo et al., 1996; Gogorcena et al., 1997). However, the duration of exposure to catalytic Fe may be an important factor. Lipids and proteins of leaves were probably exposed to catalytic Fe for longer periods in D_2 plants (days) than in PQ plants (hours). This may have contributed to the build-up of oxidized lipids and proteins in D_2 plants, especially if water stress decreases the efficiency in the mechanisms involved in the repair (de novo synthesis) and/or the degradation (lipases and proteases) of damaged molecules.

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