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Activated oxygen and antioxidant defenses in iron-deficient pea plants

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ABSTRACT

Iron (Fe) deficiency in pea leaves caused a large decrease (44-62%) in chlorophyll *a*, chlorophyll *b* and carotenoids, and smaller decreases in soluble protein (18%) and net photosynthesis (28%). Catalase, nonspecific peroxidase and ascorbate peroxidase activities declined by 51% in young Fe-deficient leaves, whereas monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase activities remained unaffected. Ascorbate peroxidase activity was highly correlated ($r^2=0.99$, $P<0.001$) with the Fe content of leaves, which allows its use as an indicator of the Fe nutritional status of the plant. Fe deficiency resulted in an increase of CuZn-superoxide dismutase but not of Mn-superoxide dismutase. The content of ascorbate decreased only by 24% and those of reduced and oxidized glutathione and vitamin E did not vary. The low-molecular-mass fraction of Fe-sufficient leaves contained 30-65 $\mu\text{g}\cdot(\text{g dry weight})^{-1}$ Mn. This concentration was 15-60 times greater than that of Fe and Cu in the same fraction, and was further enhanced (1.5 to 2.5 fold) by Fe deficiency without causing Mn toxicity. The concentration of catalytic Fe, that is, of Fe active for free radical generation, was virtually zero and that of catalytic Cu did not change with severe Fe deficiency. Because catalytic metals mediate lipid and protein oxidation *in vivo*, the above findings would explain why oxidatively-damaged lipids and proteins do not accumulate in Fe-deficient leaves.

Key-words: *Pisum sativum*; antioxidants; Fenton reaction; free radicals; iron deficiency; mineral nutrition.

Abbreviations: ASC, ascorbate; DHA, dehydroascorbate; DW, dry weight; Fe⁺/Fe⁻ plants, Fe-sufficient/Fe-deficient plants; ICP, inductively coupled argon plasma emission spectrophotometry; MDHA, monodehydroascorbate (ascorbate free radical); SOD, superoxide dismutase.

INTRODUCTION

Partially-reduced oxygen species ("active" oxygen) have been implicated in many degradative processes of plants, including aging, wounding, pathogen attack, and exposure of plants to xenobiotics or to some stress situations (Elstner 1987; Thompson, Legge & Barber 1987; Leshem 1988). Superoxide radical (O_2^-) is generated at the membrane level in most plant cell organelles, and hydrogen peroxide (H_2O_2) is the product of SOD and of several oxidases of peroxisomes, such as glycolate oxidase (Thompson *et al.* 1987; Del Río *et al.* 1992). Although generally detrimental to metabolism, the production of O_2^- and H_2O_2 may also serve useful purposes to the plant if it is rigidly controlled and compartmentalized within the cells (Williams 1985; Leshem 1988; Del Río *et al.* 1992). In contrast, two other active oxygen species, the hydroxyl radical ($\cdot OH$) and singlet oxygen ($^1\Delta_g O_2$), are highly destructive. Both species can react at nearly diffusion-controlled rates with most types of biological molecules, including chlorophylls, amino acids, polyunsaturated fatty acids, and DNA (Halliwell & Gutteridge 1989). Apparently, the $\cdot OH$ radical can be generated *in vivo* through the Fe-catalyzed Haber-Weiss reaction, in which trace amounts of Fe^{3+} are reduced by O_2^- radical, and the resulting Fe^{2+} reduces, in turn, H_2O_2 to $\cdot OH$ (Fenton

reaction). In the Haber-Weiss reaction ASC may replace O_2^- as the reductant of Fe^{3+} , and Cu^{2+} may replace Fe^{3+} as the catalytic metal ion (Halliwell & Gutteridge 1989). $^1\Delta_gO_2$ is mainly formed in photodynamic processes taking place in the chloroplasts, but it may originate also as a by-product of lipoxygenase (Thompson *et al.* 1987).

Photosynthetic plant cells are particularly exposed to active oxygen because they both consume and produce oxygen; however, they possess also more complex defensive mechanisms than other eukaryotic tissues (Scandalios 1993). Antioxidant defenses in plants include enzymes that scavenge O_2^- (various SOD isoenzymes) or H_2O_2 (catalase, enzymes of the ASC-GSH cycle, nonspecific peroxidases); small metabolites such as ASC, GSH, vitamin E (α -tocopherol), carotenoids, flavonoids, and uric acid, which are direct scavengers or quenchers of various active oxygen species; and molecules, such as phytic acid, phytoferritin and phytochelatins, which form complexes with metals in a form inactive to catalyze Fenton-like reactions.

In healthy plants, the antioxidant defenses are usually sufficient to prevent biological damage mediated by active oxygen; however, this may not occur in plants subjected to adverse environmental conditions. Such conditions may include deficiencies in mineral nutrients, but these have been seldom considered as stressing factors for plants. Thus, Mg-deficient bean leaves (Cakmak & Marschner 1992) and Mn-deficient needles of Norway spruce trees (Polle *et al.* 1992) had greater activities of several antioxidant enzymes than the controls, which may reflect a response of plants to increased free radical production. In this context, the study of Fe deficiency is particularly appealing. Firstly, Fe deficiency is a worldwide problem, enormously detrimental to plant production (Marschner 1986). Secondly, Fe is a transition metal of pivotal importance in reactions involving active oxygen (Halliwell & Gutteridge 1989). Fe is a constituent of antioxidant enzymes such as catalase, peroxidases and Fe-SOD, but it may also act as a

prooxidant factor because free or loosely-bound Fe catalyzes free radical generation in the presence of reductants and peroxides through Fenton chemistry. In this work we have investigated whether Fe deficiency causes oxidative stress in pea plants. To this end, we have conducted an extensive study on the metabolism of several biologically-relevant active oxygen species by comparing the prooxidant and antioxidant enzymes and metabolites of Fe-sufficient (Fe⁺) and Fe-deficient (Fe⁻) leaves.

MATERIALS AND METHODS

Plant culture and experimental design

Surface-disinfected pea (*Pisum sativum* L. cv Frilene) seeds were germinated in Petri dishes for 4 d. Seedlings were grown in pots containing a Perlite: Vermiculite mixture (1:1) and were watered with half-strength Hoagland nutrient solution, containing 45 mmol·m⁻³ of Fe in the form of Sequestrene-138 (6 % Fe, Ciba-Geigy). After 11 d, plants were transferred to continuously aerated hydroponic culture on the same nutrient solution. Plants were grown in 10-dm³ Fe-free plastic containers (six plants per container) in a controlled environmental chamber (ASL, Madrid, Spain) at 25/20°C (day/ night), with 65-75% relative humidity and 300 μmol photons·m⁻²·s⁻¹ for a daylength of 15 h. Nutrient solution was renewed every 5 d and maintained at a pH between 5.5 and 6.5. When plants were 25 d old, the containers were separated at random into two groups: Fe⁺ plants received half-Hoagland solution, and Fe⁻ plants received the same nutrient solution except that Fe was omitted and ≈0.3 g of CaCO₃ per container was added to accelerate the Fe deficiency process (Terry 1980). Measurements of all parameters for both Fe⁺ and Fe⁻ plants were made at 30-32 d age, after separating the shoots into three approximately equally-sized parts

(upper, medium and lower); for each of these parts leaves, but not stems, were collected (see Fig. 1). The chlorophyll concentration of leaves was estimated during the harvest with a SPAD-502 (Minolta) chlorophyll meter. These readings helped to ensure homogeneity between the harvests of the five series of plants which were needed to perform all analyses.

Metal analyses

For the determination of the total concentration of metals, leaves were dried at 85°C for 48 h and ground to a fine powder with a mortar and pestle. Dried material (0.5 g) was ashed at 450°C for 4 h and the ash residue was dissolved in 10 cm³ of a solution containing 30% HCl, 10% HNO₃, and 20 µg cm⁻³ of Mo as internal standard. This standard was omitted for Mo determination. Metals were then quantified by inductively coupled argon plasma emission spectrophotometry (ICP; Jarrell-Ash 965) using certified standards (National Bureau of Standards).

For the determination of Fe, Cu and Mn in the low-molecular-mass fraction (< 3 kDa), leaves (0.5 g) were homogenized with 6 cm³ of Chelex-treated 25 mol m⁻³ K-phosphate buffer (pH 7.0) using an ice-cold mortar and pestle. The homogenate was centrifuged at 15,000g for 20 min and the supernatant was filtered through Centricon (Amicon, Beverly, MA, USA) membranes with 3 kDa of molecular exclusion. Metals were quantified using an atomic absorption spectrophotometer (AA-670G, Shimadzu) equipped with graphite furnace atomizer (GFA-4A, Shimadzu). High-density graphite tubes were used for Fe and Cu determination, and pyrolytically-coated graphite tubes for Mn determination. In these conditions the detection limits for Fe, Cu and Mn were in the range of 2-5 ng cm⁻³.

Physiological parameters

Photosynthesis, stomatal resistance, and transpiration were measured in intact plants inside the growth chamber with a LI-6200 portable photosynthesis system equipped with a LI-6250 CO₂ analyzer (Li-COR, Lincoln, NE, USA). Leaf areas were measured with a LI-3000A portable area meter. Leaf water potentials and osmotic potentials were determined with a pressure chamber (Soil Moisture Equipment, Santa Barbara, CA, USA) and a vapor pressure osmometer 5500 (Wescor, Logan, UT, USA), respectively. Stomatal density was determined by microscopic observation of clear nail varnish prints of the adaxial (upper) and abaxial (lower) epidermis. Chlorophyll *a*, chlorophyll *b*, and total carotenoids were quantified in acetone extracts (Lichtenthaler 1987). Soluble protein was determined by the micro dye-binding assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as the standard.

Biochemical parameters

Leaves used for biochemical determinations were harvested in 0.5-g (fresh weight) samples, immediately frozen in liquid N₂, and stored at -80°C until assayed. Except otherwise indicated, all enzymes and metabolites were extracted using an ice-cold mortar and pestle, and the homogenate was filtered through one layer of Miracloth (Calbiochem, La Jolla, CA, USA) and centrifuged at 15,000*g* for 20 min.

Enzymes were extracted in optimized media as indicated previously (Moran *et al.* 1994) and initial rates of enzymatic activities were measured at 25°C with a Lambda16 UVDM (Perkin-Elmer) spectrophotometer. Glycolate oxidase (EC 1.1.3.1) was measured by the phenylhydrazine method (Baker & Tolbert 1966), catalase (EC 1.11.16) by following the decomposition of H₂O₂ at 240 nm (Aebi 1984), and nonspecific peroxidase (EC 1.11.1.7) by following guaiacol oxidation at 470 nm (Pütter 1974). ASC peroxidase (EC

1.11.1.11), MDHA reductase (EC 1.6.5.4), GSSG reductase (EC 1.6.4.2), and DHA reductase (EC 1.8.5.1) were assayed according to the procedures of Asada (1984), Dalton, Langeberg & Robbins (1992), Dalton *et al.* (1986), and Nakano & Asada (1981), respectively. Where appropriate, controls were run for correcting nonenzymatic rates, and Chelex-treated buffers and reagents were used to avoid contamination by trace amounts of transition metals. For determination of SOD (EC 1.15.1.1) activity, extracts were extensively dialyzed against 5 mol·m⁻³ K-phosphate (pH 7.8) containing 0.1 mol·m⁻³ Na₂EDTA. Total SOD activity was assayed by its ability to inhibit ferric cytochrome *c* reduction by a constant flux of O₂⁻ generated by the xanthine-xanthine oxidase system (McCord & Fridovich 1969). The reaction mixture contained 10 mmol·m⁻³ KCN to inhibit Cyt *c* oxidase, without affecting CuZn-SOD activity. CuZn-, Fe- and Mn-SODs were differentiated using 3 mol·m⁻³ KCN and 5 mol·m⁻³ H₂O₂ as selective inhibitors (Halliwell & Gutteridge 1989). Fe-SOD was not detected in any of the extracts.

ASC was extracted from leaves (0.5 g) with 5 cm³ of 5% (w/v) HPO₃. After filtration and centrifugation of the homogenate as indicated above, the pH of the supernatant was carefully adjusted to pH 7.4, and ASC was assayed by its ability to reduce Fe³⁺ at very low pH (Law, Charles & Halliwell 1983). GSH and GSSG were extracted from leaves (0.5 g) with 5 cm³ of 5% (w/v) sulfosalicylic acid. The homogenate was filtered and centrifuged, and the concentrations of total glutathione (GSH+GSSG) and GSSG were determined in two aliquots of the supernatant essentially by the method of Law *et al.* (1983). This method involves oxidation of GSH by 5,5'-dithiobis(2-nitrobenzoic acid), reduction of GSSG by GSSG reductase, and derivatization of GSH by 2-vinylpyridine at slightly acidic pH (Griffith 1980).

Vitamin E was extracted from leaves (1 g) essentially as described by Laidman *et al.* (1971), using sequentially 3 cm³ isopropanol [containing 1% (v/v) pyrogallol] and 3 cm³ chloroform, both at 40°C. The organic solvent

mixture was placed on ice and partitioned by vigorous shaking with 1 cm³ of 0.9% (w/v) NaCl. After centrifugation, 1 cm³ of the organic phase was evaporated gently under a N₂ stream and stored at -80°C under N₂. The next day, the pellet was resuspended in 150 mm³ of ethanol, filtered (0.22 µm), and injected (20 mm³) on the HPLC (System Gold, Beckman). Vitamin E was separated on an analytical C₁₈ column (4.6 x 250 mm, 5 µm, Ultrasphere), eluted with 95% methanol at 2 cm³ min⁻¹, and detected at 292 nm. The retention time of vitamin E was ≈ 19.5 min and the purity of the peak was ascertained by HPLC with photodiode-array detection (Waters).

Lipid peroxides and oxidized proteins were extracted from leaves (0.5 g) as previously described (Moran *et al.* 1994). Lipid peroxidation was estimated as the content of thiobarbituric-reactive substances (Minotti & Aust 1987), and protein oxidation was measured as the content of carbonyl groups upon reaction with 2,4-dinitrophenylhydrazine (Levine *et al.* 1990).

Statistical analyses

All values reported in this work are means of at least 3 replicates corresponding to measurements or extracts made from different plants chosen at random. The exact number of replicates is stated in the Table and Figures. Means were compared by one-way analysis of variance and Duncan's multiple range test at $P=0.05$.

Chemicals

Organic solvents, inorganic acids and salts used for preparing nutrient solutions were analytical or HPLC grade from Panreac (Barcelona, Spain). All other chemical and biochemicals were of the highest quality available from Sigma or Aldrich. Chelex-100 (200-400 mesh, Na⁺ form) resin was obtained from Bio-Rad. Single-distilled water was used for preparing the

nutrient solutions, and ultrapure water, obtained through a Milli-Q system (Millipore, Milford, MA, USA), was used for all other purposes.

RESULTS

In this study, the shoot of Fe⁺ and Fe⁻ pea plants was separated into three parts (upper, medium and lower) for determination of physiological and biochemical parameters (Fig. 1). Each of these three parts was reasonably homogenous from a metabolic viewpoint, as judged from the low variability (SEM were typically < 10 % of the mean for 4-5 replicates) observed in the values for most physiological and biochemical parameters obtained from different plants. However, there was considerable variation among the values obtained for the upper, middle and lower parts of the same shoot. This variation was due to the different leaf age and therefore independent harvest of the three parts was found to be essential for comparing Fe⁺ and Fe⁻ plants.

Metal analyses and physiological parameters

When Fe was omitted from the nutrient solution, leaves became chlorotic after 5 to 7 d. Chlorosis was very severe in upper leaves, less intense in medium leaves and not apparent in lower leaves. Visual symptoms of chlorosis were in good agreement with the readings of the chlorophyll meter (SPAD), which greatly facilitated the harvest of the three types of leaves. Readings were in the range of 13-28, 28-38 and 40-50 for the upper, medium and lower leaves of Fe⁻ plants, respectively, and in the range of 40-50 for all three types of leaves of Fe⁺ plants (Fig. 1).

ICP and flameless atomic absorption spectrophotometry were used to quantify transition metals in leaves. The Fe contents of upper, medium and

lower leaves were 40%, 53% and 71% those of the corresponding Fe⁺ leaves (Fig. 2). There was no variation in the Fe content of Fe⁺ leaves but, as expected, the upper leaves of Fe⁻ plants had much less Fe (61%) than the lower leaves. In contrast, the Cu content of Fe⁻ leaves did not differ significantly from that of Fe⁺ leaves but it increased with leaf age in parallel for both Fe⁺ and Fe⁻ plants (Fig. 2). In the free form or bound to many small chelates, such as organic acids, amino acids or nucleotides, Fe and Cu are active catalysts of Fenton-like reactions (Halliwell & Gutteridge 1989). Separation of leaves into two fractions by ultrafiltration (3 kDa exclusion limit) allowed us to calculate the contents of catalytic Fe and Cu in leaves (<3 kDa fraction). The content of catalytic Fe was virtually zero in the upper and medium leaves of Fe⁻ plants, and was drastically decreased in lower leaves (8% of that in the corresponding leaves of Fe⁺ plants). In addition, the content of catalytic Fe was 4-fold greater in lower leaves than in upper leaves. In contrast, there was no substantial change in the content of catalytic Cu in response to Fe deficiency, with the exception of the medium leaves of Fe⁻ plants, which contained greater amounts than those of Fe⁺ plants (Fig. 2).

Fe deficiency caused a large increase in the content of Mn: 2.6-fold in upper leaves and 1.5-fold in medium and lower leaves of Fe⁻ plants (Fig. 2). Likewise, the content of Mn markedly increased with leaf age. Thus, the lower leaves of Fe⁺ and Fe⁻ plants contained 2.5- and 1.4-fold more Mn than the corresponding upper leaves. The contents of Mn in the upper leaves of Fe⁻ plants and in the lower leaves of Fe⁺ plants were similar, in the order of 180 $\mu\text{g}\cdot\text{g DW}^{-1}$, and increased up to 263 $\mu\text{g}\cdot\text{g DW}^{-1}$ in the lower leaves of Fe⁻ plants (Fig. 2). To further investigate the accumulation of Mn in leaves, extracts were fractionated by ultrafiltration. The fraction containing molecules of >3 kDa mostly represents Mn associated to proteins. This Mn was not quantified by atomic absorption spectrophotometry, but its content can be

estimated by subtraction from the values of total Mn. The content of Mn in the < 3 kDa fraction of upper leaves was 2.5-fold higher in Fe⁻ plants than in Fe⁺ plants, and about 1.5-fold higher in the medium and lower leaves of Fe⁻ plants than in the corresponding leaves of Fe⁺ plants (Fig. 2). Other d-block metals were also quantified by ICP. However, Cr, Co, Cd, Ni and Mo were found at very low levels (1-5 µg·g DW⁻¹), and Zn (≈ 40 µg·g DW⁻¹) is not redox active and, hence, not relevant in this study.

The carbon and water status of leaves was assessed by measuring photosynthesis, stomatal resistance, transpiration and water potential. Other physiological parameters such as stomatal density, chlorophyll and soluble protein were also quantified (Table 1). The upper leaves exhibited photosynthetic rates 28% lower in Fe⁻ plants than in Fe⁺ plants but the medium and lower leaves had similar rates for both Fe⁻ and Fe⁺ plants. Fe deficiency had no effect on the water status of leaves because there were no changes in the values of water potential and its components nor in those of stomatal resistance and transpiration (Table 1). The stomatal density in the adaxial and abaxial epidermis was also similar (≈100 pores·mm⁻²) for both Fe⁺ and Fe⁻ leaves. As expected, there was a small, yet significant, gradient of water potential along the shoot, with upper leaves having ≈ -0.40 and -0.88 MPa and lower leaves having ≈ -0.22 and -0.79 MPa of water potential and osmotic potential, respectively, regardless of the Fe nutritional status. In Fe⁻ plants, the contents of chlorophyll *a* and *b* consistently decreased in upper leaves but that of soluble protein was much less affected (Table 1).

Biochemical parameters

The activities of catalase, nonspecific peroxidase and ASC peroxidase decreased by 51% in the upper leaves of Fe⁻ plants (Fig. 3) but glycolate oxidase activity did not vary (data not shown). The activities of the other

enzymes participating in the ASC-GSH cycle, *i.e.* DHA reductase, MDA reductase and GSSG reductase, were similar in the upper leaves of Fe⁻ and Fe⁺ plants. Another antioxidant enzyme, SOD, occurs in aerobic organisms as a mixture of isoenzymes differing in their cofactor metals. The use of the selective inhibitors CN⁻ and H₂O₂ permits to differentiate the isoenzymes (Halliwell & Gutteridge 1989). We found Mn-SOD (H₂O₂- and CN-insensitive) and CuZn-SOD (CN-sensitive) activities in pea leaves but not Fe-SOD activity (CN-insensitive, H₂O₂-sensitive). Fe deficiency did not affect Mn-SOD activity but caused a 27% increase in CuZn-SOD activity (Fig. 3). On the other hand, catalase, ASC-peroxidase, CuZn-SOD and Mn-SOD activities did not vary with leaf age, whereas nonspecific peroxidase activity decreased by 18%, DHA reductase and GSSG reductase activities decreased by 36%, and MDHA reductase activity increased by 34% (Fig. 3).

The content of total carotenoids in leaves was markedly reduced (-58%) in the upper leaves of Fe⁻ plants but did not change with leaf age (Fig. 4). The content of ASC decreased by 24% and that of GSH, GSSG and vitamin E remained constant. The GSH/GSSG ratio, indicative of the redox status of the cells, was maintained in the range of 10-14, which means that > 90 % of the total glutathione was in the form of GSH (Fig. 4). In contrast, there were large variations in the contents of low-molecular-mass antioxidants with leaf age. The contents of ASC, GSH and GSSG substantially decreased whereas that of vitamin E increased 3-fold.

DISCUSSION

Pea plants subjected to Fe deficiency contained less chlorophyll *a*, chlorophyll *b* and carotenoids (Table 1, Fig. 4). These parameters correlated with each other ($r^2 > 0.92$, $P < 0.003$) and also with the content of Fe in leaves

($r^2 > 0.64$, $P < 0.06$). The correlation between chlorophyll and Fe is explained by the known Fe requirement for the formation of δ -aminolevulinic acid and protochlorophyllide, precursors of the chlorophyll molecule (Marschner 1986). Protein synthesis is impaired under Fe deficiency and this may also account, at least in part, for the decline in the contents of chlorophylls and carotenoids. In our conditions, however, Fe deficiency affected only slightly (-13%) the content of soluble protein in leaves (Table 1) but this decrease could be much larger for the chromoproteins of chloroplasts, which are particularly susceptible to degradation under Fe deficiency (Marschner 1986).

Fe deficiency caused a 44-62% drop in content of photosynthetic pigments and a 28% inhibition of the rate of net photosynthesis in the upper leaves (Table 1). The decrease in photosynthetic activity cannot be attributed to CO₂ limitation by stomatal closure or to a lower pore number because water and osmotic potentials, stomatal resistance, transpiration and stomatal density were identical for Fe- and Fe+ leaves (Table 1). Rather, it may be related to the decline in the amount of light-harvesting and electron transport apparatus, which is probably a result of a decreased photochemical capacity (Terry 1980).

The accumulation of elevated amounts of Mn in Fe- leaves is noteworthy (Fig. 2) and may originate from an increased Mn uptake through the roots, a process that can lead to Mn toxicity (Marschner 1986). The increases in the content of Mn in the <3 kDa fraction of Fe- leaves were similar to those noticed for the contents of total Mn, which indicates that a large proportion of the Mn present in leaves is not bound to proteins and that this "low-molecular-mass" Mn is at least partly responsible for the observed increase in the Mn content of Fe- leaves.

Preparation of leaf extracts may result in decompartmentation of metals and therefore in artifactual formation of complexes between metals and small

molecules. However, average concentrations of metals in leaves can be assessed considering the percentage of water for each type of leaf (between 87 and 90% of fresh weight) and assuming homogenous distribution of metals throughout leaves. In this way, the average concentration of Mn in the < 3 kDa fraction of leaves was estimated as 70-160 μM for Fe- plants and 180-190 μM for Fe+ plants. This Mn may be present in the free state and/or bound to small molecules (chelates) and its concentration is greater than that of Fe (2.3-9.6 μM) and Cu (4.0-4.6 μM) in the same leaf fraction. Mn is capable of redox cycling when not bound to proteins and hence a potential candidate for catalyzing free radical production (Williams 1985). Unlike Fe^{2+} and Cu^+ , however, Mn^{2+} cannot reduce H_2O_2 to toxic $\cdot\text{OH}$ radicals (Asada & Takahashi 1987) nor decompose lipid peroxides into highly toxic alkoxyl and peroxy radicals (Halliwell & Gutteridge 1989). Indeed, Mn^{2+} may act as an antioxidant because it can inhibit lipid peroxidation by efficiently quenching peroxy radicals (Coassin, Ursini & Bindoli 1992). This function of Mn^{2+} as an antioxidant would explain why leaves can accumulate high levels of "low-molecular-mass" Mn, apparently with no detrimental effect on the plant.

The activities of the three hemoproteins investigated here, catalase, nonspecific peroxidase and ASC peroxidase, decreased by 51% in the upper leaves of Fe- plants (Fig. 3). The consistent decline in catalase activity, along with that of glycolate oxidase, might be conducive to an increase in the concentration of H_2O_2 in leaves because H_2O_2 rapidly crosses the peroxisomal membrane (Halliwell & Gutteridge 1989). However, we could not detect any increase in the average concentration of H_2O_2 in leaves, which suggests that either the remaining catalase activity in the upper leaves of Fe- plants is sufficient to cope with the peroxisomal generation of H_2O_2 or that the ASC peroxidase in the cytosol is being dealt with the H_2O_2 released by peroxisomes. Catalase and peroxidase activities were correlated with the Fe content in leaves, the correlation being highest for ASC peroxidase

($r^2=0.99$, $P<0.001$) and lower for guaiacol peroxidase ($r^2=0.84$, $P=0.01$) and catalase ($r^2=0.79$, $P=0.02$). These findings indicate that under severe Fe deficiency the activity (and probably the level) of these hemoproteins is determined by Fe availability, and that ASC peroxidase activity is an excellent marker of the Fe nutritional status of pea plants. Similarly, catalase and nonspecific peroxidase in tomato, cereals and other plants (Marschner 1986), and CuZn-SOD and Mn-SOD in pea (Del Río *et al.* 1978) have been used with apparent success for the diagnosis of nutritional disorders involving the respective metals.

Fe deficiency did not affect Mn-SOD activity but led to increased CuZn-SOD activity (Fig. 3). When specific activities were calculated from values of protein (Table 1), CuZn-SOD activity rose from 2.1 to 3.1 units $\cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$. As found for drought-stressed plants (Moran *et al.* 1994), this increase may represent a response of Fe- plants to enhanced O_2^- production in the chloroplasts, which contain most CuZn-SOD activity of photosynthetic tissue (Del Río *et al.* 1992). Several plant tissues, including bean leaves (Cakmak & Marschner 1993), have been reported to contain predominantly CuZn-SOD activity. Our finding of comparable activities for CuZn-SOD and Mn-SOD should probably be ascribed to the inclusion of Triton X-100 in the extraction medium (Moran *et al.* 1994), which may result in higher recoveries of Mn-SOD from the mitochondria. This explanation does not exclude additional factors, such as an abundance of mitochondria (and peroxisomes) in pea leaves relative to other plant tissues.

Low-molecular-mass antioxidants are also very important in photosynthetic cells (Thompson *et al.* 1987). With the same assumptions indicated above for metals, average concentrations of ASC and GSH in the upper leaves were estimated, respectively, as 5 mM and 0.5 mM for Fe⁺ leaves, and 4 mM and 0.5 mM for Fe⁻ leaves (Fig. 4). The concentrations in the chloroplasts were probably even higher (10-20 mM for ASC and 1-4 mM

for GSH; Halliwell & Gutteridge 1989). Consequently, Fe deficiency did not cause any significant weakening of the nonenzymatic antioxidant defenses of leaves. This contention is also supported by the maintenance of a high GSH/GSSG ratio (> 10) during Fe deficiency (Fig. 4). These highly reducing conditions are essential for chloroplast functioning because GSSG is an inhibitor of protein synthesis and inactivates several enzymes (Halliwell & Gutteridge 1989).

Proteins and membrane lipids are especially prone to attack by free radicals and are considered reliable indicators of oxidative stress in animal and plant tissues (Halliwell & Gutteridge 1989; Moran *et al.* 1994). Lipid peroxidation can be estimated as the content of substances that react with 2-thiobarbituric acid, and protein oxidation as the content of carbonyl groups, using carefully chosen controls in both cases. However, significant differences were not detected in the amounts of oxidized lipids and proteins between Fe⁺ and Fe⁻ leaves. Mean values across all types of leaves ($\pm$ standard error of mean, $n= 36$ replicates) were 110 ± 7 nmol malondialdehyde equivalents $\cdot$ (g DW)⁻¹, and 10 ± 0.2 nmol carbonyl groups $\cdot$ (mg protein)⁻¹, respectively.

The similar amount of peroxidized lipids in Fe⁻ and Fe⁺ leaves, despite the pronounced decline in the content of total carotenoids, indicates that chloroplasts are still sufficiently protected against $^1\Delta_gO_2$. This can be explained by a low rate of $^1\Delta_gO_2$ formation due to the moderate illumination used in this study and to the parallel decrease in the concentration of chlorophyll (Table 1) and of its subsequent excited triplet state. Besides, the content of vitamin E, which is also an important scavenger of $^1\Delta_gO_2$ in membranes (Halliwell & Gutteridge 1989), was not affected by Fe stress (Fig. 4).

The lack of increased oxidation of proteins and lipids in Fe⁻ leaves might be related to the demonstration that Fe⁻ leaves are virtually devoid of

catalytic Fe (Fig. 2). Thus, both lipid and protein oxidation have a strict requirement for a suitable transition metal (Halliwell & Gutteridge 1989; Stadtman & Oliver 1991). Although Fe may be the required metal for lipid peroxidation (Minotti & Aust 1987), Cu is more active than Fe in catalyzing protein oxidation (Moran *et al.* 1994). Because pea leaves have similar levels of catalytic Fe and Cu, both metals could be potential sources of activated oxygen species *in vivo*. In this context, a comparison can be made with a previous work (Moran *et al.* 1994). In pea plants subjected to drought, the concentrations of catalytic Fe and Cu increased 1.5-fold and 2.5-fold, respectively, and oxidized lipids and proteins accumulated in leaves (Moran *et al.* 1994). In contrast, in pea plants subjected to Fe deficiency, catalytic Fe was absent or drastically decreased, catalytic Cu did not increase in very chlorotic leaves, and no oxidative damage to lipids and proteins occurred. Taken together, these results lend further indirect support to our previous hypothesis that increases in the amounts of catalytic metals, especially of Fe, are required for ensuing oxygen free radical-mediated damage to plant proteins and lipids.

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Legends for Figures

Figure 1. Diagrammatic representation of the procedure followed to separate the shoots into upper, medium and lower parts. For each part, only the leaves were harvested. After growing in hydroponic culture on half-Hoagland nutrient solution, pea plants were separated into two groups, which received the same solution, omitting (-Fe) or not (+Fe) the Fe. Contents of chlorophyll *a+b* are indicated for each part of the plant as percentage of the maximum value [100%=15.4 mg·(g DW)⁻¹].

Figure 2. Contents of Fe, Cu and Mn in whole leaves and in the low-molecular-mass fraction (< 3 kDa) of leaves of Fe⁺ () and Fe⁻ () pea plants. Values are means of 4-6 replicates and those denoted by the same letter were not significantly different at *P*= 0.05.

Figure 3. Antioxidant enzymes in leaves of Fe⁺ () and Fe⁻ () pea plants. Enzymatic activities are expressed in min⁻¹·(g DW)⁻¹. One unit of SOD was the amount of enzyme which inhibited the O₂⁻-dependent reduction of ferricytochrome *c* by 50% (McCord & Fridovich 1969). Values are means of 3-6 replicates and those denoted by the same letter were not significantly different at *P*= 0.05.

Figure 4. Low-molecular-mass antioxidants in leaves of Fe⁺ () and Fe⁻ () pea plants. Values are means of 3-6 replicates and those denoted by the same letter were not significantly different at *P*= 0.05.