

**Iron-dependent oxygen free radical generation in plants subjected to environmental stress: toxicity and antioxidant protection**

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## **Abstract**

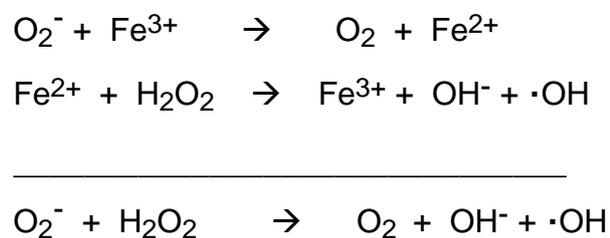
Iron has a pivotal and dual role in free radical chemistry in all organisms. On the one hand, free Fe can participate in Fenton reactions and catalyze ('catalytic Fe') the generation of hydroxyl radical and other toxic oxygen species. On the other hand, Fe is a constituent of the antioxidant enzymes catalase, ascorbate peroxidase, guaiacol peroxidase, and ferro-superoxide dismutase. Protein Fe is Fenton inactive but can be released from proteins upon attack by activated oxygen. Healthy, unstressed plants avoid the interaction of catalytic Fe and peroxides by disposing of Fe in vacuoles and apoplast, by sequestering Fe in ferritin, and by having high levels of antioxidant enzymes in most subcellular compartments. However, when plants are exposed to a variety of adverse conditions, including chilling, high light, drought and paraquat, oxidative stress ensues due primarily to the decrease in antioxidant defenses but also to the increase in free radical production mediated by catalytic Fe. The latter accumulates in many stressed plant tissues. Oxidative stress may lead to metabolic dysfunction and ultimately to plant cell death, so it needs to be estimated conveniently by quantifying the oxidation products of lipids (malondialdehyde and other cytotoxic aldehydes), proteins (total carbonyls, methionine sulfoxide, 2-oxohistidine), and DNA (8-hydroxyguanine, 5-hydroxycytosine). Protein oxidation appears to be a more sensitive and precocious marker than is lipid peroxidation, and DNA damage may also prove to be a useful marker for stress studies in plants.

*Abbreviations:* MDA - malondialdehyde, SOD - superoxide dismutase, TBA - 2-thiobarbituric acid, TBARS - thiobarbituric acid-reactive substances.

## Introduction

Iron is an essential nutrient of plants. It is involved in fundamental processes such as photosynthesis, respiration, nitrogen fixation, DNA synthesis, and hormone formation (Briat and Lobréaux, 1997). The critical role of Fe in those biological processes largely stems from its redox properties ( $\text{Fe}^{2+}/\text{Fe}^{3+}$ ), which allow its participation in electron transfer reactions at physiological pH. However, in the aerobic cellular medium, Fe insolubility and toxicity may represent a major problem and all living organisms have evolved strategies to preserve Fe homeostasis against changes in the extracellular concentration of the metal (Briat and Lobréaux, 1997). These strategies include the chelation of Fe to organic acids or transferrin for transport, the compartmentation of Fe in the apoplast space and vacuoles, storage of Fe in ferritin, and the avoidance of the reaction between Fe and peroxides by subcellular compartmentation and by the presence of high levels of antioxidants.

In cells, free  $\text{Fe}^{2+}$  is toxic because it is able to catalyze the decomposition of  $\text{H}_2\text{O}_2$  to the extremely reactive hydroxyl ( $\cdot\text{OH}$ ) radical. This is known as the Fenton reaction. The resulting  $\text{Fe}^{3+}$  can be reduced back to  $\text{Fe}^{2+}$  by the superoxide radical ( $\text{O}_2^-$ ), regenerating  $\text{Fe}^{2+}$  and allowing the reaction to continue. The sum of these two reactions is known as the Haber-Weiss reaction:



The free  $\text{Fe}^{2+}$  ions can be replaced by  $\text{Cu}^+$  (Gutteridge and Wilkins, 1983) or by  $\text{Fe}^{2+}$  bound to many small chelators (carboxylic acids, di- and triphosphate nucleotides) as Fenton catalysts (Baker and Gebicki, 1986; Floyd, 1983).

The  $O_2^-$  radical can be substituted for by ascorbate as the reductant of the metal ion. Furthermore, many pro-oxidant effects of ascorbate *in vitro*, and possibly *in vivo*, can be ascribed to its capacity to furnish  $Fe^{2+}$  for Fenton reactions (Aruoma et al., 1987; Halliwell and Gutteridge, 1990). Thiols and pyridine nucleotides can also reduce  $Fe^{3+}$  but the actual reducing species may be  $O_2^-$  rather than the metabolites themselves (Rowley and Halliwell, 1982a,b).

Because plants contain all the basic components required for *in vitro* production of  $\cdot OH$  radicals, it is most important to determine whether Fenton reactions also occur *in vivo*. In this review, we will address the dual role of Fe as a pro-oxidant factor and as a constituent of antioxidant enzymes, especially in plants. Some emphasis will be placed on current methodology to estimate oxidants, antioxidants, and oxidative damage. Whenever possible, we will refer to review articles in order to keep the bibliography within reasonable limits and to guide readers to more specialized literature.

## **Role of catalytic iron in the generation of activated oxygen**

### *Production of hydroxyl radicals mediated by catalytic iron*

The potential toxicity of catalytic Fe in biological systems can be ascribed for the most part to the formation of  $\cdot OH$  radicals. The most common techniques used to detect this and, for comparison, other activated oxygen species are indicated in Table 1. In chemical terms, the two most satisfactory methods to detect  $\cdot OH$  radicals are electron paramagnetic resonance and aromatic hydroxylation. The former measures directly the  $\cdot OH$  radicals produced in a system using a spin-trap such as 5,5'-dimethylpyrroline-*N*-oxide (DMPO). The DMPO- $\cdot OH$  adduct shows a characteristic pattern which, using adequate controls, can be considered as proof of  $\cdot OH$  production in a biological system (Buettner, 1985).

Methods based on aromatic hydroxylation are also very reliable when used with appropriate controls and have the added advantage that they do not require sophisticated equipment. Thus, the  $\cdot\text{OH}$  radical will hydroxylate salicylic acid to yield a mixture of about 11% of catechol, 49% of 2,3-dihydroxybenzoate and 40% of 2,5-dihydroxybenzoate, as measured by HPLC (Halliwell et al., 1987). Another example is the hydroxylation of phenylalanine by the  $\cdot\text{OH}$  radical to produce a mixture of the three tyrosine isomers in rather definite proportions which can be determined by HPLC (Kaur et al., 1988). Finally, the  $\cdot\text{OH}$  radicals will degrade deoxyribose to TBARS which can be quantified by fluorescence (Aruoma et al., 1987; Halliwell et al., 1987).

When using techniques based on aromatic hydroxylation, it is necessary to verify the production of  $\cdot\text{OH}$  radicals by adding a battery of compounds to the reaction mixtures. These compounds include good and poor  $\cdot\text{OH}$  radical scavengers as well as native and denatured SOD and catalase. This can be illustrated by an example from our own research (Becana and Klucas, 1992). The purpose was to demonstrate that  $\cdot\text{OH}$  radicals were formed through a Fenton reaction catalyzed by the Fe present in the plant extracts. Reaction mixtures comprised the low-molecular-mass fraction of root nodules (containing catalytic Fe), deoxyribose,  $\text{H}_2\text{O}_2$  and one of the compounds listed in Table 2. Relative to control, both SOD and catalase inhibited the reaction but the denatured enzymes did not. Desferrioxamine, a strong chelator that binds  $\text{Fe}^{3+}$  in a Fenton-inactive form, suppressed the degradation, indicating that Fe in the extracts was indeed catalyzing deoxyribose degradation. Mannitol, Hepes, thiourea and salicylate, which are good  $\cdot\text{OH}$  scavengers, partially inhibited the reaction, but urea, which is a very poor scavenger, did not inhibit (Table 2). This indicates that the  $\cdot\text{OH}$  radical was the free radical species causing deoxyribose degradation. The capacity of extracts from control and stressed plants to catalyze free radical production *in vitro* was compared using the deoxyribose and lipid degradation assays; the oxidation of deoxyribose and linolenic acid was higher in extracts from stressed tissue, which

indicates that the potential of these extracts to generate  $\cdot\text{OH}$  radicals, and probably other activated oxygen species, was also higher than that of extracts from control, healthy tissue (Becana and Klucas, 1992).

Experiments *in vivo* have demonstrated that the  $\cdot\text{OH}$  radical is formed during oxidative stress. Babbs et al. (1989) developed a technique to detect  $\cdot\text{OH}$  *in vivo*. They fed plants with dimethyl sulfoxide (DMSO) at high, but nontoxic, concentrations to ensure that all nascent  $\cdot\text{OH}$  radicals in leaves were trapped by DMSO. The reaction between DMSO and  $\cdot\text{OH}$  radical yields methanesulfinic acid, which can then be extracted from the tissue and quantified by spectrophotometry or HPLC. In this way, Babbs et al. (1989) demonstrated that  $\cdot\text{OH}$  radicals were formed at lethal concentrations in leaves sprayed with paraquat. Using the same method  $\cdot\text{OH}$  radical formation was detected in legume nodules induced to senesce by exposing the plants to prolonged darkness (Becana and Klucas, 1992).

#### *Other oxidative reactions mediated by catalytic iron*

Catalytic Fe can participate as a prooxidant factor in other reactions of biological relevance. It can promote the decomposition of lipid hydroperoxides (LOOH) to alkoxy ( $\text{LO}\cdot$ ) and peroxy ( $\text{LOO}\cdot$ ) radicals, thus contributing to the propagation of lipid peroxidation (Aust et al., 1985):



The reaction of  $\text{Fe}^{2+}$  with LOOH is an order of magnitude faster than that with  $\text{H}_2\text{O}_2$  but the reaction of  $\text{Fe}^{3+}$  with LOOH seems much slower than that with  $\text{Fe}^{2+}$  (Halliwell and Gutteridge, 1990). Finally, catalytic Fe accelerates the oxidation, mediated by  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ , of important metabolites, such as ascorbate, glutathione, and pyridine nucleotides (Halliwell and Gutteridge, 1986).

### *Measurement of catalytic iron*

To investigate the role of catalytic Fe in oxidative stress requires sensitive and accurate measurements because catalytic Fe is expected to be present at very low concentrations *in vivo* and because protein Fe is Fenton inactive (Aust and Koppenol, 1991; Halliwell and Gutteridge, 1986). Catalytic Fe can be assayed using the antitumoral antibiotic bleomycin, which forms a ternary complex with Fe and DNA. In the presence of bleomycin, Fe<sup>3+</sup>, O<sub>2</sub> and ascorbate, DNA is degraded to yield MDA, which can be quantified by colorimetry or fluorescence (Halliwell and Gutteridge, 1990). The chemistry of the reaction is very complex, but following detailed protocols, mainly aimed to avoid contamination with adventitious metals, the amount of MDA formed from DNA is proportional to the concentration (usually about 0.5-3 μM) of catalytic Fe in the sample (Aruoma et al., 1987; Halliwell et al., 1987).

The concentration of catalytic Fe in leaves and nodules of legumes has been determined using the bleomycin assay (Becana and Klucas, 1992; Escuredo et al., 1996; Gogorcena et al., 1997; Moran et al., 1994). There is virtually no catalytic Fe in control plant tissues but it can be clearly detected in senescent tissues. For example, catalytic Fe increases steadily in bean nodules induced to senesce by application of nitrate or continuous darkness, or in pea leaves exposed to drought or paraquat. In the latter case, the concentration of catalytic Fe is surprisingly high, which strongly suggests that at least part of the detrimental effect of the herbicide is mediated by the release of catalytic Fe from proteins (Iturbe-Ormaetxe et al., 1998). This hypothesis is substantiated by studies showing that pretreatment of pea plants with desferrioxamine lessens the toxic effects of paraquat (Zer et al., 1994).

## Protection by antioxidants and by iron sequestration

### *Antioxidant defenses*

So far we have examined the toxic effects of Fe. However, this metal is also a constituent of important antioxidant enzymes. In fact, all aerobic organisms, and especially plants, are endowed with an impressive array of antioxidants to cope with activated oxygen (Asada, 1996; Dalton, 1995; Foyer, 1993). The antioxidant metabolites of plants include ascorbate, glutathione, carotenoids, tocopherols, uric and lipoic acids, and ubiquinol. Glutathione reaches a concentration of 0.2-0.8 mM in nodules and 1-5 mM in chloroplasts, and ascorbate 1-2 mM in nodules and 10-50 mM in chloroplasts (Dalton, 1995; Escuredo et al., 1996; Foyer, 1993; Gogorcena et al., 1997). Antioxidant enzymes of plants include catalase, nonspecific peroxidases, SOD, and the enzymes of the ascorbate-glutathione cycle (Asada, 1996; Dalton, 1995; Foyer, 1993; Scandalios et al., 1980). This involves the enzymes ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and glutathione reductase. Ultimately, during the operation of the cycle,  $H_2O_2$  is reduced to water at the expense of NADH or NADPH (Fig. 1).

Several characteristics of plant antioxidant enzymes are indicated in Table 3. Some of these enzymes contain Fe, either in heme (catalases, peroxidases) or nonheme (Fe-SODs) form. Catalases decompose  $H_2O_2$  to water and  $O_2$ , and are mostly confined to peroxisomes and glyoxysomes, although a specific isoform (Cat-3) is present in maize mitochondria (Scandalios et al., 1980). Peroxidases also decompose  $H_2O_2$  but, unlike catalase, they rely on various organic electron donors to reduce  $H_2O_2$  to water. Plants usually contain many isoenzymes of peroxidases with nonspecific activity. These isoenzymes are involved in lignin and ethylene synthesis and in plant development and organogenesis (Campa, 1991). Ascorbate peroxidases participate in the detoxification of  $H_2O_2$  in several cellular compartments, including the stroma (soluble form) and thylakoids (membrane-

bound form) of chloroplasts, cytosol, peroxisomes, and mitochondria (Asada, 1996). Finally, Fe-SODs, initially thought to be present only in a few plant species (Bridges and Salin, 1981), have now been detected in the chloroplasts of many plants (Bowler et al., 1994) and could be also present in the peroxisomes and mitochondria of nonphotosynthetic tissue (Droillard and Paulin, 1990).

The effect of Fe deficiency on the antioxidant status of pea leaves has been investigated (Iturbe-Ormaetxe et al., 1995). Omission of Fe from the nutrient solution caused a 40% decrease in the Fe content of the upper leaves and a 50% decrease in catalase, guaiacol peroxidase and ascorbate peroxidase activities; however, Fe deficiency had no effect on the antioxidant enzymes lacking Fe. The high correlation found between Fe and enzyme activities led the authors to propose that the activity (and probably the level) of the hemoproteins was controlled by the availability of Fe in the tissue (Iturbe-Ormaetxe et al., 1995). Interestingly, very recently Fe has been reported to induce ascorbate peroxidase gene expression in rapeseed leaves (Vansuyt et al., 1997).

Pea leaves subjected to Fe deprivation had virtually no catalytic Fe and did not accumulate oxidatively-modified lipids or proteins. Due mainly to the absence of oxidative damage, despite the substantial decrease in the key H<sub>2</sub>O<sub>2</sub>-detoxification enzyme activities, these results were interpreted as indirect support for the hypothesis that catalytic Fe is involved in the oxidative damage of lipids and proteins (Iturbe-Ormaetxe et al., 1995).

### *Iron sequestration*

The Fe present in antioxidant enzymes is obviously inactive for Fenton reactions, but this may be also true for other Fe-proteins. There has been considerable controversy about whether certain Fe-proteins from mammals, including hemoglobin, myoglobin, transferrin and ferritin, can act as Fenton catalysts. It is now widely accepted that the Fe in those proteins is Fenton inactive but can become available for Fenton reactions if Fe is released from proteins following

oxidative attack (Aust et al., 1985; Gutteridge, 1986; Halliwell and Gutteridge, 1990). Soybean nodule leghemoglobin can release Fe when exposed to excess  $\text{H}_2\text{O}_2$ , and then free Fe can generate  $\cdot\text{OH}$  radicals outside or on the surface of the protein (Puppo and Halliwell, 1988). Plant ferredoxins are also attacked by  $\text{H}_2\text{O}_2$  but in this case a highly oxidizing species, not identical to the  $\cdot\text{OH}$  radical, is generated (Puppo and Halliwell, 1989).

Ferritin is a protein especially suitable for Fe sequestration and storage, so playing a critical role in Fe homeostasis. Animal and plant ferritins are encoded by nuclear genes, have similar three-dimensional structures, and are induced by Fe overload (Briat and Lobréaux, 1997). However, there are important differences between animal and plant ferritins (Proudhon et al., 1996; Briat and Lobréaux, 1997). The gene of animal ferritins contains seven introns but that of plant ferritins only three. Animal ferritins are localized in the cytosol but plant ferritins are in the plastids. Control of animal ferritin synthesis by Fe occurs at the translational level and entails binding of Fe-responsive proteins to Fe-responsive elements located within the 5' untranslated region of the ferritin mRNA. Control of plant ferritin synthesis by Fe takes place at the transcriptional (and post-transcriptional) level and involves, in maize, two independent regulatory signals. One pathway is mediated by abscisic acid and the other by an oxidative step since  $\text{H}_2\text{O}_2$ , at low Fe concentration, induces ferritin gene expression (Lobréaux et al. 1995; Briat and Lobréaux, 1997). This finding is important because it provides a link between Fe and activated oxygen metabolisms and because it shows a role of ferritin in the oxidative stress response of plants.

Plant ferritins are composed of 24 subunits forming the protein shell, and the central cavity of the holoprotein can accommodate up to 4500 atoms of Fe in a soluble, nontoxic form. Loading of Fe in ferritin requires oxidation to  $\text{Fe}^{3+}$  by the ferroxidase activity of ferritin subunits, whereas unloading involves reduction to  $\text{Fe}^{2+}$  by a physiological reductant (Briat and Lobréaux, 1997). Many cellular compounds including  $\text{O}_2^-$  and organic radicals, ascorbate, flavins, and nitric oxide

have been proposed for the 'reductive mobilization' of Fe (Aust et al., 1985; Halliwell and Gutteridge, 1986; Reif, 1992). Despite the criticality of this step, it is still unclear what physiological reductant is involved in the release of Fe from the ferritin core. Ascorbate seems to be a plausible candidate. At concentrations greater than 2.5 mM, ascorbate promotes Fe release from pea seed ferritin *in vitro*, whereas at lower concentrations it favors uptake (Lobréaux et al., 1995). The high ascorbate concentrations present in chloroplasts may explain why there is no (or very little) ferritin in mature, fully photosynthetic leaves under normal conditions; in contrast, under Fe overload conditions, which induce ferritin synthesis, ascorbate concentration decreases and this would favor Fe uptake by ferritin (Laulhere and Briat, 1993; Lobréaux et al., 1995).

Because the Fe released from ferritin is catalytic, it will have to be incorporated rapidly in other proteins and chloroplasts will have to avoid any build-up of H<sub>2</sub>O<sub>2</sub> (and its precursor, the O<sub>2</sub><sup>-</sup> radical) that is formed continuously during photosynthesis. This may be another good reason why chloroplasts have high levels of ascorbate peroxidase and other enzymes of the ascorbate-glutathione cycle. Similarly, young legume nodules contain abundant ascorbate, protein and nonprotein Fe, and ferritin (Becana and Klucas, 1992; Ragland and Theil, 1993; Dalton, 1995). Because in nodules ferritin expression is developmentally regulated (Ragland and Theil, 1993), it will be interesting to know also whether there is a relationship between ascorbate and ferritin during nodule ontogeny.

In any case, much investigation still needs to be done to ascertain how plants manage to mobilize Fe from ferritin or other Fe reservoirs for the synthesis of Fe-proteins while avoiding the risk of free radical production. For example, it is still unclear whether the intracellular 'transit Fe pool' is redox active (Halliwell and Gutteridge, 1986; Aust and Koppenol, 1991) and whether it can react with the low steady-state concentrations (low  $\mu\text{M}$  range) of H<sub>2</sub>O<sub>2</sub> reported to occur in plant tissues (Warm and Laties, 1982).

The antioxidant content of young, healthy plant tissues is usually sufficient to prevent the incipient formation of free radicals or to destroy them once they have been formed. However, in aging plants or in plants exposed to stressful conditions such as drought, high light, or chilling, the rate of formation of free radicals may overwhelm the plant's antioxidant capacity. This oxidative stress results in damage to plant tissues. Hence, it is critical to develop reliable techniques to measure oxidative damage, and this is commonly performed using marker molecules.

## **Oxidative stress**

### *Lipid damage*

There is a vast array of techniques to quantify lipid peroxidation (Gutteridge and Halliwell, 1990). These include the measurement of diene conjugation, hydrocarbon gases, electrolyte leakage, and cytotoxic aldehydes such as MDA (Fig. 2a, Table 4). The aldehydes formed during lipid peroxidation can be measured by the TBA test. The sample is heated with TBA at low pH to yield an adduct which can be quantified by colorimetry or fluorescence. The results are expressed as TBARS (Table 4). This test is simple and easy to perform but requires stringent controls to correct for the artifactual formation of TBARS during the extraction and assay procedure. For instance, traces of free Fe (originating from buffer contaminants or from extracted plant tissue) may catalyze the decomposition of lipid peroxides during the heating step of the assay and produce high levels of TBARS (Gutteridge and Halliwell, 1990). Some of these pitfalls can be circumvented by including the antioxidant butylated hydroxytoluene in the extraction and reaction media, and by running parallel controls omitting TBA (replaced by NaOH) and controls omitting samples (replaced by extraction medium).

To avoid many interferences, it is best to separate the TBA<sub>2</sub>MDA adduct by HPLC (Draper et al., 1993). This technique allows for differentiation of MDA from

other cytotoxic aldehydes such as 4-hydroxynonenal. We have adapted an HPLC method to quantify MDA in plant tissues (Iturbe-Ormaetxe et al., 1998). Briefly, MDA is extracted from leaves or root nodules with metaphosphoric acid and a small amount of butylhydroxytoluene; the extract is centrifuged and the supernatant is reacted with TBA; finally, the adduct is extracted in butanol, dried under N<sub>2</sub>, and resuspended in HPLC solvent (15% acetonitrile and 0.6% tetrahydrofuran in 5 mM potassium phosphate pH 7.0). Separation of the adduct by HPLC with photodiode-array detection allowed us to identify unequivocally the peak by comparison of its retention time and visible spectrum with those of authentic MDA. Using this technique, the MDA content of pea leaves was found to increase during water stress (Iturbe-Ormaetxe et al., 1998).

#### *Protein damage*

Free radical attack on proteins results in a variety of products (Fig. 2c,d) which include methionine sulfoxide (Ferguson and Burke, 1994) and 2-oxohistidine (Lewisch and Levine, 1995). However, protein damage is usually estimated as the total content of carbonyl groups (Fig. 2b, Table 4). The technique involves precipitation of contaminating nucleic acids with streptomycin sulfate and derivatization of protein carbonyl groups with phenylhydrazine (Levine et al., 1990). The oxidative reaction leading to a carbonyl group proceeds through a site-specific mechanism (Stadtman, 1992). This is illustrated by the attack of a lysyl residue. The  $\epsilon$ -amino group binds Fe<sup>2+</sup> which is oxidized by H<sub>2</sub>O<sub>2</sub> with formation of  $\cdot$ OH radical according to Fenton chemistry. The resulting  $\cdot$ OH radical abstracts a hydrogen atom from the carbon adjacent to the amino group forming an alkyl radical and the terminal carbon is converted to carbonyl group (Fig. 2b). The residues most prone to oxidation to carbonyl derivatives are lysine, arginine, and proline (Stadtman, 1992).

The content of oxidatively-modified proteins in leaves and nodules invariably increases with stress (Escuredo et al., 1996; Gogorcena et al., 1997; Moran et al.,

1994). For instance, the application of a moderate water stress to peas (cultivar Frilene) caused an almost 2-fold increase in the protein carbonyl content of leaves (Table 4). Also, the content of protein carbonyl groups increases in legume nodules induced to senesce by exposure to nitrate or darkness, whereas no increase in total lipid peroxides was found under the same conditions (Escuredo et al., 1996; Gogorcena et al., 1997). This finding strongly suggests that protein oxidation is a more sensitive marker of oxidative stress in plants than is lipid peroxidation.

### *DNA damage*

Oxidative stress can be also assessed by measuring DNA damage. Certainly one of the mechanisms of DNA damage involves the  $\cdot\text{OH}$  radical, which is known to cause strand breakage, deoxyribose fragmentation, and extensive base modification; in contrast,  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  do not react with DNA (Halliwell and Aruoma, 1991). This is important because, as reviewed throughout this work, formation of  $\cdot\text{OH}$  radicals in biological systems requires a catalytic transition metal ion and the reaction is site-specific. Therefore, if DNA is damaged *in vivo* by the  $\cdot\text{OH}$  radical, we need to postulate that catalytic metals, mostly Fe but possibly also Cu, may bind nuclear and organellar DNA during oxidative stress.

In mammals, DNA damage can be measured as steady-state damage (*i.e.* the balance between damage and repair) or as total damage (*i.e.* that which has occurred but has been repaired). About 20 oxidation products from all DNA bases are known to be formed upon free radical attack (Fraga et al., 1990). The relative proportions of many of these products can be determined by gas chromatography-mass spectrometry with selected-ion-monitoring (GC-MS-SIM). This was used to measure steady-state levels of DNA damage in a number of pathological conditions and as a diagnostic 'fingerprint' for  $\cdot\text{OH}$  attack (Aruoma et al., 1989; Halliwell and Aruoma, 1991; Wiseman and Halliwell, 1996). For many purposes, however, and considering some inherent limitations, DNA damage in animal and human tissues can be estimated using 8-hydroxyguanine (Fig. 2e) or its nucleoside, 8-

hydroxydeoxyguanosine, as markers. These and other oxidized bases such as thymine glycol and 5-hydroxycytosine can be quantified by HPLC with electrochemical detection (Halliwell and Aruoma, 1991; Wagner et al., 1992; Wiseman and Halliwell, 1996). Irrespective of the type of measurement (GC-MS-SIM or HPLC), results have shown that there is a link between DNA damage and many degenerative processes, including aging, inflammatory disease, and cancer (Fraga et al., 1990; Wagner et al., 1992; Wiseman and Halliwell, 1996). This is not surprising because free radicals can be mutagenic, affect cytoplasmic and nuclear signal transduction, and modulate the activity of proteins and genes that are involved in the response to stress conditions (Wiseman and Halliwell, 1996).

Contrary to the enormous amount of work performed in animal and humans systems, there is, to the best of our knowledge, only one report analyzing the effects of stress on DNA damage in plants. Using HPLC with electrochemical detection, Floyd et al. (1989) quantified 8-hydroxyguanine in chloroplast DNA from bean and pea plants previously exposed to ozone (Table 4). The conclusion was clear-cut. Ozone-exposed plants had, in general, twice as much 8-hydroxyguanine as control plants; when the ozone treatment was performed in isolated chloroplasts instead of in intact plants, the levels were 7-fold higher than those of untreated chloroplasts (Table 4). These findings strongly suggest that DNA damage is involved in the detrimental effects of stress in plants at the molecular level.

## **Concluding remarks**

Iron is essential for many vital processes of plants. However, free Fe is Fenton active and plants have evolved strategies to minimize the risk of free radical production while maintaining the required steady-state levels of 'low-molecular-mass Fe' for biosynthetic purposes. Some of these strategies, such as the storage of Fe in ferritin and the avoidance of the interaction between peroxides and catalytic Fe, have been reviewed. Although significant progress has been made to understand the signal transduction pathways linking Fe and oxidative stress and to quantify the antioxidants of plants, whether healthy or stressed, at the tissue, cellular and subcellular levels, many key questions still remain. These include the complete characterization of the 'low-molecular-mass Fe' pool, the identification of the molecular mechanisms for ferritin loading and unloading, and the determination of the sequence of molecular events triggering oxidative damage in plants exposed to stress.

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## References

- Aruoma OI, Grootveld M and Halliwell B 1987 The role of iron in ascorbate-dependent deoxyribose degradation. Evidence consistent with a site-specific hydroxyl radical generation caused by iron ions bound to the deoxyribose molecule. *J. Inorg. Biochem.* 29, 289-299.
- Aruoma OI, Halliwell B and Dizdaroglu M 1989 Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hypoxanthine/xanthine oxidase. *J. Biol. Chem.* 264, 13024-13028.
- Asada K 1984 Chloroplasts: formation of active oxygen and its scavenging. *Methods Enzymol.* 105, 422-429.
- Asada K 1996 Radical production and scavenging in the chloroplasts. *In* *Photosynthesis and the Environment*. Ed. NR Baker. pp 123-150. Kluwer, Dordrecht.
- Aust SD and Koppenol WH 1991 Transition metals in oxidative stress: an overview. *In* *Oxidative Damage and Repair*. Ed. KJA Davies. pp. 802-807. Pergamon Press, Oxford.
- Aust SD, Morehouse LA and Thomas CE 1985 Role of metals in oxygen radical reactions. *Free Rad Biol Med* 1, 3-25.
- Babbs CF, Pham JA and Coolbaugh RC 1989 Lethal hydroxyl radical production in paraquat-treated plants. *Plant Physiol.* 90, 1267-1270.
- Baker MS and Gebicki JM 1986 The effect of pH on yields of hydroxyl radicals produced from superoxide by potential biological iron chelators. *Arch. Biochem. Biophys.* 246, 581-588.
- Becana M and Klucas RV 1992 Transition metals in legume root nodules: iron-dependent free radical production increases during nodule senescence. *Proc. Natl. Acad. Sci. USA* 89, 8958-8962.
- Brennan T and Frenkel C 1977 Involvement of hydrogen peroxide in the regulation of senescence in pear. *Plant Physiol.* 59, 411-416.

- Briat JF and Lobréaux S 1997 Iron transport and storage in plants. *Trends Plant Sci.* 2, 187-193.
- Bridges SM and Salin ML 1981 Distribution of iron-containing superoxide dismutase in vascular plants. *Plant Physiol.* 68, 275-278.
- Bowler C, Van Camp W, Van Montagu M and Inzé D 1994 Superoxide dismutase in plants. *Crit. Rev. Plant Sci.* 13, 199-218.
- Buettner GR 1985 Spin trapping of hydroxyl radical. *In* CRC Handbook of Methods for Oxygen Radical Research. Ed. RA Greenwald. pp 151-155. CRC Press, Boca Raton, Fla.
- Campa A 1991 Biological roles of plant peroxidases: known and potential function. *In* Peroxidases in Chemistry and Biology, Vol II. Eds J Everse, KE Everse and MB Grisham. pp 25-50. CRC Press, Boca Raton.
- Dalton DA 1995 Antioxidant defenses of plants and fungi. *In* Oxidative Stress and Antioxidant Defenses in Biology. Ed. S Ahmad. pp 298-355. Chapman and Hall, New York.
- Del Río LA, Sandalio LM, Palma JM, Bueno P and Corpas FJ 1992 Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Rad. Biol. Med.* 13, 557-580.
- Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S and Hadley M 1993 A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. *Free Rad. Biol. Med.* 15, 353-363.
- Droillard MJ and Paulin A 1990 Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence. *Plant Physiol.* 94, 1187-1192.
- Droillard MJ, Paulin A and Massot JC 1987 Free radical production, catalase and superoxide dismutase activities and membrane integrity during senescence of petals of cut carnations (*Dianthus caryophyllus*). *Physiol. Plant.* 71, 197-202.

- Escuredo PR, Minchin FR, Gogorcena Y, Iturbe-Ormaetxe I, Klucas RV and Becana M 1996 Involvement of activated oxygen in nitrate-induced senescence of pea root nodules. *Plant Physiol.* 110, 1187-1195.
- Ferguson DL and JJ Burke 1994 Methionyl sulfoxide content and protein-methionine-S-oxide reductase activity in response to water deficits or high temperature. *Physiol. Plant.* 90, 253-258.
- Floyd RA 1983 Direct demonstration that ferrous ion complexes of di- and triphosphate nucleotides catalyze hydroxyl free radical formation from hydrogen peroxide. *Arch. Biochem. Biophys.* 225, 263-270.
- Floyd RA, West MS, Hogsett WE and Tingey DT 1989 Increased 8-hydroxyguanine content of chloroplast DNA from ozone-treated plants. *Plant Physiol.* 91, 644-647.
- Foyer CH 1993 Ascorbic acid. *In* Antioxidants in Higher Plants. Ed. RG Alscher and JL Hess. pp 31-58. CRC Press, Boca Raton, Fla.
- Fraga CG, Shigenaga MK, Park JW, Degan P and Ames BN 1990 Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci. USA* 87, 4533-4537.
- Gogorcena Y, Gordon AJ, Escuredo PR, Minchin FR, Witty JF, Moran JF and Becana M 1997 N<sub>2</sub> fixation, carbon metabolism, and oxidative damage in nodules of dark-stressed common bean plants. *Plant Physiol.* 113, 1193-1201.
- Gutteridge JMC 1986 Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett.* 201, 291-295.
- Gutteridge JMC and Halliwell B 1990 The measurement and mechanism of lipid peroxidation in biological systems. *Trends Biol. Sci.* 15, 129-135.
- Gutteridge JMC and Wilkins S 1983 Copper salt-dependent hydroxyl radical formation: damage to proteins acting as antioxidants. *Biochim. Biophys. Acta* 759, 38-41.
- Halliwell B and Aruoma OI 1991 DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett.* 281, 9-19.

- Halliwell B, Grootveld M and Gutteridge JMC 1987 Methods for the measurement of hydroxyl radicals in biochemical systems: deoxyribose degradation and aromatic hydroxylation. *Methods Biochem. Anal.* 33, 59-90.
- Halliwell B and Gutteridge JMC 1986 Iron and free radical reactions: two aspects of antioxidant protection. *Trends Biochem. Sci.* 11, 372-375.
- Halliwell B and Gutteridge JMC 1990 Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* 186, 1-85.
- Haworth P and Hess FD 1988 The generation of singlet oxygen ( $^1O_2$ ) by the nitrodiphenyl ether herbicide oxyfluorfen is independent of photosynthesis. *Plant Physiol.* 86, 672-676.
- Jiménez A, Hernández JA, Del Río LA and Sevilla F 1997 Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol.* 114, 275-284.
- Iturbe-Ormaetxe I, Escuredo PR, Arrese-Igor C and Becana M 1998 Oxidative damage in pea plants exposed to water deficit or paraquat. *Plant Physiol.* 116, 173-181.
- Iturbe-Ormaetxe I, Moran JF, Arrese-Igor C, Gogorcena Y, Klucas RV and Becana M 1995 Activated oxygen and antioxidant defences in iron-deficient pea plants. *Plant Cell Environ.* 18, 421-429.
- Kaur H, Fagerheim I, Grootveld M, Puppo A and Halliwell B 1988 Aromatic hydroxylation of phenylalanine as an assay for hydroxyl radicals: application to activated human neutrophils and to the heme protein leghemoglobin. *Anal. Biochem.* 172, 360-367.
- Laulhere JP and Briat JF 1993 Iron release and uptake by plant ferritin: effects of pH, reduction and chelation. *Biochem. J.* 290, 693-699.
- Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S and Stadtman ER 1990 Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* 186, 464-478.

- Lewis SA and Levine RL 1995 Determination of 2-oxohistidine by amino acid analysis. *Anal. Biochem.* 231, 440-446.
- Lobréaux S, Thoiron S and Briat JF 1995 Induction of ferritin synthesis in maize leaves by an iron-mediated oxidative stress. *Plant J.* 8, 443-449.
- McRae DG and Thompson JE 1983 Senescence-dependent changes in superoxide anion production by illuminated chloroplasts from bean leaves. *Planta* 158, 185-193.
- Mittler R and Zilinskas BA 1991 Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiol.* 97, 962-968.
- Moran JF, Becana M, Iturbe-Ormaetxe I, Frechilla S, Klucas RV and Aparicio-Tejo P 1994 Drought induces oxidative stress in pea plants. *Planta* 194, 346-352.
- Murphy TM and Huerta AJ 1990 Hydrogen peroxide formation in cultured rose cells in response to UV-C radiation. *Physiol. Plant.* 78, 247-253.
- Popham PL and Novacky A 1991 Use of dimethyl sulfoxide to detect hydroxyl radical during bacteria-induced hypersensitive reaction. *Plant Physiol.* 96, 1157-1160.
- Proudhon D, Wei J, Briat JF and Theil EC 1996 Ferritin gene organization: differences between plants and animals suggest possible kingdom-specific selective constraints. *J. Mol. Evol.* 42, 325-336.
- Puppo A and Halliwell B 1988 Generation of hydroxyl radicals by soybean nodule leghaemoglobin. *Planta* 173, 405-410.
- Puppo A and Halliwell B 1989 On the reaction of plant ferredoxins with hydrogen peroxide. What reactive oxidants are generated? *Phytochemistry* 28, 3265-3270.
- Ragland M and Theil EC 1993 Ferritin (mRNA, protein) and iron concentrations during soybean nodule development. *Plant Mol. Biol.* 21, 555-560.
- Reif DW 1992 Ferritin as a source of iron for oxidative damage. *Free Rad. Biol. Med.* 12, 417-427.

- Rich PR and Bonner WD 1978 The sites of superoxide anion generation in higher plant mitochondria. *Arch. Biochem. Biophys.* 188, 206-213.
- Rich PR, Boveris A, Bonner WD and Moore AL 1976 Hydrogen peroxide generation by the alternate oxidase of higher plants. *Biochem. Biophys. Res. Commun.* 7, 695-703.
- Rowley DA and Halliwell B 1982a Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds. *FEBS Lett.* 138, 33-36.
- Rowley DA and Halliwell B 1982b Superoxide-dependent formation of hydroxyl radicals from NADH and NADPH in the presence of iron salts. *FEBS Lett.* 142, 39-41.
- Scandalios JG, Tong WF and Roupakias DG 1980 *Cat3*, a third gene locus coding for a tissue-specific catalase in maize: genetics, intracellular location, and some biochemical properties. *Mol. Gen. Genet.* 179, 33-41.
- Simontacchi M and Puntarulo S 1992 Oxygen radical generation by isolated microsomes from soybean seedlings. *Plant Physiol.* 100, 1263-1268.
- Stadtman ER 1992 Protein oxidation and aging. *Science* 257, 1220-1224.
- Vansuyt G, Lopez F, Inzé D, Briat JF and Fourcroy P (1997) Iron triggers a rapid induction of ascorbate peroxidase gene expression in *Brassica napus*. *FEBS Lett.* 410, 195-200.
- Wagner JR, Hu CC and Ames BN 1992 Endogenous oxidative damage of deoxycytidine in DNA. *Proc. Natl. Acad. Sci. USA* 89, 3380-3384.
- Warm E and Laties GG 1982 Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. *Phytochemistry* 21, 827-831.
- Wise RR and Naylor AW 1987 Chilling-enhanced photooxidation. *Plant Physiol.* 83, 278-282.
- Wiseman H and Halliwell B 1996 Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.* 313, 17-29.

- Yruela I, Pueyo JJ, Alonso PJ and Picorel R 1996 Photoinhibition of photosystem II from higher plants. Effect of copper inhibition. *J. Biol. Chem.* 271, 27408-27415.
- Zer H, Peleg I and Chevion M 1994 The protective effect of desferrioxamine on paraquat-treated pea (*Pisum sativum*). *Physiol. Plant.* 92, 437-442.

## Legends for Figures

*Fig. 1.* Antioxidant enzymes of higher plants. The scheme depicts the ascorbate-glutathione cycle as well as the reactions catalyzed by catalase and SOD.

*Abbreviations:* ASC=ascorbate; DHA=dehydroascorbate; GSH/GSSG=reduced/oxidized glutathione; MDHA=monodehydroascorbate (ascorbate free radical).

*Fig. 2.* Marker molecules commonly used to diagnose oxidative stress in biological systems. The figure shows the oxidation of **(a)** lipid hydroperoxides to MDA; **(b)** a lysyl residue of proteins to a carbonyl derivative; **(c)** methionine to methionine sulfoxide; **(d)** histidine to 2-oxohistidine; and **(e)** guanine to 8-hydroxyguanine.