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Iron induction of lipid peroxidation and effects on antioxidative enzyme activities in rice leaves

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Abstract

Lipid peroxidation in relation to toxicity of detached rice leaves caused by excess iron ($FeSO_4$) was investigated. Excess $FeSO_4$, which was observed to induce toxicity, enhanced the content of lipid peroxidation but not the content of H_2O_2. Superoxide dismutase activity was reduced by excess $FeSO_4$. Ascorbate peroxidase and glutathione reductase activities were increased by excess $FeSO_4$. Free radical scavengers, such as mannitol and reduced glutathione, inhibited excess iron-induced toxicity and at the same time inhibited excess iron-enhanced lipid peroxidation, suggesting that lipid peroxidation enhanced by excess iron is mediated through free radicals.

Abbreviations: APOD – ascorbate peroxidase, DR – dry weight, FW – fresh weight, GR – glutathione reductase, GSH – reduced glutathione, MDA – malondialdehyde, SOD – superoxide dismutase

Introduction

Wetland rice is grown in anaerobic soil or paddy-soil. Under this condition, the soluble Fe^{2+} ion is stable. Excess uptake of Fe^{2+} by rice may cause iron toxicity. Bronzing and deposition of brown pigments in leaves is a typical nutritional disorder in wetland rice caused by iron toxicity (Ponnamperuma et al. 1955; Tanaka et al. 1996). This symptom can be produced artificially by dipping the stem of de-rooted rice leaves (Yamauchi and Peng 1995), the cut end of detached rice leaves (Peng et al. 1996), or cut-root plants of *Nicotiana plumbaginifolia* (Kampfenkel et al. 1995) in $FeSO_4$ or Fe-EDTA, respectively.

Reactive oxygen species have been implicated in many degradative processes of plants, including aging, wounding, pathogen attack, and exposure of plants to some stress conditions (Elstner 1987; Inzé and Van Montagu 1995; Leshem 1988; Thompson et al. 1987). The superoxide radicals are generated at the membrane level in most plant cell organelles, and H_2O_2 is the product of SOD and of several oxidases of peroxisomes, such as glycolate oxidase (Del Rio et al. 1992; Thompson et al. 1987) and of polyamine metabolism, such as di- and polyamine oxidases (Smith 1985). The superoxide can serve as a source to generate more reactive hydroxyl radicals by Haber-Weiss and Fenton reactions (Naqui and Chance 1986). As a transition metal, iron is able to accelerate Haber-Weiss and Fenton reactions (Gutterida et al. 1981). Although iron-catalysed Haber-Weiss and Fenton reactions have been known for many years, the involvement of oxidative stress in iron-induced toxicity in plant tissues has received little attention. It has been reported that excess iron causes oxidative stress in *Nicotiana plumbaginifolia* (Kampfenkel et al. 1995) and sunflower leaves (Gallego et al. 1996) and induces free radicals in soybean roots (Caro and Puntarulo 1996) and drought plants (Price and Hendry 1991). In addition, Fe^{2+} was shown to stimulate hydrogen peroxide-induced lipid peroxidation (Degousee et al. 1994). Detoxification of reactive oxygen species can be achieved by antioxidative enzymes including superoxide dismutase (SOD) or enzymes of the ascorbate-glutathione cycle or non-specific peroxidases (Foyer et al. 1994; Inzé and Van Montagu

1995; Leshem 1988; Thompson et al. 1987). In the present paper, we have conducted a study of the effect of excess $FeSO_4$ on rice leaf oxidative stress and the activities of some antioxidative enzymes, as well as its prevention by free radical scavengers, such as mannitol and reduced glutathione (GSH).

Materials and methods

Rice (*Oryza sativa* L. cv. Taichung Native 1) was cultured as described previously (Lin et al. 1999). The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 ml of test solution. Incubation was carried out at 27 °C in the light (40 μmol m^{-2} s^{-1}).

Chlorophyll was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein extraction, leaf segments were homogenised in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 g for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976) and for enzyme assays. Malondialdehyde (MDA) was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968).

For the determination of Fe, leaf segments were dried at 65 °C for 48 h. Dried material was ashed at 550 °C for 20 h. Ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 72 °C for 2 h, and dissolved in 0.1 N HCl. Fe was then quantified using an atomic absorption spectrophotometer (Model AA-680, Shimadzu, Kyoto).

The H_2O_2 content was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenising 50 mg leaf tissue with 3 ml of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000 g for 25 min. To determine H_2O_2 level, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium sulphate in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6,000 g for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 μmol^{-1} cm^{-1}.

SOD was determined according to Paoletti et al. (1986). Ascorbate peroxidase (APOD) was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as the decline in optical density at 290 nm and activity was calculated using the extinction coefficient (2.8 mM cm^{-1} at 290 mm) for ascorbate. Glutathione reductase (GR) was determined by the method of Foster and Hess (1980). One unit of SOD, APOD and GR were defined as the amount of enzyme which inhibits by 50% the rate of NADH oxidation observed in control, breaks down 1 μmol of ascorbate per min, and decreases 1 A_{340} unit per min, respectively.

Chlorophyll, protein, H_2O_2 and MDA contents were expressed per g fresh weight (FW). Fe contents were expressed per g dry weight (DW). Enzyme activities were expressed as units per mg protein.Absolute levels of each measurement varied among experiments because of seasonal effects. However, the patterns of response to $FeSO_4$ were reproducible. For all measurements, each treatment was repeated four times. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

Results

The effect of $FeSO_4$ on toxicity, judged by the loss of chlorophyll and protein in detached rice leaves, is shown in Figure 1. Chlorophyll and protein contents in detached rice leaves were not affected by $FeSO_4$ in the light at 0.01 – 0.1 mM, but decreased at 1 – 10 mM. To be sure that the described toxicity was related to an increase in the leaf Fe content, Fe concentrations were determined in detached rice leaves treated with various $FeSO_4$ concentrations. Fe content in detached rice leaves was not affected by $FeSO_4$ at 0.01 – 0.1 mM, but increased at 1 – 10 mM (Figure 1).

Changes in the contents of chlorophyll and protein in detached rice leaves floating on water or 10 mM $FeSO_4$ are shown in Figure 2. It is clear that the loss of chlorophyll and protein was evident two days and one day, respectively, after the addition of $FeSO_4$.

It is generally accepted that rice discoloration characterised by bronzing and deposition of brown pigments is a typical symptom of iron toxicity (Ponnamperuma et al. 1955; Tanaka et al. 1996). In the present study, these typical symptoms were also observed when detached rice leaves were treated with 1 – 10 mM $FeSO_4$ in the light (data not shown). Since the loss of chlorophyll was less pronounced than that of protein (Figures 1 and 2), the loss of protein was

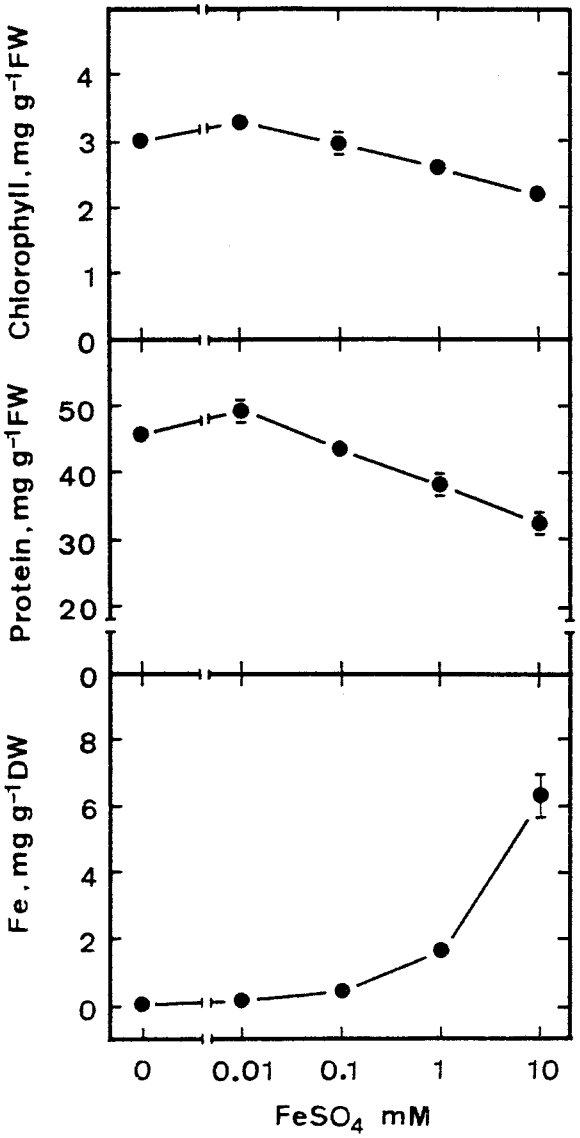

Figure 1. Effects of $FeSO_4$ on chlorophyll, protein and Fe contents in detached rice leaves. Chlorophyll and protein contents were measured 3 days after treatment in the light. Vertical bars represent SE (n = 4). Only those SE larger than symbol size are shown.

used as an indicator of iron toxicity in the subsequent experiments.

MDA is routinely used as an indicator of lipid peroxidation. MDA content in $FeSO_4$-treated detached rice leaves was observed to be higher than the water-treated controls throughout the entire duration of incubation (Figure 2). This shows that $FeSO_4$ induced toxicity of detached rice leaves is linked to lipid peroxidation. Figure 2 also shows that H_2O_2 content increased significantly in detached rice leaves incubated

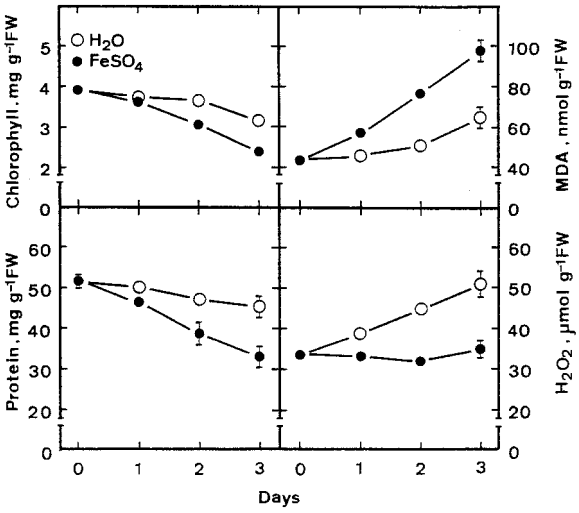

Figure 2. Time courses of the changes in chlorophyll, protein, MDA and H_2O_2 contents in detached rice leaves treated with $FeSO_4$. Detached rice leaves were treated with either water or 10 mM $FeSO_4$ in the light. Vertical bars represent SE (n = 4). Only those SE larger than symbol size are shown.

in water. However, H_2O_2 content in $FeSO_4$-treated detached rice leaves remained unchanged throughout the entire duration of incubation (Figure 2). Moran et al. (1994) also reported that H_2O_2 content did not accumulate in drought-treated pea leaves.

Lipid peroxidation is a free radical mediated process (Thompson et al. 1987). The striking increase in lipid peroxidation in $FeSO_4$-treated detached rice leaves may be a reflection of the decline of antioxidative enzymes. As shown in Figure 3, $FeSO_4$-treated detached rice leaves had lower activity of SOD than the controls. However, APOD activity was observed to be higher in $FeSO_4$-treated than water-treated leaves (Figure 3). $FeSO_4$ treatment also resulted in a higher GR activity than water control during 3 days of incubation (Figure 3).

The effects of free radical scavengers such as GSH, and mannitol on $FeSO_4$-induced loss of protein and lipid peroxidation of detached rice leaves are shown in Figure 4. It is clear that all tested free radical scavengers reduced toxicity caused by $FeSO_4$ and at the same time reduced $FeSO_4$-induced lipid peroxidation. As expected, free radical scavengers were found to reduce the decrease of SOD activity and the increase of APOD and GR activities caused by $FeSO_4$ (Table 1).

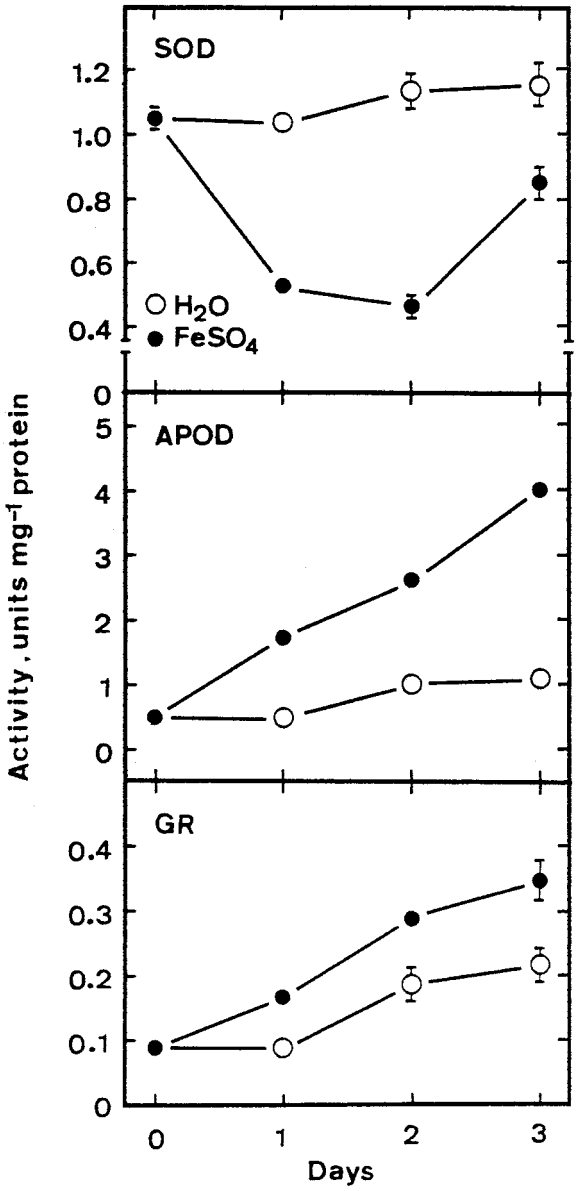

Figure 3. Time courses of the changes in SOD, APOD, and GR activities in detached rice leaves treated with $FeSO_4$. Detached rice leaves were treated with either water or 10 mM $FeSO_4$ in the light. Vertical bars represent SE (n = 4). Only those SE larger than symbol size are shown.

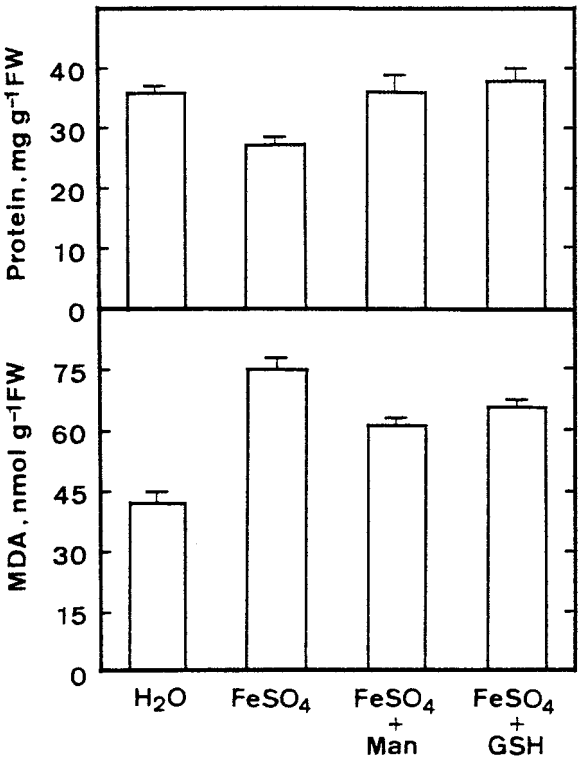

Figure 4. Effects of free radical scavengers on protein and contents in detached rice leaves in the presence of $FeSO_4$ in the light. Detached rice leaves were incubated for 2 days with 10 mM $FeSO_4$ alone, or simultaneously with 1 mM mannitol (Man) or 1 mM GSH. Vertical bars represent SE (n = 4).

Table 1. Effect of free radical scavengers on antioxidative enzymes activities in detached rice leaves in the presence of $FeSO_4$ in the light.

Treatment	Enzyme activity, units mg^{-1} protein		
	SOD	APOD	GR
H_2O	1.13 ± 0.04	1.04 ± 0.05	0.20 ± 0.04
$FeSO_4$	0.51 ± 0.07	2.70 ± 0.10	0.32 ± 0.01
$FeSO_4$ + mannitol	0.92 ± 0.04	2.02 ± 0.04	0.21 ± 0.03
$FeSO_4$ + GSH	0.81 ± 0.05	1.89 ± 0.01	0.19 ± 0.01

Detached rice leaves were incubated for 2 days with 10 mM $FeSO_4$ alone, or simultaneously with 1 mM mannitol or 1 mM GSH. Data are the means ± SE (n = 4).

Discussion

It is generally considered that the critical toxicity contents of Fe are above 0.7 mg per g leaf DW (Marschner 1995). In the present investigation, the contents were observed to be above 0.7 mg per g DW in detached rice leaves treated with 1 and 10 mM $FeSO_4$ for 3 days in the light (Figure 1), which showed the typical Fe toxicity. It has been demonstrated that sunflower leaf segments showed Fe toxicity at 0.5 mM $FeSO_4$ (Gallego et al. 1996). However, only slight Fe toxicity was observed in detached rice leaves treated with 1 mM $FeSO_4$ (Figure 1). It is obvious that detached rice leaves are less sensitive to $FeSO_4$ than detached sunflower leaves, which may be due to less effective in Fe uptake by detached rice leaves.

It has been demonstrated that excess Fe treatment increases lipid peroxidation or induces oxidative stress in plant tissues (Caro and Puntarulo 1996; Gallego et al. 1996; Kampfenkel et al. 1995; Price and Hendry 1991). In the present investigation, the addition of excess Fe (10 mM) to the incubation medium induced toxicity and increased the lipid peroxidation (Figure 2). These results support the possibility that $FeSO_4$-induced toxicity is mediated through oxidative stress. This conclusion supported further by the observations that (a) the increase in MDA content in detached rice leaves preceded the decrease of both chlorophyll and protein contents (toxicity) (Figure 2) and (b) free radical scavengers were able to reduce $FeSO_4$-induced MDA (Figure 4). The effect of $FeSO_4$ on the toxicity of detached rice leaves could have resulted from the effect of free radicals produced by the treatment of iron ions.

The reducing effect of free radical scavengers on $FeSO_4$-induced toxicity (Figure 4) is unlikely caused by the blockage of iron uptake by free radical scavengers, because the effect is also observed when detached rice leaves are exposed to free radical scavengers and $FeSO_4$ separately (data not shown).

SOD, APOD, and GR are important antioxidative enzymes in plant leaves (Elstner 1987; Inzé and Van Montagu 1995; Leshem 1988; Thompson et al. 1987). It has been reported that excess Fe decreased the activities of SOD, APOD, and GR (Gallego et al. 1996), increased the activity of APOD (Kampfenkel et al. 1995), and did not affect the activity of antioxidative enzymes (Caro and Puntarulo 1996). However, the results that we obtained with detached rice leaves in the light showed that excess Fe decreased the activity of SOD and increased the activities of APOD and GR (Figure 3). The decrease of SOD activity and the increase of APOD and GR activities in detached rice leaves caused by $FeSO_4$ were partially restored by addition of the free radical scavengers such as GSH and mannitol (Table 1), indicating that $FeSO_4$ treatment resulted in oxidative damage in detached rice leaves.

Activity of catalase, the enzyme responsible for eliminating H_2O_2, was not affected by $FeSO_4$ in detached rice leaves (data not shown). However, H_2O_2 did not accumulate in $FeSO_4$-treated detached rice leaves (Figure 2). SOD converts superoxide radicals to H_2O_2. Overproduction of SOD has been reported to result in enhanced oxidative stress tolerance in transgenic plants (Inzé and Van Montagu 1995). It has been shown that the activity of SOD was decreased by excess iron in sunflower leaves (Gallego et al. 1996), but was not affected by excess iron in soybean roots (Caro and Puntarulo 1996). In detached rice leaves, we also observed that excess iron caused a decrease in SOD activity (Figure 3). The decrease of SOD activity may lead to the accumulation of superoxide radicals. The superoxide radicals can react with H_2O_2 to form more powerful hydroxyl radicals in the presence of iron (Elstner 1987; Thompson et al. 1987). It is possible that H_2O_2 is being utilised by reacting with superoxide radicals in $FeSO_4$-treated detached rice leaves. This would explain why H_2O_2 did not accumulate in detached rice leaves exposed to excess iron. APOD uses ascorbate as the electron donor for the reduction of H_2O_2 and is well known to be important in the detoxification of H_2O_2 (Foyer 1993; Inzé and Van Montagu 1995). It has been shown that overexpression of APOD gene in plants increases protection against oxidative stress (Inzé and Van Montagu 1995; Wang et al. 1999). Since $FeSO_4$ treatment resulted in an increase in APOD activity in detached rice leaves (Figure 3), the possibility that H_2O_2 is being removed by APOD activity in $FeSO_4$-treated detached rice leaves can not be excluded.

Since detached rice leaves were used as experimental system, one may argue that the changes in lipid peroxidation and antioxidative enzyme activities as we observed in the present investigation are probably a wound response. However, in the present investigation, each long and narrow rice leaf was cut once transversely, the area of wounding was very small. Therefore, the changes in lipid peroxidation and antioxidative enzymes was unlikely resulted from wounding.

Acknowledgements

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