ELSEVIER

Plant Science 158 (2000) 71–76

PLANT SCIENCE

www.elsevier.com/locate/plantsci

Enhanced peroxidase activity in rice leaves in response to excess iron, copper and zinc

Wei-Ching Fang, Ching Huei Kao*

Department of Agronomy, National Taiwan University, Taipei, Taiwan, People's Republic of China

Received 16 March 2000; received in revised form 3 May 2000; accepted 2 June 2000

Abstract

The effect of excess Fe^{2+} on the peroxidase (POD) activity in detached rice leaves was investigated. $FeSO_4$ was effective in stimulating POD activity in detached rice leaves under both light and dark conditions. $FeSO_4$ but not K_2SO_4 induced POD activity, indicating that POD activity is induced by Fe^{2+}. $FeSO_4$-induced POD activity is not specific for the rice cultivar used in this study. $CuSO_4$ and $ZnSO_4$ were also observed to induce POD activity in detached rice leaves. Cycloheximide blocked the enhanced activity of POD by Fe^{2+}, Cu^{2+} or Zn^{2+}, indicating de novo biosynthesis of the enzyme. Paraquat treatment resulted in a decrease in POD activity. H_2O_2 had no effect on POD activity in detached rice leaves. It seems that Fe^{2+}-, Cu^{2+}- or Zn^{2+}-induced POD may not be mediated by free radicals. Using isoelectric focusing to separate POD, it was found that excess Fe^{2+}, Cu^{2+} or Zn^{2+} induced both quantitative and qualitative metal-specific changes in POD isozyme pattern in detached rice leaves. A new POD isozyme with a p*I* of 4.81 can be induced by Fe^{2+}, Cu^{2+} and Zn^{2+} in detached rice leaves.

Keywords: Copper; Iron; *Oryza sativa*; Peroxidase

1. Introduction

Peroxidases (POD, EC 1.11.1.7) belong to a large family of enzymes able to oxidize several different substrates in the presence of H_2O_2. These enzymes have been involved in several physiological and biochemical processes, such as cell growth and expansion [1,2], differentiation and development [3–5], auxin catabolism [6], lignification [7–9], as well as abiotic and biotic stress responses [2,10,11].

POD induction is a general response of higher plants to uptake of toxic amounts of metals. It has been observed in roots and leaves of various species after application of toxic doses of Zn^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} [12]. Iron toxicity is a nutritional disorder of rice associated with high ferrous iron in flooded soil [13]. Application of excess ferrous iron was found to induce POD activity in rice leaves [14,15]. The isozyme patterns of POD has been shown to be also affected by Zn^{2+}, Cd^{2+}, Ni^{2+} and Cu^{2+} [16]. Recently, Ezaki et al. [17] reported that a moderately anionic POD (approximately p*I* 6.7) was activated by Al stress in tobacco cells. It appears that toxic metals change POD activity both quantitatively and qualitatively. It also appears that the increase in POD activity is a defensive response to most if not all metals which may cause damage or disturb normal function of the plants. Relatively little is known about the inductive mechanism of POD by metals. Peng et al. [15] demonstrated that stimulation of POD activity in rice leaves by iron may be mediated by de novo synthesis of the enzyme at translational level.

The aim of the present study is to investigate the induction of POD activity and isozyme patterns by iron, copper and Zinc. The possible inductive mechanism of POD was also examined.

Abbreviations: CHI, cycloheximide; FW, fresh weight; POD, peroxidase; PQ, paraquat.

* Corresponding author. Fax: +886-2-23620879.

E-mail address: kaoch@ccms.ntu.edu.tw (C.H. Kao).

0168-9452/00/$ - see front matter

PII: S0168-9452(00)00307-1

2. Materials and methods

Rice (*Oryza sativa* cv. Taichung Native 1) was cultured as previously described [18]. 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 27°C in the light (40 μmol m^{-2} s^{-1}) or in darkness.

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).

For extraction of enzyme, leaf tissues were homogenized with 100 mM sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12 000 × *g* for 20 min and the resulting supernatant was used for determination of POD activity. The whole extraction procedure was carried out at 4°C. POD activity was measured using modification of the procedure of McAdam et al. [19]. Guaiacol was used as the substrate. POD activity was measured in a reaction mixture (3 ml) that contained 0.1 ml enzyme extract, 12 mM H_2O_2, and 7.2 mM guaiacol in 50 mM phosphate buffer, pH 5.8. The kinetics of the reaction were followed at 470 nm. Activity was calculated using extinction coefficient (26.6 mM^{-1} cm^{-1} at 470 nm) for tetraguaiacol [20] and expressed as units per gram of fresh weight (FW). One unit of POD activity was defined as 1 μmol tetraguaiacol produced per minute. Protein content in enzyme extracts was determined by the method of Bradford [21].

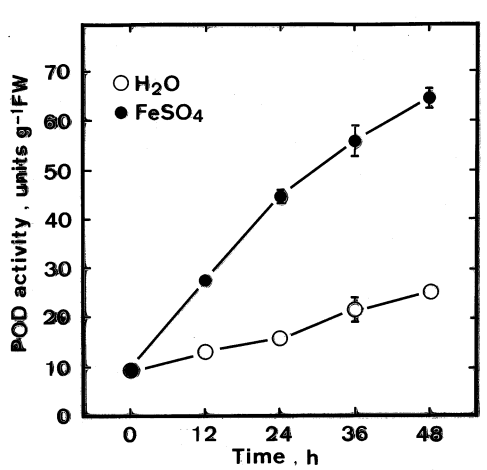

Fig. 1. Time course of $FeSO_4$ affect on POD activity in detached rice leaves in the light. Detached rice leaves were incubated in water or 10 mM $FeSO_4$. Vertical bars represent standard errors ($n = 4$). Only those standard errors larger than the symbol were shown.

POD isozymes were separated by isoelectric focusing using 10% polyacrylamide gel containing 5% ampholytes (pH 3–10), based on the method described by Nakanishi and Fujii [22] with some modifications. The p*I* markers, ranging from 3.5 to 9.3 were co-electrophoresed to determine the p*I* of POD isoforms. Aliquots of enzyme extracts (25 μg protein; depending on treatments, POD specific activity ranges from 0.5 to 2 units mg^{-1} protein) were applied on the gel. The voltage was increased stepwise; 200 V for 30 min, 400 V for 30 min, 600 V for 30 min, 800 V for 30 min and 1000 V for 30 min. The gels were stained for POD activity with 0.1% benzidine in acetic buffer (100 mM, pH 4.5) and 2 mM H_2O_2, rinsed with distilled water, then dried at room temperature.

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.

3. Results and discussion

It is generally considered that the critical iron toxicity contents are above 500 μg Fe per g leaf dry weight [23]. Fe content was 6390 μg per gram dry weight in detached rice leaves treated with 10 mM $FeSO_4$ for 3 days in the light, which showed the typical Fe toxicity (data not shown). Thus, 10 mM $FeSO_4$ was used in the present investigation to examine its effect on POD activity in detached rice leaves.

Changes in POD activity in detached rice leaves floating on water or 10 mM $FeSO_4$ in the light are shown in Fig. 1. Only slight increase in POD activity was observed in detached rice leaves treated with distilled water. It is clear that POD activity in detached rice leaves incubated in $FeSO_4$ solution is higher than that in distilled water. POD activity increased about threefold in detached rice leaves treated with $FeSO_4$ for 48 h in the light compared with the control. When the effect of $FeSO_4$ on POD activity in detached rice leaves was

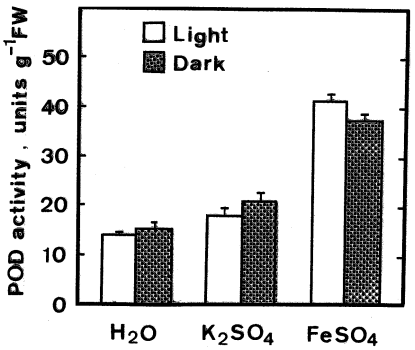

Fig. 2. Effects of light and dark on POD activity in detached rice leaves were treated with K_2SO_4 or $FeSO_4$. Detached rice leaves were treated with water, 10 mM K_2SO_4 or 10 mM $FeSO_4$ for 24 h in the light or in the dark. Vertical bars represent standard errors ($n = 4$).

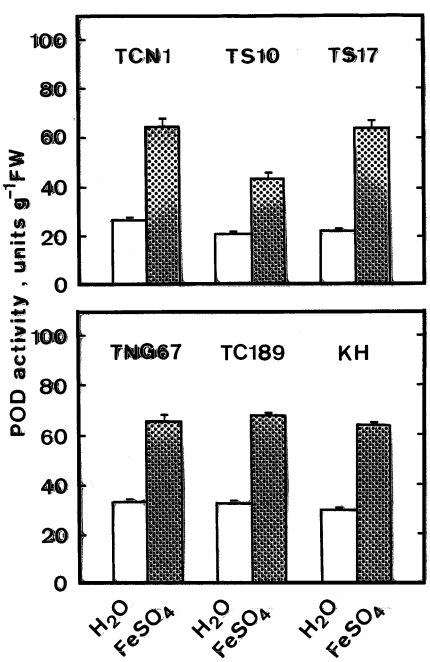

Fig. 3. Effect of $FeSO_4$ on POD activity of six rice cultivars. Cultivars in the upper panel are Indica varieties, whereas those in the lower panel are Japonica varieties. TCN1, Taichung Native 1: TS10, Taichung Sen 10, TS17, Taichung Sen 17; TNG 67, Tainung 67; TC 189, Taichung 189; KH, Koshihikari. Detached rice leaves were incubated in water (open columns) or 10 mM $FeSO_4$ (shaded columns) for 24 h in the light. Bars represent standard errors ($n = 4$).

compared with that of K_2SO_4 in the light or in the dark, it was found that $FeSO_4$ but not K_2SO_4 was able to increase POD activity in both light and dark conditions (Fig. 2), indicating that POD activity is induced by Fe ions rather than SO_4^{2-}.

The effect of $FeSO_4$ on POD activity is unlikely to be specific for the particular cultivar (Taichung Native 1, TCN1) used in this study, since $FeSO_4$ also increased POD activity in detached rice leaves of five other cultivars of rice (Fig. 3). It appears that the induction of POD activity is a general response of rice leaves to uptake of toxic amounts of iron ions.

Fig. 4 shows the effect of various divalent metals on POD activity in detached rice leaves. It is clear that sulfate salts of Co^{+2}, Mn^{2+} and Mg^{2+} did not affect POD activity in detached rice leaves. Ni^{2+}, on the contrary, was found to decrease POD activity in detached rice leaves. It is also obvious from Fig. 4 that Cu^{2+} was more effective in inducing POD activity than Fe^{2+}; and Zn^{2+} treatment showed a slight increase in POD activity.

It has been reported that excess Fe^{2+} induces the formation of free radicals in several plant species [24–26]. Excess Cu^{2+} is also known to mediate free radical formation in isolated chloroplasts [27], intact roots [28], in detached leaves [29,30], and in intact leaves [31]. Prasad et al. [32] reported that free radical generation was promoted in plants exposed toxic levels of Zn^{2+}. They suggest that free radical- induced lipid peroxidation is part of the overall expression of Fe^{2+}, Cu^{2+} or Zn^{2+} toxicity. It is suggested that generated lipid hydroperoxides are toxic for cells and should be scavenged through the function of POD as soon as possible. Thus, free radical generation is most likely involved in induction of POD activity by Fe^{2+}, Cu^{2+} or Zn^{2+}. To test this possibility, detached rice leaves were treated with a well

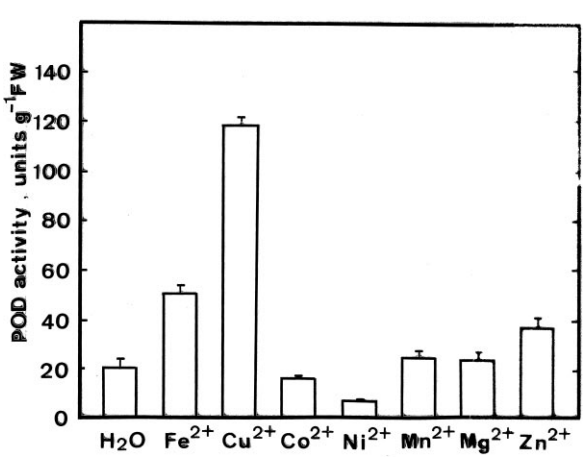

Fig. 4. Effect of various divalent metals on POD activity in detached rice leaves. POD activity was determined 24h after treatment of sulfate salts of various metals (10 mM) in the light. Vertical bars represent standard errors ($n = 4$).

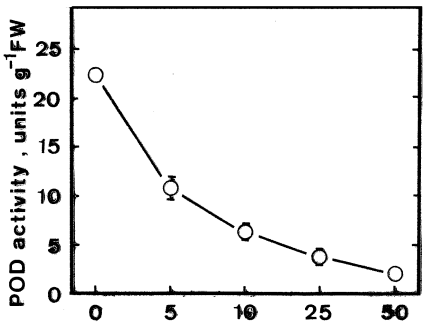

Fig. 5. Effect of paraquat (PQ) on POD activity in detached rice leaves. POD activity was determined 24 h after treatment in the light. Vertical bars represent standard errors ($n = 4$). Only those standard errors larger than the symbol are shown.

Table 1
Effect of H_2O_2 on POD activity and cycloheximide (CHI) on the induction of POD by $FeSO_4$, $CuSO_4$ or $ZnSO_4$[a]

Treatment	POD activity, units g^{-1} FW
H_2O	15.7 ± 0.7
$FeSO_4$	44.8 ± 1.0
H_2O_2	16.5 ± 0.9
$FeSO_4$+CHI	21.7 ± 0.5
H_2O	23.1 ± 2.5
$CuSO_4$	130.0 ± 2.8
$ZnSO_4$	45.9 ± 0.6
$CuSO_4$+CHI	72.9 ± 5.1
$ZnSO_4$+CHI	26.0 ± 0.7

[a] The concentration of $FeSO_4$, $CuSO_4$, $ZnSO_4$ and H_2O_2 was 10 mM, whereas that of CHI was 20 μM. POD activity was determined 24 h after treatment in the light. Data are the means ± standard errors ($n = 4$).

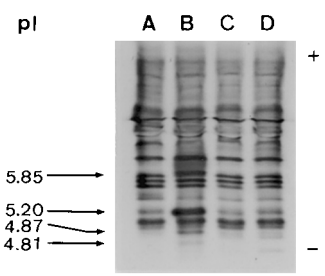

Fig. 6. Effect of $FeSO_4$ and cycloheximide (CHI) on POD isozymes in detached rice leaves, Detached rice leaves were incubated in water (A), 10 mM $FeSO_4$ (B), 20 μM CHI (C), or 10 mM $FeSO_4$ plus 20 μM CHI (D) for 24 h in the light.

known free radical-generating chemical paraquat (PQ) and H_2O_2. However, increasing PQ concentrations from 5 to 50 μM progressively decreased POD activity in detached rice leaves (Fig. 5). Furthermore, external application of H_2O_2 had no effect on POD activity in detached rice leaves (Table 1). This is probably indicated that free radicals were not involved in the induction of POD by Fe^{2+}, Cu^{2+} or Zn^{2+}. Peng et al. [15] also reported that Fe^{2+}-induced POD in rice leaves was not mediated by free radicals. Furthermore, Foster and Heath [33] found that oxygen toxicity, which is closely related to the action of oxygen free radicals, also could not induce POD activity in maize plants.

Effect of cycloheximide (CHI), an inhibitor of protein biosynthesis [34], on the induction of POD activity by Fe^{2+}, Cu^{2+}, or Zn^{2+} in detached rice leaves are shown in Table 1. CHI significantly blocked the induction of POD by Fe^{2+}, Cu^{2+} or Zn^{2+}, suggesting that the induction of POD activity is due to de novo POD biosynthesis. This result is in agreement with that of Peng et al. [15] who reported that the stimulated POD activity by Fe^{2+} resulted from inducing de novo biosynthesis of the enzyme.

In order to know whether toxic Fe^{2+} changes POD isozyme patterns, POD isozymes were separated by isoelectric focusing. Two new POD bands (p*I* 4.87 and 4.81) were identified in the Fe^{2+}-treated leaves compared with the control (Fig. 6). The expression of these two bands was inhibited by CHI (Fig. 6). Two other POD staining bands (p*I* 5.85 and 5.20) were also found to be increased by Fe^{2+} and inhibited by CHI (Fig. 6).

POD isozymes in detached rice leaves in response to Cu^{2+} or Zn^{2+} are shown in Fig. 7. Three new POD bands with p*I* of 6.43, 6.26 and 4.81 were detected which could not be seen in untreated leaf preparation. Cu^{2+} also increased the activity of POD isozymes with p*I* of 7.70, 5.85, 5.20 and 5.14. All these Cu^{2+}-increased POD

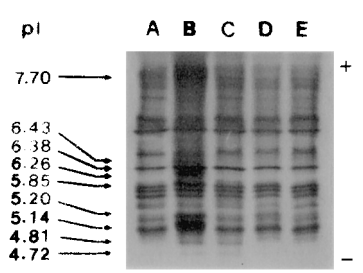

Fig. 7. Effect of $CuSO_4$, $ZnSO_4$ and cycloheximide (CHI) on POD isozymes in detached rice leaves. Detached rice leaves were incubated in water (A), 10 mM $CuSO_4$ (B), 10 mM $ZnSO_4$ (C), 10 mM $CuSO_4$ plus 20 μM CHI (D), or 10 mM $ZnSO_4$ plus 20 μM CHI (E) for 24 h in the light.

isozyme activities were inhibited by CHI. Only a new POD isozyme (p*I* 4.81) in Zn^{2+}-treated leaves was observed. The expression of this POD isozyme was also inhibited by CHI. Clearly, excessive uptake of toxic metals Fe^{2+}, Cu^{2+} and Zn^{2+} induces both quantitative and qualitative metal-specific changes in POD isozyme patterns in rice leaves. Van Assche et al. [16] reached the similar conclusion using *Phaseolus vulgaris*.

It is highly interesting that a new POD isozyme with a p*I* of 4.81 can be induced by Fe^{2+}, Cu^{2+} and Zn^{2+} in detached rice leaves. However, Peng et al. [15] reported that a POD isozyme with a p*I* of about 9.8 was enhanced in rice (cv. IR 50) leaves treated with Fe^{2+}. It is well known that POD isozymes fall into three subgroups (anionic, moderate anionic and cationic). Although the precise role that anionic POD plays in plant growth, development, and stress tolerance remains uncertain, there is significant evidence that anionic POD is involved in host defense and stress-induced lignification [5]. Therefore, what function the induced POD isozyme with a p*I* of 4.81 may perform in coping with Fe $^{2+}$, Cu^{2+} and Zn^{2+} toxicity remains to be further clarified.

Acknowledgements

This work was supported by grant NSC 89-2313-B-002-111 from the National Science Council of the Republic of China.

References

[1] G. Wallace, S.C. Fry, Phenolic components of the cell wall: Dynamic aspects, Int. Rev. Cytol. 151 (1994) 229–267.

[2] C.C. Lin, C.H. Kao, NaCl induced changes in ionically bound peroxidase activity in roots of rice seedlings, Plant Soil 216 (1999) 147–153.

[3] T. Gaspar, C. Penel, D. Hagage, H. Greppin, Peroxidases in plant growth, differentiation and development processes, in: J.H. Lobarzawsky, H. Greppin, C. Penel, T. Gaspar (Eds.), Biochemical, Molecular, and Physiological Aspects of Plant Peroxidases, University de Geneve, Geneve, 1991, pp. 249–280.

[4] I.E. Mansouri, J.A. Mercado, N. Santiago-Domenech, F. Pliego-Alfaro, V. Valpuesta, M.A. Quesada, Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase, Physiol. Plant 106 (1999) 355–362.

[5] L.M. Lagrimini, V. Gingas, F. Finger, S. Rothstein, T.-T.Y. Liu, Characterization of antisense transfromed plant deficient in the tobacco anionic peroxidase, Plant Physiol. (1997) 1187–1196.

[6] L.M. Largimini, R.J. Joly, J.R. Dunlap, T.-T.Y. Liu, The consequence of peroxidase overexpression in transgenic plants on root growth and development, Plant Mol. Biol. 33 (1997) 887–895.

[7] Y. Sato, M. Sugiyama, R.J. Gorecki, H. Fukuda, A. Komamine, Interrelationship between lignin deposition and the activities of peroxidase isoenzymes in differentiating tracheary elements of *Zinnia*. Analysis using L-α-aminooxy-β-phenylpropionic acid and 2-aminoindan-2phosphonic acid, Planta 189 (1993) 584–589.

[8] F. Sitbon, S. Hennion, C.H.A. Little, B. Sundberg, Enhanced ethylene production and peroxidase activity in IAA-overproducing transgenic tobacco plants is associated with increased lignin content and altered lignin composition, Plant Sci. 141 (1999) 165–171.

[9] T. Otter, A. Polle, Characterisation of acidic and basic apoplastic peroxidases from needles of Norway spruce (*Picea abies*, L., Karsten) with respect to lignifying substrates, Plant Cell Physiol. 38 (1997) 595–602.

[10] R. Mohan, A.M. Bajar, P.E. Kolettukudy, Induction of a tomato anionic peroxidase gene (*tap 1*) by wounding in transgenic tobacco and activation of *tap 1*/GUS and *tap 2*/GUS chimeric gene fusions in transgenic tobacco by wounding and pathogen attack, Plant Mol. Biol. 21 (1993) 341–354.

[11] M.I. Medina, M.A. Quesada, F. Pilego, M.A. Botella, V. Valpuesta, Expression of the tomato peroxidase gene *TPX 1* in NaCl-adapted and unadapted suspension cells, Plant Cell Rep. 18 (1999) 680–683.

[12] F. Van Assche, H. Clijsters, Effects of metals on enzyme activity in plants, Plant Cell Environ. 13 (1990) 195–206.

[13] F.N. Ponnamperuma, R. Bradfield, M. Reech, Physiological disease of rice attributable to iron toxicity, Nature 175 (1995) 265.

[14] X.X. Peng, M. Yamauchi, Ethylene production in rice bronzing leaves induced by ferrous iron, Plant Soil 149 (1993) 227–234.

[15] X.X. Peng, X.L. Yu, M.Q. Li, M. Yamauchi, Induction of proxidase by Fe^{2+} in detached rice leaves, Plant Soil 180 (1996) 159–163.

[16] F. Van Assche, C. Put, H. Clijsters, Heavy metals induce specific isozyme patterns of peroxidase in *Phaseolus vulgaris* L, Arch Int. Physiol. Biochim. 94 (1986) 60.

[17] B. Ezaki, S. Tsugita, H. Matsumoto, Expression of a moderately anionic peroxidase is induced by aluminum treatment in tobacco callus: Possible involvement of peroxidase isozymes in aluminum ion stress, Physiol. Plant. 96 (1996) 21–28.

[18] J.-N. Lin, J.-W. Wang, C.H. Kao, Effect of abscisic acid and water stress on the senescence of detached rice leaves, Biol. Plant. 42 (1999) 313–316.

[19] J.W. MacAdam, C.J. Nelson, R.E. Sharp, Peroxidase activity in the leaf elongation zone of tall fescue, Plant Physiol. 99 (1992) 872–878.

[20] M. Kato, S. Shimizu, Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing to-

bacco leaves; phenolic-dependent peroxidative degradation, Can. J. Bot. 65 (1987) 729–735.
[21] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[22] F. Nakanishi, T. Fujii, Appearance of peroxidase isozymes in floral-initiated shoot apices of *Pharbitis nil*, Physiol. Plant. 86 (1992) 197–201.
[23] H. Marscher, Mineral Nutrition of Higher Plants, Academic Press, San Diego, 1995, p. 324.
[24] K. Kempfenkel, M. Van Montagu, D. Inze, Effects of iron excess on *Nicotiana plumbaginifolia* plants. Implications to oxidative stress, Plant Physiol. 107 (1995) 725–735.
[25] S.M. Gallego, M.P. Benovides, M.L. Tomaro, Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress, Plant Sci. 121 (1996) 151–159.
[26] A. Caro, S. Puntarulo, Effect of in vivo iron supplementation on oxygen radical production by soybean roots, Biochem. Biophys. Acta 1291 (1996) 245–251.
[27] G. Scandmann, P. Boger, Copper-induced lipid peroxidation processes in photosynthetic membranes, Plant Physiol. 66 (1980) 797–800.
[28] C.H.R. De Vos, W.M. Ten Bookum, R. Vooijs, H. Schat, L.J. De Kok, Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*, Plant Physiol. Biochem. 31 (1993) 148–151.
[29] C.M. Luna, C.A. Gonalez, V.S. Trippi, Oxidative damage caused by an excess of copper in oat leaves, Plant Cell Physiol. 35 (1994) 11–15.
[30] L.-M. Chen, C.H. Kao, Effect of excess copper on rice leaves: evidence for involvement of lipid peroxidation, Bot. Bull. Acad. Sin. 40 (1999) 283–287.
[31] J.E.J. Weckx, H.M.M. Clijsters, Oxidative damage and defense mechanisms in primary leaves of Phaseolus vulgaris as a result of toxic amounts of copper, Physiol. Plant. 96 (1996) 506–512.
[32] K.V.S.K. Prasad, P. Paradha Sarachi, P. Sharmila, Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*, Environ. Exp. Bot. 42 (1999) 1–10.
[33] J. Foster, J.L. Hess, Oxygen effects on maize leaf superoxide dismutase and glutathione reductase, Phytochemistry 21 (1982) 1527–1532.
[34] U. Luttge, A. Lauchli, E. Ball, M.G. Pitman, Cycloheximide: a specific inhibitor of protein synthesis and intercellular ion transport in plant roots, Experientia 30 (1974) 470–471.