DOI: 10.1007/s10535-017-0720-0 BIOLOGIA PLANTARUM 61 (4): 733-740, 2017

Zinc-alleviating effects on iron-induced phytotoxicity in roots of *Triticum aestivum*

T. MA[1], X.H. DUAN[1], Y.Y. YANG[1]*, J. YAO[1], and T.P. GAO[2]

School of Life Science, Northwest Normal University, Lanzhou 730070, P.R. China[1]
Centre of Urban Ecology and Environmental Biotechnology, Lanzhou City University, Lanzhou 730070, P.R. China[2]

Abstract

The mechanisms of growth inhibition and antioxidative response were investigated in wheat roots exposed to 300 μM iron together with different zinc concentrations (0, 50, and 250 μM). All Zn concentrations decreased Fe content but increased Zn content in the roots and leaves of Fe-treated seedlings. Compared with Fe stress alone, 50 or 250 μM Zn + Fe treatment stimulated root growth, and increased cell viability but decreased malondialdehyde content, which were correlated with the decreases of total and apoplastic hydrogen peroxide and superoxide anion radical ($O_2^{\cdot-}$) content along with apoplastic hydroxyl radical content. Generation of $O_2^{\cdot-}$ in response to 10 μM diphenylene iodonium suggested that NADPH oxidase activity was lower in Zn + Fe-treated roots than in other roots. In addition, cell wall-bound peroxidase, diamine oxidase, and polyamine oxidase in Fe-treated roots were insensitive to Zn addition. Further study showed the stimulation of total superoxide dismutase and glutathione reductase (GR) activities as well as apoplastic catalase, ascorbate peroxidase, and GR in Zn + Fe-stressed roots in comparison with Fe-alone-treated ones. Taken together, Zn could alleviate iron-inhibitory effect on root growth, which might be associated with the decrease of lipid peroxidation, the increase of cell viability and the reductions of reactive oxygen species generation.

Additional key words: antioxidative enzymes, lipid peroxidation, reactive oxygen species, root growth.

Introduction

Heavy metals are nowadays among the most important pollutants because of their phytotoxicity and potential risk for human health. Among those, iron is an essential element and plays important roles in such plant biological processes as photosynthetic electron transport, cell wall metabolism, and oxidative stress response (Sinha *et al.* 2009, Tewari *et al.* 2013). Moreover, Fe is the most abundant in biological systems and is able to promote hydroxyl radical ($^{\cdot}OH$) formation through Fenton reaction (Halliwell and Gutteridge 1993). Zinc is also essential for plant growth and development because Zn is an important component of a number of enzymes associated with the saccharide metabolism, proteins synthesis, and gene expression and regulation (Cherif *et al.* 2011). However, these two metals at high concentrations are toxic, altering biochemical and physiological characteristics and inhibiting plant growth (Nenova 2006, De Oliveira Jucoski *et al.* 2013).

Plants produce a high amount of reactive oxygen species (ROS), such as H_2O_2, $^{\cdot}OH$, and superoxide anion radical ($O_2^{\cdot-}$), as part of their normal metabolism, and they are important signals, *e.g.*, during pollen germination and pollen-tube growth (Pasqualini *et al.* 2015). However, excessive ROS generation is one of the major

Submitted 5 September 2015, *last revision* 6 June 2016, *accepted* 14 June 2016.
Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DAO - diamine oxidase; DPI - diphenylene iodonium; EDTA - ethylenediaminetetraacetic acid; GR - glutathione reductase; H_2O_2 - hydrogen peroxide; MDA - malondialdehyde; NADPH - nicotinamide adenine dinucleotide; NBT - nitrobluetetrazolium; $O_2^{\cdot-}$ - superoxide anion radical; $^{\cdot}OH$ - hydroxyl radical; PAO - polyamine oxidase; PBS - phosphate buffer solution; PM - plasma membrane; POD - peroxidase; PVP - polyvinylpyrrolidone; ROS - reactive oxygen species; SOD - superoxide dismutase.
Acknowledgments: This work was financially supported by the National Natural Science Foundation of China (Nos. 31470464, 31360094, 31460142), and the Science and Technology Plan Project of Gansu Province (144FKCA059). The first two authors contributed equally to the paper.
* Corresponding author; fax: (+86) 0931 7971575, e-mail: xbsfxbsdyang@163.com

responses of plants to different stresses. If they are not effectively and rapidly removed, high ROS accumulation has the potential to damage macromolecules including lipids, proteins, and nucleic acids (Halliwell *et al.* 1999). Antioxidant enzyme superoxide dismutase (SOD) can catalyze the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2, and catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR) make up main elimination systems of H_2O_2. To reduce functional and structural damages, these enzymes counteract also negative effects induced by heavy metals (Cherif *et al.* 2011, Israr *et al.* 2011, Turhan *et al.* 2012). In addition, cell wall-bound POD, diamine oxidase (DAO), and polyamine oxidase (PAO) may be involved in the regulation of H_2O_2 content in plants (Ranieri *et al*. 2001, Karuppanapandian *et al.* 2011, Talaat *et al.* 2012).

The interaction between essential elements may be important for understanding heavy metal toxicity mechanisms (Upadhyay and Panda 2010). Recently, Zn-alleviating effects on other metal-induced oxidative stresses have been investigated in some plant species. For example, Zn addition reversed Cu-induced oxidative damage and growth inhibition in duckweed (Upadhyay and Panda 2010). In addition, Zn at low concentration has an alleviating effect on Cd toxicity in tomato (Cherif *et al.* 2011). Moreover, Zn is an integral component of numerous functional proteins in a wide range of physiological systems, for which the membrane lipid is protected from ROS (Vallee and Falchuk 1993). However, few detailed studies were conducted to evaluate the mechanism of Fe toxicity, and even less information is available about the effect of Zn on Fe-induced phytotoxicity. More recently, we observed such Fe-phytotoxicity as growth inhibition in wheat seedlings exposed to 100, 300, and 500 μM Fe alone (Li *et al.* 2012a). In the present study, the influence of Zn on Fe toxicity was investigated in wheat seedlings by analyzing element absorption, root growth, ROS generation, and related enzyme activities.

Materials and methods

Wheat (*Triticum aestivum* L., cv. Xihan 3) seeds were purchased from Gansu Agricultural Academy. The seeds were surface-sterilized with $HgCl_2$ and germinated in the dark at 25 ± 1.5 °C. Uniformly germinated seeds were transferred into Petri dishes, treated with 1/4 Hoagland nutrient solution supplied with 0 (control) or 300 μM $FeCl_3$ (Fe), 50 μM $ZnCl_2$ (Zn) + 300 μM Fe or 250 μM Zn + 300 μM Fe and grown at 25 ± 1.5 °C, an irradiance of 300 μmol m^{-2} s^{-1}, and a 12-h photoperiod. Root length and other indexes were measured 6 d later.

Roots and leaves were prepared for measuring Fe and Zn content using the method of Achary *et al.* (2008) with some modifications. Plant material was thoroughly washed with deionized water and dried to constant mass at 80 °C. The dry samples were dissolved in a solution containing concentrated nitric acid (HNO_3), hydrofluoric acid, and H_2O_2 (30 %, v/v), and were digested in a closed microwave digestion system (*Multiwave 3000*, *Anton Paar*, Graz, Austria) for 2.5 h. The chilled samples were transferred to polytetrafluoroethylene beaker and evaporated to dryness. The resulting ash residue was dissolved in 1 % HNO_3, and total Fe and Zn content was measured with flame atomic absorption spectrophotometry (*WFX210*, *Zhejiang Nade Scientific Instruments*, Zhejiang, China).

Malondialdehyde (MDA) content was determined according to Zhou (2001) and the absorbance of the supernatant was measured at 450, 532, and 600 nm. The loss of cell viability was assayed by Evans blue staining (Zanardo *et al.* 2009). H_2O_2 content was calculated by use of the standard curve prepared with known H_2O_2 concentrations according to Sergiev *et al.* (1997). The generation of $^{\cdot}OH$ was analyzed according to the method of Halliwell *et al.* (1987). $O_2^{\cdot-}$ generation was determined using the method of Achary *et al.* (2012).

Cell walls were prepared by homogenizing roots in 50 mM phosphate buffer solution (PBS) (pH 5.8) as described by Lee and Lin (1995) with some modifications. The homogenate was centrifuged at 1 000 *g* for 10 min and washed at least twice with 50 mM PBS (pH 5.8), and then the pellets were incubated in 1 M NaCl. Then, it was centrifuged at 1 000 *g* for 10 min, and the supernatant containing cell wall-bound POD was collected to analyze enzyme activity according to Dos Santos *et al.* (2008). One unit (U) of cell wall-bound POD activity was defined as the absorbance change of 0.01 min^{-1}. Diamineoxidase (DAO) activity was calculated according to Naik *et al.* (1981) by measuring the generation of H_2O_2. Polyamine oxidase (PAO) activity was assayed by using spermidine as the substrate (Asthir 2002). One unit of enzyme activity was defined as a change in absorbance of 0.001 units. Total SOD activity was estimated based on the method described by Dhindsa and Matowe (1981). One unit of enzyme activity was defined as the quantity of SOD which produced 50 % of the maximum competition against nitrobluetetrazolium (NBT). A modification of the method of Aebi (1984) was used to assay CAT activity. The enzyme extract was added to 50 mM PBS (pH 7.0) and incubated at 25 °C for 5 min. The reaction was started by the addition of 6 μM H_2O_2 and the absorbance changes were recorded at 240 nm for 2 min. An absorbance change of 0.1 unit min^{-1} was defined as one unit (U) of CAT activity. Soluble POD activity was measured following the method of Rao *et al.* (1996). One unit of POD activity was defined as an absorbance change of 0.01 unit min^{-1}. Ascorbate

peroxidase (APX) activity was determined following the method of Nakano and Asada (1981). Glutathione reductase (GR) activity was measured by monitoring the oxidation of NADPH according to Schaedle and Bassham (1977). One unit of APX or GR activity was defined as an absorbance change of 0.1 unit min^{-1}. All enzyme activities were expressed as U mg^{-1}(protein) and the amounts of soluble proteins were estimated according to Bradford (1976).

Apoplastic fluid was extracted with the vacuum infiltration-centrifugation technique which was reported by Córdoba-Pedregosa *et al.* (2005) with some modifications. Root tissue was quickly washed in distilled water, placed in test tubes in 10 mM PBS (pH 6) containing 1.5 % polyvinylpyrrolidine (PVP), 1 mM EDTA, and then submitted to vacuum at 4 °C for 40 min. Then, centrifuged at 12 000 *g* for 10 min and the apoplastic fluid was collected. Apoplastic H_2O_2 content was measured on the basis of method of Sergiev *et al.* (1997). Apoplastic $\cdot OH$ content and $O_2^{\cdot -}$ generation was assayed according to the above mentioned methods. Apoplastic antioxidant enzyme activities were also estimated based on the aforementioned measurement of total antioxidant enzyme activities, and all enzyme activities were expressed as U g^{-1}(f.m.).

Each experiment was set up in at least 3 repetitions, and each sample was measured 3 times. The data were expressed as the average ± standard error (SE). Statistical comparisons were carried out with *SPSS 13.0* software. After analysis of variance (one-way *ANOVA*) and Duncan`s multiple comparisons were performed. The significant differences were indicated at $P < 0.05$.

Results

Root length exhibited about 28 % reduction in the Fe-stressed seedlings compared with the control. Both Zn concentrations could block Fe-induced inhibition of root growth, and 250 μM Zn-alleviating effect was more significant than 50 μM Zn ones (Fig. 1*A*).

The constitutive amount of Fe was 103.85 ± 1.52 and 141.61 ± 3.22 μg g^{-1}(d.m.) in the roots and leaves of untreated seedlings, respectively. Compared with the control, a significant enhancement of Fe content was found in 300 μM Fe-treated roots. The presence of Zn blocked Fe-induced enhancement of Fe content in wheat roots, with the lowest content at 250 μM Zn. However, addition of 300 μM Fe did not affect Fe content in the leaves, but both Zn concentrations reduced Fe content in the leaves of Fe-treated seedlings, in comparison with the control (Table 1). In comparison with the control, root and leaf Zn content in 300 μM Fe-treated seedlings increased by about 31 and 21 %, respectively. Different Zn concentrations further increased Zn content in the roots and leaves of Fe-treated seedlings in a concentration-dependent manner (Table 1).

Compared with the control, root MDA content significantly increased in seedlings stressed with 300 μM Fe, and the addition of 50 μM or 250 μM Zn alleviated Fe-induced effect on MDA content. The uptake of Evans blue rose by about 35 % in 300 μM Fe-treated roots in comparison with untreated roots, indicated that Fe stress resulted in the loss of cell viability (Table 1). When different Zn concentrations were applied, the uptake of Evans blue was significantly lowered in Fe-stressed roots in comparison with Fe-alone-treated ones.

Compared with the control, treatment with 300 μM Fe caused about 93 % increase in total H_2O_2 content in roots (Table 1). The application of 50 μM or 250 μM Zn significantly alleviated Fe-induced elevation of total H_2O_2 content in roots. Furthermore, roots treated with Fe exhibited a significant increase in apoplastic H_2O_2 content by 1.21-folds compared with the control and both Zn concentrations reduced apoplastic H_2O_2 production in Fe-stressed roots in comparison with Fe-alone-treated

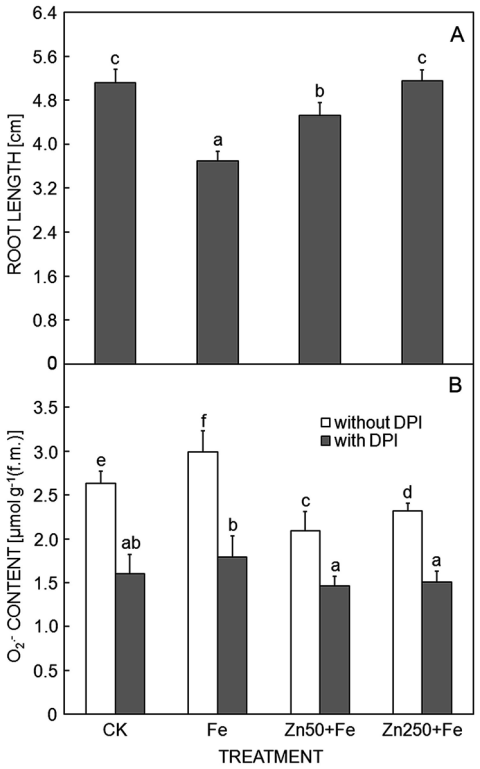

Fig. 1. Zinc induced changes of root growth (*A*) and $O_2^{\cdot -}$ content (*B*) in roots of 300 μM Fe-treated wheat seedlings. CK - control, Fe - 300 μM Fe, Zn50+Fe - 50 μM Zn + 300 μM Fe, Zn250+Fe - 250 μM Zn + 300 μM Fe. "with DPI" means the addition of diphenylene iodium in the reaction solution, and "without DPI" means the absence of DPI in the reaction solution. Means ± SE ($n \geq 3$), different letters indicate significant differences ($P < 0.05$).

ones (Table 1). Compared with the control, the amount of total and apoplastic $^{\cdot}OH$ rose by about 32 and 22 % in Fe-treated roots, respectively (Table 1). The 50 μM Zn did not obviously affect Fe-induced elevation of total $^{\cdot}OH$ production, but 250 μM Zn led to the decrease of this parameter in comparison with Fe treatment alone. And also, the presence of 50 or 250 μM Zn significantly blocked Fe-caused enhancement of apoplastic $^{\cdot}OH$ content (Table 1). Total $O_2^{\cdot-}$ content increased to 126 % of the control value in roots exposed to Fe stress, but in comparison with Fe-alone-treated roots, this parameter significantly lowered in wheat roots under Fe treatment along with different Zn concentrations (Fig. 1*B*). When 10 μM diphenylene iodonium (DPI) was applied, total $O_2^{\cdot-}$ generation exhibited a notable decrease in all roots compared with the absence of DPI (Fig. 1*B*). Similarly, apoplastic $O_2^{\cdot-}$ content elevated remarkably in roots compared with the control, but this parameter decreased by about 13 and 25 % under 50 μM Zn + Fe and 250 μM Zn + Fe treatments, respectively, in comparison with Fe stress alone (Table 1; Fig. 1*B*)

A significant reduction of cell wall-bound POD activity was found in 300 μM Fe-stressed roots compared with the control (Table 1). 50 μM Zn significantly stimulated cell wall-bound POD in Fe-treated roots. However, the application of 250 μM did not affect cell wall-bound POD activity. In comparison with untreated seedlings, root DAO activity rose in Fe-stressed seedlings insignificantly. In addition, Fe together with 50 μM or 250 μM Zn treatment did not affect root DAO activity compared with the control. When seedlings were exposed to 300 μM Fe, root PAO activity elevated to 121 % of the control value (Table 1). Fe treatment in combination with 250 μM Zn resulted in a significant decrease in PAO activity in wheat roots in comparison with Fe stress alone.

Table 1. Changes of iron, zinc, and malondialdehyde (MDA) content, cell viability, hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), superoxide anion radical ($O_2^{\cdot-}$) content, cell wall-bound (peroxidase) POD, diamine oxidase (DAO), and polyamine oxidase (PAO) activities, and total and apoplastic activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione reductase (GR) in wheat seedlings under 300 μM Fe treatment with or without Zn. Means ± SEs, $n \geq 3$, different letters indicate significant differences ($P < 0.05$).

Parameters	Control	300 μM Fe	50 μM Zn + Fe	250 μM Zn +Fe
Root Fe content [μg g^{-1}(d.m.)]	103.85 ± 1.52a	1590.41 ± 5.83d	972.02 ± 6.45c	594.97 ± 3.38b
Leaf Fe content [μg g^{-1}(d.m.)]	141.61 ± 3.22c	146.00 ± 2.68c	122.30 ± 2.13b	106.63 ± 3.53a
Root Zn content [μg g^{-1}(d.m.)]	42.03 ± 0.61a	55.24 ± 1.33b	136.71 ± 0.42c	303.78 ± 3.07d
Leaf Zn content [μg g^{-1}(d.m.)]	20.39 ± 1.05a	24.65 ± 0.09b	28.86 ± 0.58c	33.64 ± 1.44d
MDA [μmol g^{-1}(f.m.)]	9.02 ± 0.19ab	10.75 ± 0.05c	9.43 ± 0.13b	8.87 ± 0.07a
Cell viability [A_{600}]	0.06 ± 0.002b	0.08 ± 0.003c	0.06 ± 0.002b	0.04 ± 0.002a
Total H_2O_2 [μmol g^{-1}(f.m.)]	100.73 ±5.62a	194.83 ± 5.36d	126.83 ± 2.90b	167.76 ± 1.27c
Total $^{\cdot}OH$ [nmol g^{-1}(f.m.)]	3.88 ± 0.13a	5.13 ± 0.17b	5.29 ± 0.06b	3.94 ± 0.20a
Apoplast H_2O_2 [μmol g^{-1}(f.m.)]	0.13 ± 0.002b	0.15 ± 0.012c	0.11 ± 0.008a	0.14 ± 0.009bc
Apoplast $^{\cdot}OH$ [nmol g^{-1}(f.m.)]	27.61 ± 0.50a	33.57 ± 0.50b	29.10 ± 0.50a	29.60 ± 1.00a
Apoplast $O_2^{\cdot-}$ [μmol g^{-1}(f.m.)]	1.07 ± 0.09a	1.35 ± 0.03b	1.18 ± 0.03a	1.02 ± 0.02a
Cell-wall bound POD [U mg^{-1}(protein)]	42.14 ± 0.72c	26.18 ± 0.57a	31.63 ± 1.24b	27.56 ± 1.33a
DAO [U mg^{-1}(protein)]	3.57 ± 0.19a	3.70 ± 0.06a	3.78 ± 0.02a	3.47 ± 0.06a
PAO [U mg^{-1}(protein)]	7.42 ± 0.20a	8.95 ± 0.47c	8.28 ± 0.16bc	7.73 ± 0.05b
Total SOD [U mg^{-1}(protein)]	10.81 ± 0.36a	12.26 ± 0.64b	14.37 ± 1.14c	28.89 ± 1.80d
Total CAT [U mg^{-1}(protein)]	4.45 ± 0.30a	5.00 ± 0.21b	4.49 ± 0.29a	9.88 ± 0.23c
Total POD [U mg^{-1}(protein)]	127.83 ± 1.15a	143.14 ± 1.57b	156.87 ± 1.77c	147.21 ± 1.59b
Total APX [U mg^{-1}(protein)]	68.20 ± 1.32ab	71.77 ± 2.94b	71.36 ± 2.28b	63.27 ± 2.14a
Total GR [U mg^{-1}(protein)]	2.14 ± 0.07a	2.44 ± 0.12b	3.27 ± 0.04c	3.54 ± 0.14c
Apoplast SOD [U g^{-1}(f.m.)]	6.42 ± 0.17a	6.75 ± 0.25ab	6.25 ± 0.14a	7.17 ± 0.22b
Apoplast CAT [U g^{-1}(f.m.)]	0.47 ± 0.02a	0.57 ± 0.01b	0.84 ± 0.02c	1.21 ± 0.02d
Apoplast POD [U g^{-1}(f.m.)]	47.80 ± 0.60a	107.33 ± 6.30c	48.27 ± 0.18a	93.67 ± 0.98b
Apoplast APX [U g^{-1}(f.m.)]	1.29 ± 0.04ab	1.55 ± 0.01b	1.16 ± 0.06a	2.40 ± 0.08c
Apoplast GR [U g^{-1}(f.m.)]	0.09 ± 0.001c	0.05 ± 0.002a	0.08 ± 0.002b	0.15 ± 0.009d

In comparison with the control, an insignificant elevation in root SOD activity was found when seedlings were treated with 300 μM Fe. In contrast, Fe treatment together with 50 μM or 250 μM Zn resulted in about 17 and 136 % increases of this enzyme activity, respectively, compared with single Fe treatment (Table 1). Root CAT activity was stimulated by 300 μM Fe. Compared with the control, no significant change in CAT activity was found in 50 μM Zn + Fe-treated roots. Differently, root CAT activity increased to 222 % of the control value in

250 μM Zn + Fe-stressed seedlings, respectively. Root POD activity resulting from 300 μM Fe treatment increased about 1.12-folds compared with the control. This enzyme activity further rose in 50 μM Zn + Fe-treated roots significantly, whereas 250 μM Zn did not affect Fe-induced changes of this parameter (Table 1). Compared to the control, root APX activity did not significantly change in wheat seedlings when exposed to 300 μM Fe with or without Zn (Table 1). A 14 % enhancement of GR activity was observed in 300 μM Fe-stressed roots in comparison with the control. When the seedlings were treated with 50 or 250 μM Zn and Fe in combination, root GR activity rose by about 34 and 45 %, respectively, in comparison with Fe treatment alone.

Compared with the control, apoplastic SOD activity was elevated in 300 μM Fe-stressed roots slightly. All Zn concentrations did not significantly alter Fe-induced effect on apoplastic SOD activity. After roots were treated with 300 μM Fe, apoplastic CAT activity increased to 121 % of the control values. Roots exposed to Fe stress together with 50 or 250 μM Zn exhibited about 47 and 112 % increases of apoplastic CAT activity in comparison with Fe-alone-treated roots, respectively. Apoplastic POD activity was 47.80 ± 0.60 U g^{-1} (f.m.) in untreated roots. In comparison with the control, apoplastic POD activity in roots remarkably increased in response to Fe treatment. Differently, its activity decreased by about 55 and 13 % in roots treated with 50 μM Zn + Fe and 250 μM Zn + Fe, respectively, in comparison with Fe-alone-treated ones. Apoplastic APX activity increased by about 20 % in roots in response to Fe stress compared with the control. 50 μM Zn + Fe did not affect this parameter, but 250 μM application led to about 86 % increase of apoplastic APX activity in Fe-treated roots, respectively, compared with the untreated ones. Compared with the control, Fe stress significantly inhibited apoplastic GR activity in wheat roots. All Zn concentrations obviously retarded Fe-induced inhibition of apoplastic GR activity in Fe-treated roots, with the highest GR activity at 250 μM Zn + Fe treatment (Table 1).

Discussion

Iron stress induced the decrease of relative growth rate and the increase of Fe content in young *Eugenia uniflora* roots (De Oliveira Jucoski *et al.* 2013). Similarly, reduced root length and increased Fe accumulation were observed in wheat seedlings exposed to different Fe concentrations including 300 μM Fe (Li *et al.* 2012b). Furthermore, in this study, the presence of 50 and 250 μM Zn could alleviate 300 μM Fe-induced inhibition of root growth. This was consistent with the study of Upadhyay and Panda (2010), which demonstrated that Zn application weakened Cu toxicity and promoted the growth of *Spirodela polyrhiza*. Metal-induced phytotoxicity is generally associated with high metal accumulation because cells have to spend extra energy to cope with high metal content in plant tissues (Greger 1999). Here, we observed that the application of different Zn concentrations obviously decreased Fe content but increased Zn content in Fe-stressed seedlings. In consistency with our results, Cu uptake gradually declined due to 10, 50, or 100 μM Zn in aquatic duckweed (Upadhyay and Panda 2010), and Zn supply clearly reduced Cd accumulation and increased Zn content in the leaves of tomato plants under Cd stress (Cherif *et al.* 2011). These phenomena might result from the competition of these metals for the same transport system during uptake process. This could be supported by the study of Zhao *et al.* (2012) demonstrating that Zn was a competitor for Fe absorption. Differently, the presence of Zn did not affect Cd accumulation in Cd-treated *Chara australis* (Clabeaux *et al.* 2013).

Plants treated with heavy metals produce ROS, which in turn can induce oxidative damage and even cell death (Maheshwari and Dubey 2009, Liu *et al.* 2012). MDA is an indicator of lipid peroxidation (Halliwell and Gutteridge 1993). In the present study, the changes of MDA content might be associated with alleviating effects of Zn on Fe-induced increases in total and apoplastic H_2O_2 and $O_2^{\cdot-}$ content together with apoplastic $^{\cdot}OH$ in wheat roots (Table 1). It was proposed that H_2O_2 played an important role in growth inhibition of heavy metal-stressed plants (Chen *et al.* 2000). A previous study demonstrated that oxidative stress might be responsible for the inhibition of root growth due to heavy metal stress (Tamás *et al.* 2009). Interestingly, the changes of MDA content in this study suggested that increased root length in 50 or 250 μM Zn + Fe-treated seedlings might be due to alleviating effect of Zn on lipid peroxidation induced by Fe stress. ROS accumulation and cell death resulting from Cd and Pb stress were reported in aerial roots of *Ficus microcarpa* (Liu *et al.* 2012). In this study, a declining trend in the loss of cell viability due to 50 μM or 250 μM Zn + Fe treatment might be responsible for the retarding effect of Zn on Fe-induced inhibition of root growth in wheat seedlings. This could be supported by the study of Finger-Teixeira *et al.* (2010) demonstrating that the reduction of root length was followed by the loss of cell viability in soybean plants under heavy metal treatment.

One enzymatic source of extracellular $O_2^{\cdot-}$ is plasma membrane (PM) NADPH oxidase (Matsumoto and Motoda 2012), and the stimulation of this enzyme may be responsible for extracellular $O_2^{\cdot-}$ or H_2O_2 accumulation when plants are exposed to different heavy metal stresses (Remans *et al.* 2010). DPI is a widely used selective

NADPH oxidase inhibitor (Auh and Murphy 1995). Decreased $O_2^{\cdot-}$ content in response to DPI supplement indicated that PM NADPH oxidase activity in Zn + Fe-stressed roots was lower than in other roots. Previous studies suggested that extracellular $O_2^{\cdot-}$ generation was required for root growth in different plants (Dunand *et al.* 2007, Kranner *et al.* 2010), but according to our study, the changes of $O_2^{\cdot-}$ generation by PM NADPH oxidase seemed to suggest that this enzyme might not be associated with the regulation of root growth in wheat seedlings under Fe treatment alone or together with Zn. Besides, cell wall-bound POD can mediate extracellular H_2O_2 generation through oxidation of NADH (Ranieri *et al.* 2001). Recent studies revealed that cell wall-bound POD activity was triggered and correlated with increased H_2O_2 content under heavy metal stresses (Verma *et al.* 2008, Ma *et al.* 2012, Wen *et al.* 2012). In the present study, the changes of cell wall-bound POD activity indicated that cell wall-bound POD was not responsible for increased H_2O_2 content in wheat seedlings. In addition to NADPH oxidase and cell wall-bound POD, it is believed that DAO and PAO participate in the production of extracellular H_2O_2 *via* catabolism of polyamines (Cvikrová *et al.* 2012, Talaat and Shawky 2012). Meanwhile, their activities could be triggered in plants exposed to different heavy metal stresses (Yang *et al.* 2010, Qiao *et al.* 2015). The present data suggested that PAO but not DAO might be involved in H_2O_2 generation in Fe-treated roots.

ROS content in plant tissues may also decrease with the increase of antioxidant enzyme activities including SOD, CAT, POD, APX, and GR. For example, increased CAT and APX activities, which might result in the reduction of H_2O_2 content, were found in Zn + Cu-treated duckweed (Upadhyay and Panda 2010). However, the increase of H_2O_2 generation and the stimulation of CAT, POD, and APX are caused by a heavy metal treatment in some plants (Wang *et al.* 2010, Thounaojam *et al.* 2012). These results supported the conclusion that heavy metals could stimulate ROS generation, leading to the increases of antioxidant enzyme activities as a defence system (Fariduddin *et al.* 2009, Azooz *et al.* 2012). The present data suggested that the reduction of H_2O_2 and $O_2^{\cdot-}$ content in response to 50 μM or 250 μM Zn +Fe treatment resulted from the stimulation of major ROS-scavenging enzymes because the increases of total SOD, CAT, and GR activities along with the stimulation of apoplastic CAT, APX and GR in Zn + Fe-treated roots compared with Fe-alone-treated ones were observed (Table 1). Similarly, in other plant species the addition of Zn further stimulated Cd- or Cu-induced increases of SOD, CAT, POD and GR compared with single metal stresses (Upadhyay and Panda 2010, Cherif *et al.* 2011). However, Zn presence did not affect Cu-induced changes of SOD, APX, and GR activities in *Sesbania drummondii* (Israr *et al.* 2011).

Conclusion

In comparison with Fe-alone-treated seedlings, the decrease of Fe content and the increase of Zn content in Zn + Fe-treated wheat seedlings revealed a competition of these two metals for the same membrane transporters. Low Zn concentration alleviated Fe-inhibitory effects on root growth, which might be linked to the decrease in MDA content, the increase of cell viability, the reduction of total H_2O_2 and $O_2^{\cdot-}$, as well as apoplastic H_2O_2, $^{\cdot}OH$, and $O_2^{\cdot-}$ accumulation. Moreover, the reduced ROS generation was correlated with the increases of total SOD, CAT, and GR activities along with apoplastic CAT, APX, and GR activities in Zn + Fe-treated roots compared with Fe-alone-treated ones. However, cell wall-bound POD, DAO, and PAO activities in Fe-treated roots were insensitive to Zn addition.

References

Achary, V.M.M., Jena, S., Panda, K.K., Panda, B.B.: Aluminium induced oxidative stress and DNA damage in root cells of *Allium cepa* L.. - Ecotoxicol. Environ. Safety **70**: 300-310, 2008.

Achary, V.M.M., Patnaik, A.R., Panda, B.B.: Oxidative biomarkers in leaf tissue of barley seedlings in response to aluminum stress. - Ecotoxicol. Environ. Safety **75**: 16-26, 2012.

Aebi, H.: Catalase *in vitro*. - Method Enzymol. **105**: 121-126, 1984.

Asthir, B., Duffus, C.M., Smith, R.C., Spoor, W.: Diamine oxidase is involved in H_2O_2 production in the chalazal cells during barley grain filling. - J. exp. Bot. **53**: 677-682, 2002.

Auh, C.K., Murphy, T.M.: Plasma membrane redox enzyme is involved in the synthesis of $O_2^{\bullet-}$ and H_2O_2 by *Phytophthora* elicitor-stimulated rose cells. - Plant Physiol. **107**: 1241-1247, 1995.

Azooz, M.M., Abou-Elhamed, M.F., Al-Fredan, M.A.: Biphasic effect of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of wheat (*Triticum aestivum* cv. Hasaawi) at early growing stage. - Aust. J. Crop Sci. **6**: 688-694, 2012.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chen, L.M., Lin, C.C., Kao, C.H.: Copper toxicity in rice seedlings: changes in antioxidative enzyme activities, H_2O_2

level, and cell wall peroxidase activity in roots. - Bot. Bull. Acad. sin. **41**: 99-103, 2000.

Cherif, J., Mediouni, C., Ammar, W.B., Jemal, F.: Interactions of zinc and cadmium toxicity in their effects on growth and in antioxidative systems in tomato plants (*Solarium lycopersicum*). - J. environ. Sci. **23**: 837-844, 2011.

Clabeaux, B.L., Navarro, D.A., Aga, D.S., Bisson, M.A.: Combined effects of cadmium and zinc on growth, tolerance, and metal accumulation in *Chara australis* and enhanced phytoextraction using EDTA. - Ecotoxicol. Environ. Safety **98**: 236-243, 2013.

Córdoba-Pedregosa, M.C., Villalba, J.M., Córdoba, F., González-Reyes, J.A.: Changes in intracellular and apoplastic peroxidase activity, ascorbate redox status, and root elongation induced by enhanced ascorbate content in *Allium cepa* L.. - J. exp. Bot. **56**: 685-694, 2005.

Cvikrová, M., Gemperlová, L., Dobrá, J., Martincová, O., Prásil, I., Gubis, J., Vanková, R.: Effect of heat stress on polyamine metabolism in proline-over-producing tobacco plants. - Plant Sci. **182**: 49-58, 2012.

De Oliveira-Jucoski, G., Cambraia, J., Riveiro, C., Alves-De Oliveira, J., Oliveira-De Paula, S., Oliva, M.A.: Impact of iron toxicity on oxidative metabolism in young *Eugenia uniflora* L. plants. - Acta Physiol. Plant. **35**: 1645-1657, 2013.

Dhindsa, R.S., Matowe, W.: Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation. - J. exp. Bot. **32**: 79-91, 1981.

Dos Santos, W.D., Ferrarese, M.L.L., Nakamura, C.V., Mourão, K.S.M., Mangolin, C.A., Ferrarese-Filho, O.: Soybean (*Glycine max*) root lignification induced by ferulic acid. The possible mode of action. - J. chem. Ecol. **34**: 1230-1241, 2008.

Dunand, C., Crèvecoeur, M., Penel, C.: Distribution of superoxide and hydrogen peroxide in *Arabidopsis* root and their influence on root development: possible interaction with peroxidases. - New Phytol. **174**: 332-341, 2007.

Fariduddin, Q., Yusuf, M., Hayat, S., Ahmad, A.: Effect of 28-homobrassinolide on antioxidant capacity and photosynthesis in *Brassica juncea* plants exposed to different levels of copper. - Environ. exp. Bot. **66**: 418-424, 2009.

Finger-Teixeira, A., Ferrarese, M.L.L., Soares, A.R., De Silva, D., Ferrarese-Filho, O.: Cadmium-induced lignification restricts soybean root growth. - Ecotoxicol. Environ. Safety **73**: 1959-1964, 2010.

Greger, M.: Metal availability and bioconcentration in plants. - In: Hagemeyer, J. (ed.): Heavy Metals Stress in Plants. From Molecules to Ecosystems. Pp. 1-27. Springer, Berlin - Heidelberg 1999.

Halliwell, B., Gutteridge, J., Aruoma, O.I.: The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. - Anal. Biochem. **165**: 215-219, 1987.

Halliwell, B., Gutteridge, J.M.C.: Free Radicals in Biology and Medicine. - Clarendon Press, Oxford 1993.

Halliwell, B., Gutteridge, J.M.C.: The chemistry of free radicals and related 'reactive species'. - Free Radicals Biol. Med. **3**: 220, 1999.

Israr, M., Jewell, A., Kumar, D., Sahi, S.V.: Interactive effects of lead, copper, nickel and zinc on growth, metal uptake and antioxidative metabolism of *Sesbania drummondii*. - J. Hazard. Mater. **186**: 1520-1526, 2011.

Karuppanapandian, T., Moon, J.C., Kim, C., Manoharan, K., Kim, W.: Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. - Aust. J. Crop. Sci. **5**: 709-725, 2011.

Kranner, I., Roach, T., Beckett, R.P., Whitaker, C., Minibayeva, F.V.: Extracellular production of reactive oxygen species during seed germination and early seedling growth in *Pisum sativum*. - J. Plant Physiol. **167**: 805-811, 2010.

Lee, T.M., Lin, Y.H.: Changes in soluble and cell wall-bound peroxidase activities with growth in anoxia-treated rice (*Oryza sativa* L.) coleoptiles and roots. - Plant Sci. **106**: 1-7, 1995.

Li, X.N., Ma, H.Z., Jia, P.X., Wang, J., Jia, L.Y., Zhang, T.G., Yang, Y.L., Chen, H.J., Wei, X.: Responses of seedling growth and antioxidant activity to excess iron and copper in *Triticum aestivum* L.. - Ecotoxicol. Environ. Safety **86**: 47-53, 2012a.

Li, X.N., Yang, Y.L., Zhang, J., Jia, L.Y., Li, Q.X., Zhang, T.G., Qiao, K.X., Ma, S.C.: Zinc induced phytotoxicity mechanism involved in root growth of *Triticum aestivum* L.. - Ecotoxicol. Environ. Safety **86**: 198-203, 2012b.

Liu, N., Lin, Z.F., Mo, H.: Metal (Pb, Cd, and Cu)-induced reactive oxygen species accumulations in aerial root cells of the Chinese banyan (*Ficus microcarpa*). - Ecotoxicology **21**: 2004-2011, 2012.

Ma, B., Gao, L., Zhang, H., Gui, J., Shen, Z.: Aluminum-induced oxidative stress and changes in antioxidant defenses in the roots of rice varieties differing in Al tolerance. - Plant Cell Rep. **31**: 687-696, 2012.

Maheshwari, R., Dubey, R.S.: Nickel-induced oxidative stress and the role of antioxidative defense in rice seedlings. - Plant Growth Regul. **59**: 37-49, 2009.

Matsumoto, H., Motoda, H.: Aluminum toxicity recovery processes in root apices. Possible association with oxidative stress. - Plant Sci. **185**: 1-8, 2012.

Naik, B.I., Goswami, R.G., Srivastawa, S.K.: A rapid and sensitive colorimetric assay of amine oxidase. - Anal. Biochem. **111**: 146-148, 1981.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. - Plant Cell Physiol. **22**: 867-880, 1981.

Nenova, V.: Effect of iron supply on growth and photosystem II efficiency of pea plants. - Gen. appl. Plant Physiol. **2006** Special issue: 81-90, 2006.

Pasqualini, S., Cresti, M., Del Casino, C., Faleri, C., Frenguelli, G., Tedeschini, E., Ederli, L.: Roles for NO and ROS signalling in pollen germination and pollen-tube elongation in *Cupressus arizonica*. - Biol. Plant. **59**: 735-744, 2015.

Qiao, X.Q., Zheng, Z.Z., Zhang, L.F., Wang, J.H., Shi, G.X., Xu, X.Y.: Lead tolerance mechanism in sterilized seedlings of *Potamogeton crispus* L.: Subcellular distribution, polyamines and proline. - Chemosphere **120**: 179-187, 2015.

Ranieri, A., Castagna, A., Baldan, B., Soldatini, G.F.: Iron deficiency differently affects peroxidase isoforms in sunflower. - J. exp. Bot. **354**: 25-35, 2001.

Rao, M.V., Paliyath, G., Ormrod, D.P.: Ultraviolet-B-and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. - Plant Physiol. **110**: 125-136, 1996.

Remans, T., Opdenakker, K., Smeets, K., Matgijsen, D., Vangronsveld, J., Cuypers, A.: Metal-specific and NADPH oxidase dependent changes in lipoxygenase and NADPH

oxidase gene expression in *Arabidopsis thaliana* exposed to cadmium or excess copper. - Funct. Plant Biol. **37**: 532-544, 2010.

Schaedle, M., Bassham, J.A.: Chloroplast glutathione reductase. - Plant Physiol. **59**: 1011-1012, 1977.

Sergiev, I., Alexieva, V.: Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. - Compt. rend. Acad. Bulg. Sci. **51**: 121-124, 1997.

Sinha, S., Basant, A.: Iron-induced oxidative stress in a macrophyte: a chemometric approach. - Ecotoxicol. Environ. Safety **72**: 585-595, 2009.

Talaat, N.B., Shawky, B.T.: 24-Epibrassinolide ameliorates the saline stress and improves the productivity of wheat (*Triticum aestivum* L.). - Environ. exp. Bot. **82**: 80-88, 2012.

Tamás, L., Dudiková, J., Ďurčeková, K., Halušková, L., Huttová, J., Mistrík, I.: Effect of cadmium and temperature on the lipoxygenase activity in barley root tip. - Protoplasma **235**: 17-25, 2009.

Tewari, R.K., Hadacek, F., Sassmann, S., Lang, I.: Iron deprivation-induced reactive oxygen species generation leads to non-autolytic PCD in *Brassica napus* leaves. - Environ. exp. Bot. **91**: 74-83, 2013.

Thounaojam, T.C., Panda. P., Mazumeder, P., Kumar, D., Sharma, G.D., Sahoo, L., Panda, S.K.: Corrigendum to "Excess copper induced oxidative stress and response of antioxidants in rice". - Plant Physiol. Biochem. **53**: 33-39, 2012.

Turhan, E., Aygogan, C., Baykul, A., Akoglu, A., Evrennosoglu, Y., Ergin, S.: Apoplastic antioxidant enzymes in the leaves of two strawberry cultivars and their relationship to cold-hardiness. - Not. Bot. Hort. Agrobot. **40**: 114-122, 2012.

Upadhyay, R.K., Panda, S.K.: Zinc reduces copper toxicity induced oxidative stress by promoting antioxidant defense in freshly grown aquatic duckweed *Spirodela polyrhiza* L.. - J. Hazard. Mater. **175**: 1081-1084, 2010.

Vallee, B.L., Falchuk, K.H.: The biochemical basis of zinc physiology. - Physiol. Rev. **73**: 79-118, 1993.

Verma, K., Shekhawat, G.S., Sharma, A., Mehta, S.K., Sharma, V.: Cadmium induced oxidative stress and changes in soluble and ionically bound cell wall peroxidase activities in roots of seedling and 3-4 leaf stage plants of *Brassica juncea* (L.) Czern. - Plant Cell Rep. **27**: 1261-1269, 2008.

Wang, J., Li, W.H., Zhang, C.B., Ke, S.S.: Physiological responses and detoxific mechanisms to Pb, Zn, Cu and Cd in young seedlings of *Paulownia fortunei*. - J. environ. Sci. **22**: 1916-1922, 2010.

Wen, J.F., Deng, M.H., Gong, M.: Cd^{2+} stress induces two waves of H_2O_2 accumulation associated with ROS-generating system and ROS-scavenging system in cultured tobacco cells. - Aust. J. Crop Sci. **6**: 846-853, 2012.

Yang, H.Y., Shi, G.X., Wang, H.X., Xu, Q.S.: Involvement of polyamines in adaptation of *Potamogeton crispus* L. to cadmium stress. - Aquat. Toxicol. **100**: 282-288, 2010.

Zanardo, D.I.L., Lima, R.B., Ferrarese, M.L.L., Bubna, G.A., Ferrarese-Filho, O.: Soybean root growth inhibition and lignification induced by p-coumaric acid. - Environ. exp. Bot. **66**: 25-30, 2009.

Zhao, H.J., Wu, L.Q., Chai, T.Y., Zhang, Y.X., Tan, J.J., Ma, S.W.: The effects of copper, manganese and zinc on plant growth and elemental accumulation in the manganese-hyperaaccumulator *Phytolacca americana*. - J. Plant Physiol. **169**: 1243-1252, 2012.

Zhou, Q.: The measurement of malondialdehyde in plants. - In: Zhou, Q. (ed.): Methods in Plant Physiology. Pp. 173-174. Agricultural Press, Beijing 2001.