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Vol. 8(16), pp. 1466-1471, 2 May, 2013
DOI: 10.5897/AJAR12.073
ISSN 1991-637X ©2013 Academic Journals
http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

Plasma membrane NADPH oxidase and apoplastic POD can be responsible for accumulation of H_2O_2 in mesophyll cells of *Elsholtzia haichowensis* under copper stress

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Accepted 15 June, 2012

Under copper (Cu) stress, the activities of some enzymes involved in the antioxidant defense system in leave cells of Cu accumulator *Elsholtzia haichowensis* were investigated. Our results showed that Cu treatment significantly increased the activities of plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, apoplastic peroxidase (POD), apoplastic superoxide dismutase (SOD), symplastic SOD and symplastic ascorbate peroxidase (APX) in leaves of *E. haichowensis*, when additional treatment with *N-N*-diethyldithiocarbamate as an inhibitor of SOD, NaN_3 as an inhibitor of peroxidase, diphenyleneiodonium as an inhibitor of NADPH oxidase and 1, 2-dihydroxybenzene-3, 5-disulphonic acid as an O_2^- scavenger and Cu-induced activities of antioxidant enzymes in leave cells of *E. haichowensis* were significantly inhibited. The results suggested that plasma membrane NADPH oxidase and apoplastic POD can be responsible for the accumulation of H_2O_2 in mesophyll cells of *E. haichowensis* under copper stress. In the leaf symplast, however, Cu-induced activities of SOD and APX may be important in avoiding the build-up of toxic H_2O_2 concentrations.

Key words: *Elsholtzia haichowensis*, leave, copper, inhibitor, antioxidant enzyme.

INTRODUCTION

Copper (Cu) is an essential micronutrient for plant, being associated with proteins and enzymes involved in electron transfer and redox reactions (Marschner, 1995), However, excess Cu interferes with numerous biochemical and physiological processes including photosynthesis, pigment synthesis, nitrogen and protein metabolism, membrane integrity, and mineral uptake (Luna et al., 1994; Nielsen et al., 2003; Demirevska-Kepova et al., 2004). Being a redox-active metal, Cu^+ can catalyze superoxide anion (O_2^-) formation and subsequently results in hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) production through Fenton-type reactions (Schützendübel and Polle, 2002). To avoid the deleterious effect of ROS, plant cells have evolved antioxidant defense mechanisms. These include enzymatic components, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), and glutathione reductase (GR), as

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well as, non-enzymatic components, such as ascorbic acid (ASC) and glutathione (GSH) (Mittler, 2002). These mechanisms of defense against oxidative damage have been observed in plants exposed to excess Cu (Chongpraditnum et al., 1992; Gupta et al., 1999; Morelli and Scarano, 2004; Lombardi and Sebastiani, 2005; Tewari et al., 2006; Sgherri et al., 2007; Zhang et al., 2008, 2010).

Elsholtzia haichowensis, of the family Lamiaceae, is a Cu-accumulator plant widely distributed in Cu-mining wastes and Cu-contaminated soils in China (Lou et al., 2004; Song et al., 2004). Previous studies showed that *E. haichowensis* was able to tolerate Cu in shoots at 160 µg g^{-1} dry weight from plants grown under hydroponic conditions when supplied with 500 µM exogenous Cu (Lou et al., 2004; Qian et al., 2005), which was much higher than the toxicity threshold (20 to 30 µg g^{-1} DW) of most other crop species (Marschner, 1995). Therefore, *E. haichowensis* must employ some internal mechanism in order to tolerate the elevated levels of Cu in shoot tissues.

Cytochemical analysis demonstrated that the accumulation of H_2O_2 was primarily localized in the cell walls and extracellular space of mesophyll cells of *E. haichowensis*, as a consequence of Cu treatment (Zhang et al., 2010). In this paper, we studied the changes in the activities of some of the enzymes involved in the antioxidant defense system in leave cells of *E. haichowensis* under Cu stress. We also investigated the effect of additional treatment with some inhibitors, such as *N*-*N*-diethyldithiocarbamate (DDC) as an inhibitor of SOD, NaN_3 as an inhibitor of peroxidase, diphenyleneiodonium (DPI) as an inhibitor of NADPH oxidase and 1, 2-dihydroxybenzene-3, 5-disulphonic acid (Tiron) as an O_2^- scavenger on the antioxidant enzymes, which may help us to assess the possible link between these subcellular antioxidant enzymes in leaves of *E. haichowensis*.

MATERIALS AND METHODS

Plant materials and treatment with inhibitors

Seeds of *E. haichowensis* were collected from a copper-contaminated site at Tongling (Anhui Province), China, in December 2009 and were germinated at room temperature (20 to 30°C) in plastic dishes filled with vermiculite. After seven days, the seedlings were selected for uniformity and transferred to vessels containing Hoagland nutrient solution. The 30 day-old seedlings were treated for 6 days with 100 µM Cu supplied in the nutrient solution as $CuSO_4 \cdot 5H_2O$. The experimental design was completely randomized, with three replicate vessels each containing 20 seedlings. The solution pH was adjusted to 5.3 with NaOH or HCl upon renewal of the nutrient solution every 2 days. Shoots from control and Cu-treated plants for six days were additionally treated with distilled water or inhibitors: 10 mM DDC, 1 mM NaN_3, 50 µM DPI, or 10 mM Tiron. The root was excised at the base of the stem before inhibitors treatment to avoid its influence. After 8 h incubation, the leaves were homogenized and assayed for detection enzyme activities by spectrophotometry.

Isolation of plasma membranes and determination of NADPH oxidase activity

Leaf plasma membranes were isolated using the two-phase aqueous polymer partition system according to the method of Larsson et al. (1987), with some modifications. Briefly, fresh leaves were homogenized in four volumes of extraction buffer containing 50 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 1 mM ASC, 1 mM EDTA, 0.6% polyvinylpyrrolidone, and 1 mM PMSF. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was recovered and centrifuged at 100,000 × g for 40 min at 4°C to obtain a microsomal membrane pellet. The pellet was suspended in 0.33 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8). The plasma membrane fraction was isolated by adding the microsomal suspension to an aqueous two-phase polymer system to give a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) PEG 3350, 0.33 M sucrose, 3 mM KCl, and 5 mM potassium phosphate (pH 7.8). Three successive rounds of partitioning followed. The resulting upper phase was diluted five-fold in Tris–HCl dilution buffer (10 mM, pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 1 mM ASC, and 1 mM PMSF. All procedures were carried out at 4°C. Protein content of plasma membranes was determined according to the method of Bradford (1976), with bovine serum albumin (BSA) as a standard.

The NADPH-dependent O_2^- generating activity in isolated plasma membrane vesicles was determined by following the reduction of sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium] -bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) by O_2^- (Sagi and Fluhr, 2001). One milliliter of assay mixture contained 50 mM Tris–HCl buffer (pH 7.5), 0.5 mM XTT, 100 µM NADPH, and 15 to 20 µg of membrane protein. The reaction was initiated by the addition of NADPH, and XTT reduction was determined at 470 nm. Corrections were made for background production in the presence of 50 U SOD. Rates of O_2^- generation were calculated using an extinction coefficient of $2.16 \times 10^4\ M^{-1}\ cm^{-1}$.

Isolation of apoplastic and symplastic fractions and assays of antioxidant enzyme activities

Apoplastic fluid was extracted by a vacuum infiltration–centrifugation technique essentially as reported by Vanacker et al. (1998) but with some modifications (Zhang et al., 2010). About 2 g of fresh leaves were quickly washed in distilled water, placed in petri dishes in 10 mM sodium phosphate buffer (pH 6) containing 1% (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM EDTA, and 0.5 mM phenylmethylsulphonyl fluoride (PMSF), and then submitted to a vacuum for 10 min at 4°C. Afterwards, leaves were carefully dried with filter paper and placed in syringes, which were then placed in centrifuge tubes and centrifuged at 1500 × *g* for 5 min and the apoplastic fluid recovered at the bottom of the tubes. The remaining tissue was used to obtain the soluble symplastic fraction after homogenization in the same buffer and centrifugation at 15000 × *g* for 20 min. According to the activity of the cellular marker enzyme glucose-6-phosphate dehydrogenase (G6PDH) in the apoplastic fluid, the contamination of the apoplastic fluid by cytosolic protein was less than 1%. Soluble proteins were extracted with ice-cold extraction buffer (50 mM potassium phosphate + 1 mM EDTA, pH 7.0) with 1% PVPP (w/v). APX activity was measured by monitoring the decrease in the absorbance at 290 nm as ascorbate was oxidized, as described by Nakano and Asada (1981). POD was determined according to Zheng and Van Huystee (1992), the reaction was followed at A_{470}. SOD activity was determined by the ability of SOD to inhibit the formation of nitroblue formazan from nitroblue tetrazolium (NBT), according to the method of Giannopolitis and Ries (1977). In this assay, SOD activity was

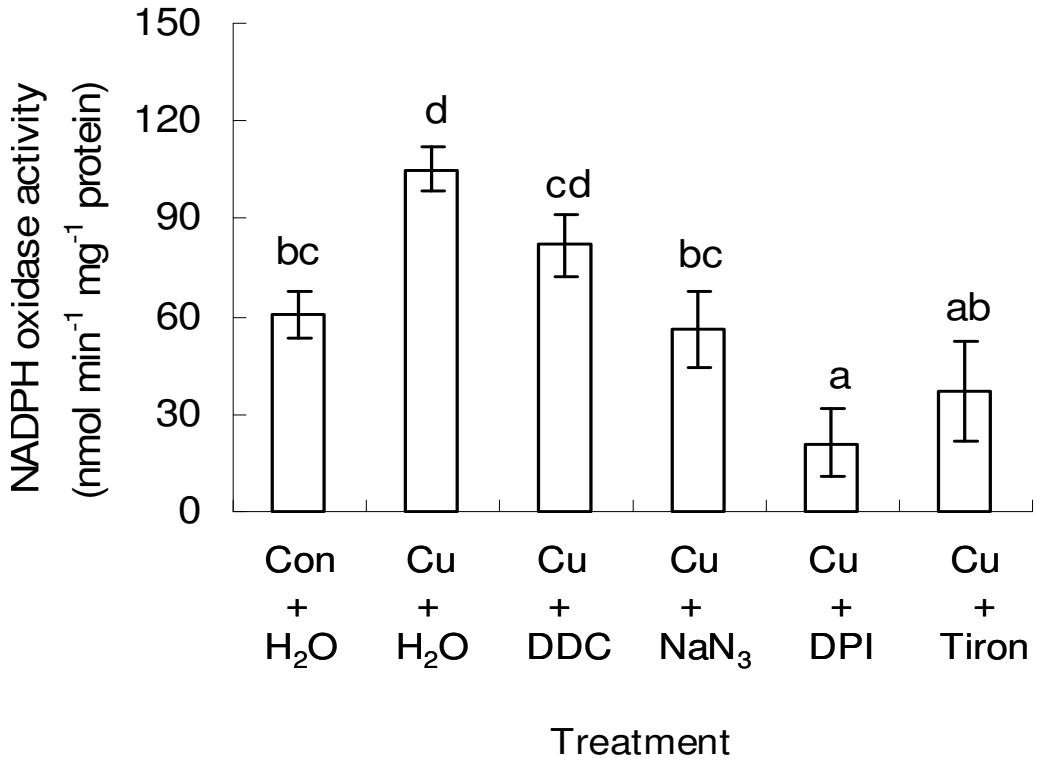

Figure 1. Cu treatment and additional treatment with DDC, NaN_3, DPI and Tiron induced changes in plasma membrane–bound NADPH oxidase activities in the leaves of *E. haichowensis*. Plants were exposed to control treatment (Con) or 100 µM Cu treatment for six days and their shoots were subsequently treated with distilled water or 10 mM DDC, or 1 mM NaN_3, or 50 µM DPI or 10 mM Tiron. Excised leaves were homogenized and enzyme activity assayed by spectrophotometry. Values are means ± SE (n = 3) of three separate experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

expressed in units per milligram of protein. One unit of SOD is defined as the amount required to inhibit photoreduction of NBT by 50%. Protein content was estimated according to the method of Bradford (1976), using BSA as a standard.

RESULTS

Effect of Cu and inhibitors on the activity of plasma membrane NADPH oxidase

Under biotic and abiotic stress conditions, plasma membrane–bound NADPH oxidases have been accepted as being responsible for O_2^- production, and this enzyme can use cytosolic NADPH to produce O_2^-, which is quickly dismutated to H_2O_2 by SOD (Mittler, 2002). Compared with control, 100 µM Cu treatment significantly increased the activity of plasma membrane NADPH oxidase. Treatment with DPI, an inhibitor of NADPH oxidase, significantly decreased the activity of plasma membrane NADPH oxidase, but NaN_3 as an inhibitor of peroxidase and Tiron as an O_2^- scavenger significantly also decreased the activities of Cu-induced NADPH oxidase (Figure 1).

Effect of Cu and inhibitors on the activity of apoplastic and symplastic antioxidant enzymes

The activities of the antioxidant enzymes SOD, POD and APX, were analyzed in the apoplastic and symplastic compartments of *E. haichowensis* leaves. The total activities of SOD, POD, and APX in the leaf symplast were significantly higher than those in the apoplast when calculated on a fresh-weight basis (Figure 2). In the apoplastic space of *E. haichowensis* leaves, the activity of SOD accounted for about 2.0% of the total foliar SOD activity, APX for 1.3 to 2.0%, and POD for 10.1 to 12.2% (Table 1). Compared with the control, treatment with 100 µM Cu significantly increased the total activities of apoplastic and symplastic SOD, apoplastic POD, and symplastic APX (Figure 2). Treatment with DDC, NaN_3, DPI and Tiron significantly inhibited the activities of Cu-induced apoplastic SOD and symplastic APX, treatment with NaN_3, DPI and Tiron significantly inhibited the activities of Cu-induced apoplastic POD and treatment with DDC, NaN_3 and Tiron significantly inhibited the activities of Cu-induced symplastic SOD (Figure 2).

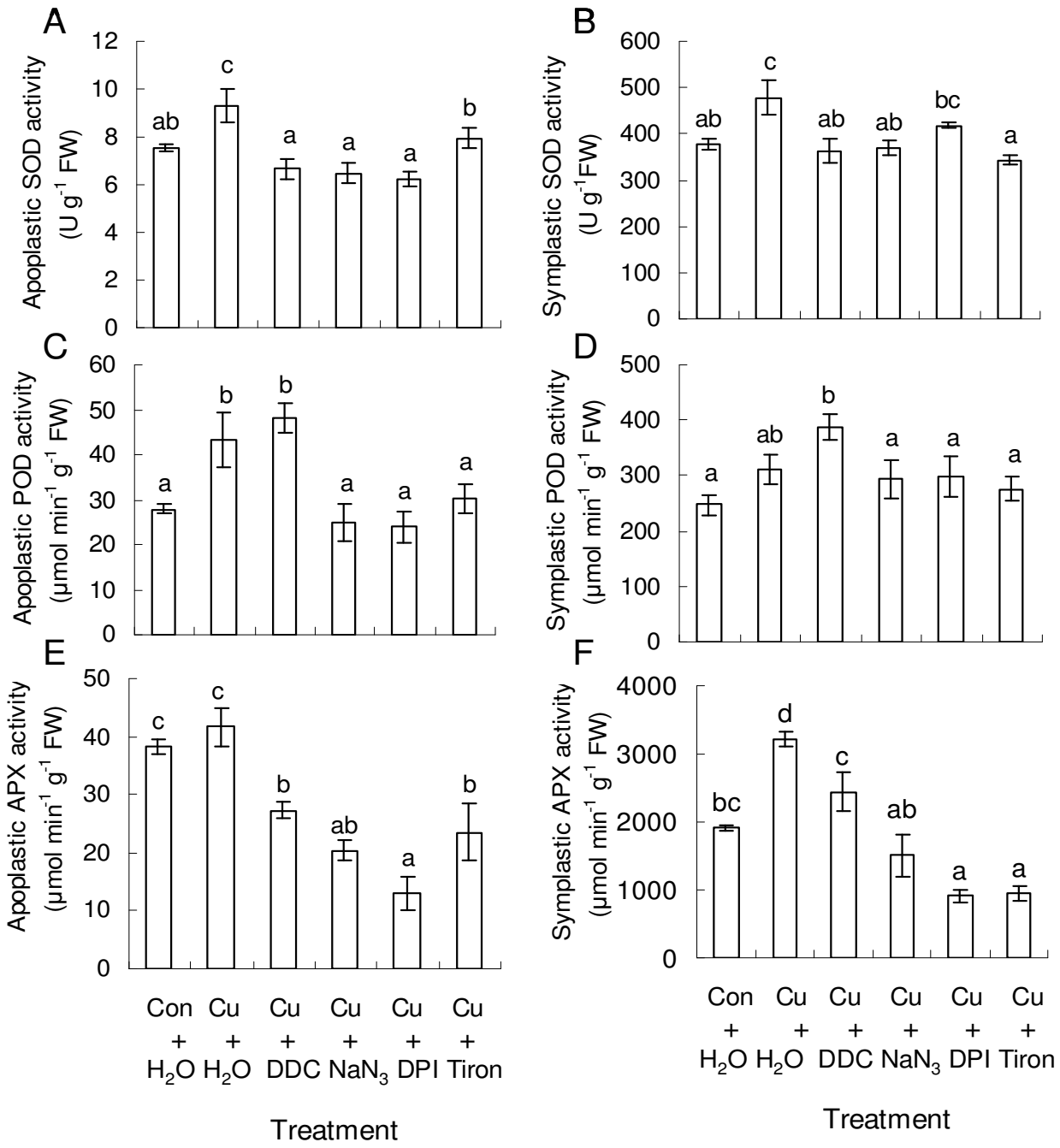

Figure 2. Effects of Cu treatment and additional treatment with DDC, NaN_3, DPI and Tiron on apoplastic and symplastic APX, POD, and SOD activities in the leaves of *E. haichowensis*. Plants were exposed to control treatment (Con) or 100 μM Cu treatment for six days and their shoots were subsequently treated with distilled water or r 10 mM DDC, or 1 mM NaN_3, or 50 μM DPI, or 10 mM Tiron. Excised leaves were homogenized and enzyme activity assayed by spectrophotometry. Values are means ± SE (n = 3) of three separate experiments. Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan's multiple range test.

DISCUSSION

In previous experiments, cytochemical analysis demonstrated that the H_2O_2-dependent $CeCl_3$ precipitates were primarily localized in the cell walls of mesophyll cells of *E. haichowensis*, whereas the O_2^- dependent DAB precipitates were mainly localized in the chloroplasts of mesophyll cells after Cu treatment (Zhang et al., 2010). Accumulation of H_2O_2 in Cu-stressed plants may be the consequence of a distribution of balance

Table 1. The percent of POD, APX and SOD activities in the apoplast, with respect to the total present in the whole leaves of *E. haichowensis*.

Cu (μM)	Apoplastic enzyme activities /total enzyme activities		
	POD (%)	APX (%)	SOD (%)
0.32	10.1	2.0	2.0
100	12.2	1.3	2.0

between its production and scavenging. H_2O_2 can be generated by specific enzymes such as SOD, NADPH oxidase, amine oxidase, and a cell-wall peroxidase (Neill et al., 2002). It has been reported that plasma membrane NADPH oxidase and apoplastic POD may be involved in H_2O_2 production under some stresses (Romero-Puertas et al., 2004; Hsu and Kao, 2007; Yeh et al., 2007; Fecht-Christoffers et al., 2006). In this study, we observed that Cu treatment significantly increased the activity of plasma membrane NADPH oxidase and apoplastic POD (Figures 1 and 2). Furthermore, the inhibition of NADPH oxidase by DPI, NaN_3 as an inhibitor of peroxidase and the scavenging of O_2^- by Tiron, significantly decreased Cu-induced activities of NADPH oxidase and apoplastic POD in the leaves of *E. haichowensis*. These results showed that the source of H_2O_2 in the cell walls of *E. haichowensis* leaves could be partly attributed to the activity of NADPH oxidase, and the enzyme can use cytosolic NADPH to produce O_2^- which quickly dismutates to H_2O_2 in a reaction catalyzed by apoplastic SOD. The activity of apoplastic POD can also be partly responsible for the accumulation of H_2O_2 in the cell walls of mesophyll cells of *E. Haichowensis*.

The accumulation of H_2O_2 is prevented by CAT, GR, POD, and APX. It is interesting to note that induction of the apoplastic SOD activity coincided with changes in the activity of plasma membrane NADPH oxidase and the activity of apoplastic POD in plants exposed to 100 μM Cu, whereas the total symplastic SOD activity coincided with that of APX. SODs, which catalyze the dismutation of O_2^- to H_2O_2 and O_2, are another key group of enzymes involved in protection of cells against oxidative stress. This result is of some significance, because the expected increase in H_2O_2 as a result of the SOD reaction was accompanied by an increased enzymatic capacity to decompose it. Results also suggested that SOD and APX are major scavengers of ROS in the symplastic spaces of *E. haichowensis* leaves.

Treatment with NaN_3 and Tiron significantly inhibited the activities of all Cu-induced antioxidant enzymes while treatment with DDC only inhibited the activities of Cu-induced SOD and symplastic APX, Treatment with DPI had not significantly effect on the activities of Cu-induced symplastic APX (Figure 2). These results showed that Cu-induced increase in the activities of various antioxidant enzymes in different cell compartments may represent a defense mechanism against oxidative stress.

In conclusion, our results suggest that the activity of plasma membrane-bound NADPH oxidase, apoplastic SOD and apoplastic POD may play an important role in the antioxidant defense system in the leaves of apoplast of *E. haichowensis*. In the symplast, SOD and APX can eliminate H_2O_2 accumulation as a result of Cu treatment.

ACKNOWLEDGEMENTS

This research project was supported by the Scientific and Technological Research Projects in Henan Province (112102310661).

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