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Copper toxicity in rice seedlings: Changes in antioxidative enzyme activities, H_2O_2 level, and cell wall peroxidase activity in roots

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Abstract. The changes in lipid peroxidation, antioxidative enzyme activity, H_2O_2 level, and cell wall peroxidase activity in Cu-stressed roots of rice seedlings and their relation with root growth inhibition were investigated. $CuSO_4$ was effective in inhibiting root growth but not shoot growth. Treatment with $CuSO_4$ resulted in an increase in lipid peroxidation and modulated antioxidative enzyme activities in rice roots. $CuSO_4$ increased the activities of superoxide dismutase, ascorbate peroxidase, glutathione reductase, and peroxidase, but had no effect on catalase. $CuSO_4$ also increased H_2O_2 level and cell wall peroxidase in roots of rice seedlings. Exogenous application of H_2O_2 resulted in an inhibition of root growth. It appears that growth inhibition of root caused by Cu is associated with H_2O_2 dependent peroxidase-catalyzed formation of cross-linking among cell wall polymers.

Keywords: Copper; Lipid peroxidation; *Oryza sativa*; Oxidative stress; Root growth.

Abbreviations: **APOD,** ascorbate peroxidase; **CAT,** catalase; **FW,** fresh weight; **GR,** glutathione reductase; **MDA,** malondialdehyde; **POD,** peroxidase; **SOD,** superoxide dismutase.

Introduction

Copper (Cu) is an essential element for plant growth (Arnon and Stout, 1939), and it is important in various biochemical processes, but at toxic concentrations it interferes with numerous physiological processes (Fernandes and Henriques, 1991).

Cu is known to damage cell membranes by binding to the sulphydryl groups of membrane proteins and by inducing lipid peroxidation (De Vos et al., 1989; De Vos et al., 1992). Cu-mediated free radical formation has been demonstrated in isolated chloroplasts (Sandmann and Boger, 1980), in intact roots (De Vos et al., 1993), in leaf segments (Chen and Kao, 1999; Gallego et al., 1996; Luna et al., 1994) and in intact leaves (Weckx and Clijsters, 1996).

Cellular damage caused by free radicals might be reduced or prevented by a protective metabolism involving antioxidative enzymes such as SOD, APOD, GR, CAT and POD. SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H_2O_2. APOD reduces H_2O_2 to water, with ascorbate as electron donor (Asada, 1992). GR plays a part in the control of endogenous H_2O_2 through an oxido-reduction cycle involving glutathione and ascorbate (Foyer and Halliwell, 1976; Smith et al., 1989). CAT and POD are implicated in removal of H_2O_2. It has been reported that Cu increases the activities of antioxidative enzymes such as SOD (Chongpraditnum et al., 1992; Rama Devi and Prasad, 1998), POD (Karataglis et al., 1991), CAT (Rama Devi and Prasad, 1998), and APOD (Rama Devi and Prasad, 1998). It is well known that CAT and APOD play an important role in preventing oxidative stress by catalyzing the reduction of H_2O_2 (Weckx and Clijsters, 1996). Rama Devi and Prasad (1998) found that CAT and APOD activities were increased by Cu, suggesting that excess Cu may increase the production of H_2O_2. H_2O_2 is a necessary substrate for the cell wall stiffening process catalyzed by cell wall POD (Elstner and Heupel, 1976; Hohl et al., 1995; Schopfer, 1996), which is considered to be one of the mechanisms resulting in growth inhibition (Fry, 1986). The present investigation was designed to study the change in lipid peroxidation, antioxidative enzyme activities, H_2O_2 level and cell wall POD activity in Cu-stressed roots of rice seedling and their relation with root growth inhibition.

Materials and Methods

Rice (*Oryza sativa* L. cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed thoroughly with distilled water. These seeds were then germinated in a Petri dish (20 cm) containing distilled water at 37°C in the dark. After a 1-day incubation, uniformly germinated seeds were selected and transferred to Petri dishes (9.0 cm) containing two sheets of Whatman No. 1 filter paper moistened with 10 ml of distilled water

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or test solution. Each Petri dish contained 20 germinated seeds. Each treatment was replicated 4 times. The germinated seeds were allowed to grow at 27°C in darkness, and 3 ml of distilled water or test solution was added to each Petri dish on day 3 of growth. All experiments described here were performed three times. Similar results of identical trends were obtained each time. The data reported here are from a single experiment.

The level of lipid peroxidation products in samples was expressed as 2-thiobarbituric acid reactive materials [aldehydes, mainly malondialdehyde (MDA) and endoperoxides (Buege and Aust, 1978)]. 2-Thiobarbituric acid-reactive materials in samples were assayed according to the modified method of Heath and Packer (1968).

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 50 mg roots with 3 ml of phosphate buffer (50 mM, pH 6.8). The homogenate was centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of the yellow colour of the supernatant at 410 nm was measured. H_2O_2 level was calculated using the extinction coefficient 0.28 $\mu mol^{-1} cm^{-1}$.

For extraction of antioxidative enzymes, roots were homogenized with 0.1 M 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 enzyme activity. The whole extraction procedure was carried out at 4°C. CAT, POD, SOD, APOD and GR were assayed as described previously (Chang and Kao, 1998).

Cell walls were prepared by homogenizing roots in ice cold phosphate buffer (50 mM, pH 5.8) using a pestle and mortar. The homogenate was centrifuged at 1,000 *g*, and washed at least four times with 50 mM phosphate buffer (Lee and Lin, 1995). The pellet was collected and used as cell wall fraction. POD ionically bound to the cell walls was extracted with 1 M NaCl. Cell walls prepared as described above were incubated in 1 M NaCl for 2 h with shaking at 30°C and centrifuged at 1,000 *g*. The supernatant was used to assay POD activity. POD activities were measured using a modification of the procedure described by Curtis (1971). The assay medium contained 50 mM phosphate buffer (pH 5.8), 7.2 mM guaiacol, 11.8 mM H_2O_2 and 0.1 ml enzyme extract in a final assay volume of 3.0 ml. The reaction was initiated by the addition of H_2O_2 and the change in absorbance at 470 nm was measured. Activity was calculated using the extinction coefficient (26.6 $mM^{-1} cm^{-1}$ at 470 nm) for tetraguaiacol per min.

Results

Figure 1 shows the effect of $CuSO_4$ on the growth of rice seedlings. Increasing concentrations of $CuSO_4$ from 20 to 50 μM progressively decreased root length. However, no reduction of shoot length by $CuSO_4$ was observed. The differential effect of Cu on root and shoot growth could be accounted for by the fact that Cu is accumulated mainly in roots and to a minor extent in shoots (Fernandes and Henriques, 1991). Figure 2 shows time courses of the effect of $CuSO_4$ (30 μM) on root length and root FW. As judged by root length and root FW, the reduction of root growth was evident 2 days after treatment.

Figure 3 demonstrates that $CuSO_4$ treatment resulted in a significant increase in MDA level, an indicator of lipid peroxidation. Also shown in Figure 3 is the increase in POD, SOD, APOD and GR activities in roots of rice seedlings treated with $CuSO_4$. However, $CuSO_4$ had no effect on the activity of CAT in roots of rice seedlings. Similar results were obtained when enzyme activities were expressed on the basis of dry weight (data not shown).

Figure 4 shows time courses of endogenous H_2O_2 level and cell wall POD activity in roots of rice seedlings in the presence and absence of $CuSO_4$ (30 μM). It is clear that $CuSO_4$-treated roots had higher H_2O_2 level and cell wall POD activity than water controls. Exogenous application of H_2O_2 (10 mM) resulted in a reduction of root growth and an increase in endogenous H_2O_2 level in roots of rice seedlings.

Discussion

Although Cu can interfere with a number of physiological processes, the primary site of Cu toxicity is probably at the cell membrane level (De Vos et al., 1989). This is also evident from our study, in which Cu treatment resulted in an increase in lipid peroxidation in rice roots. The metal-induced lipid peroxidation is mostly attributed to increased production of free radicals (Halliwell and Gutteridge, 1984; Aust et al., 1985). Our results indicate that excess Cu increased oxidative stress, as is evident from increased lipid peroxidation (Figure 3). De Vos et al. (1989), Rama Devi and Prasad (1998), and Mazhoudi et al. (1997) reported a similar increase in lipid peroxidation when plants were treated with Cu.

The protective mechanisms adapted by plants to scavenge free radicals and peroxides include several antioxidative enzymes such as SOD, APOD, GR, CAT and POD. The antioxidative enzymes are important components in preventing the oxidative stress in plants as is based on the fact that the activity of one or more of these enzymes is generally increased in plants when exposed to stressful conditions (Allen, 1995). Overexpression of genes encoding these enzymes in several transgenic plant species conferring protection against free radicals has also been demonstrated (Allen, 1995). In the present study, Cu treatment resulted in an increase in the activities of SOD, APOD, GR and POD (Figure 3), which can be considered as an indirect evidence for enhanced production of free radicals under Cu stress. The increase of SOD, APOD and POD has been reported with Cu (Chongpraditnum et al., 1992; Karataglis et al., 1991; Mazhoudi et al., 1997; Rama Devi and Prasad, 1998). However, Mazhoudi et al. (1997) reported that CAT and

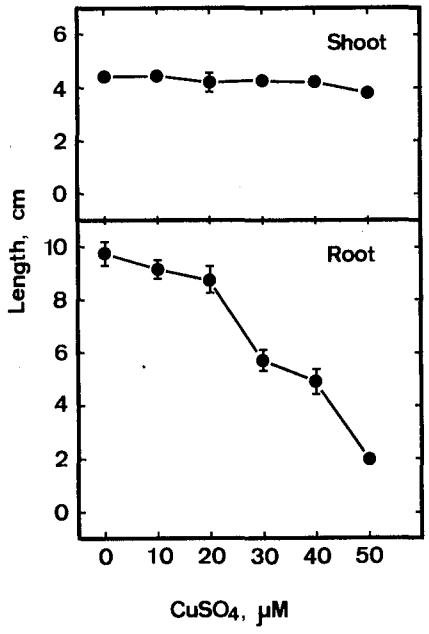

Figure 1. Effect of $CuSO_4$ on the growth of rice seedlings. Seedling growth was measured after 5 days of treatment. Vertical bars represent standard errors.

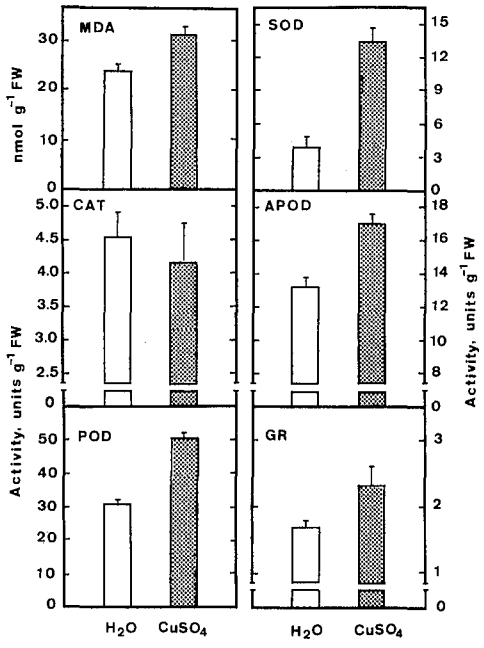

Figure 3. Effect of $CuSO_4$ (30 μM) on the level of MDA, and the activities of CAT, POD, SOD, APOD and GR in roots of rice seedlings. MDA level and antioxidative enzyme activities were measured after 5 days of treatment. Vertical bars represent standard errors.

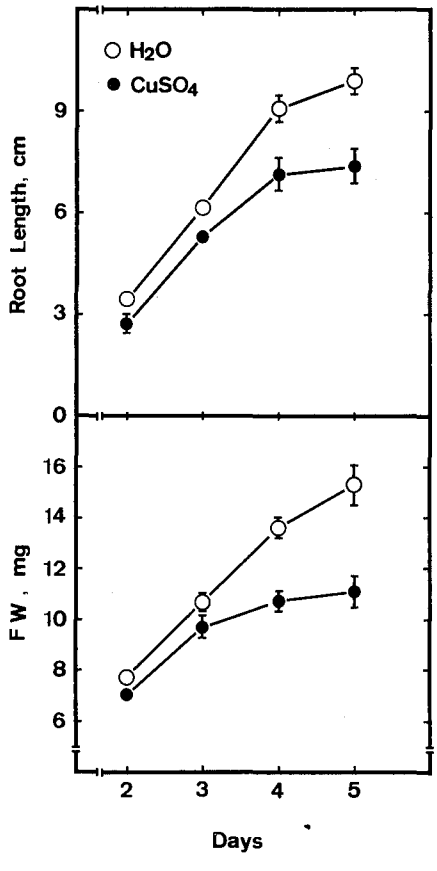

Figure 2. Time course of $CuSO_4$ effect on root length and root FW of rice seedlings. Rice seedlings were treated with distilled water or 30 μM $CuSO_4$. Vertical bars represent standard errors.

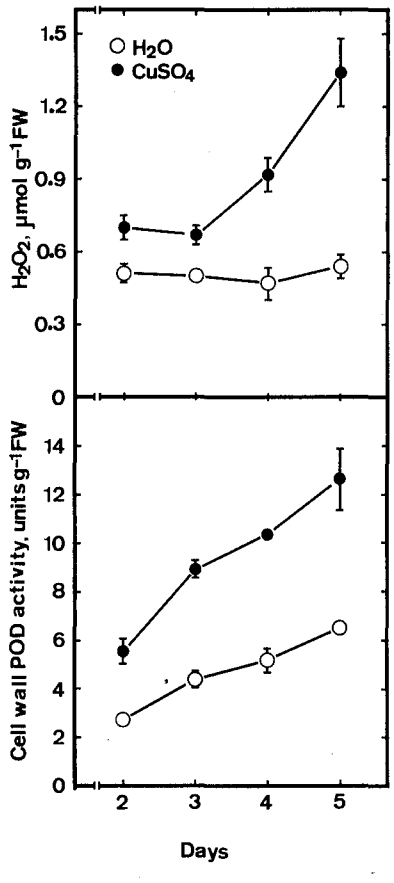

Figure 4. Changes in H_2O_2 level and cell wall POD activity in roots of rice seedlings in the presence and absence of $CuSO_4$ (30 μM). Vertical bars represent standard errors.

APOD activities were not affected by Cu. We also found no change in CAT activity (Figure 3). Such a variation in response of these enzymes to Cu stress could be due to the variability of plant species in producing free radicals (Mazhoudi et al., 1997). APOD and POD are known to play an important role in reducing oxidative stress by catalyzing the reduction of H_2O_2 (Weckx and Clijsters, 1996). Thus, the increase in the activities of APOD and POD by Cu (Figure 3) suggests increased production of H_2O_2. In the present study, $CuSO_4$ treatment indeed increases the production of H_2O_2 in roots (Figure 4).

It has been postulated that the action of POD located in the cell wall would be to confer rigidity to the cell wall and prevent later expansion involved in growth (Fry, 1986). This cell wall stiffening process appears to involve oxidative coupling, dependent on H_2O_2 (Fry, 1986). $CuSO_4$ treatment increases cell wall POD activity and H_2O_2 level in roots of rice seedlings (Figure 4). It has been shown that H_2O_2 caused a rapid cross-linking of cell wall polymers (Schopfer, 1996). If H_2O_2 plays an important role in the cell wall stiffening process, it is expected that H_2O_2 would inhibit root growth. This is indeed the case. Exogenous application of H_2O_2 inhibited root growth of rice seedlings (Figure 5). Thus, $CuSO_4$-induced inhibition in root growth of rice seedlings is likely due to cell wall stiffening related to H_2O_2 dependent POD-catalyzed formation of cross-linking among cell wall polymers.

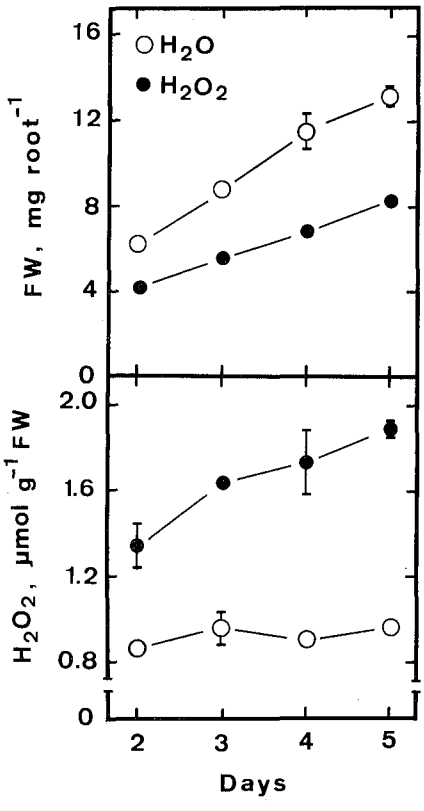

Figure 5. Changes in root growth and endogenous H_2O_2 level in roots of rice seedlings grown in the presence or absence of exogenous H_2O_2 (10 mM). Vertical bars represent standard errors.

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水稻幼苗銅之毒害：抗氧化酵素活性、過氧化氫含量與細胞壁過氧化酵素活性之變化

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本研究主要探討銅處理水稻幼苗對根內脂質過氧化作用、抗氧化酵素活性、過氧化氫含量與細胞壁內過氧化酵素活性變化之影響，同時探討這些變化與銅抑制根生長間之關係。硫酸銅處理可增加水稻幼苗根脂質過氧化作用與改變抗氧化酵素之活性。硫酸銅可增加水稻幼苗根 superoxide dismutase，ascorbate peroxidase，glutathione reductase 與 peroxidase 活性，但不影響 catalase 活性。硫酸銅處理同時可增加根之過氧化氫含量與根內細胞壁過氧化酵素活性。外加過氧化氫處理可顯著的抑制水稻幼苗根生長。銅所抑制之水稻幼苗根生長很可能與經由過氧化氫及過氧化酵素所催化之細胞壁變硬有關。

關鍵詞：銅；脂質過氧化作用；水稻；氧化逆境；根生長。