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Cadmium-Induced Changes in Antioxidant Enzyme Activities in Rice (*Oryza sativa* L. cv. Dongjin)

Mohammad Babar Ali, Hyun Sik Chun, Byung Ki Kim, and Chin Bum Lee*
Department of Biology, Dong-eui University, Busan 614-714, Korea

We studied how the relationship between cadmium (Cd) toxicity and oxidative stress influenced the growth, photosynthetic efficiency, lipid peroxidation, and activity of ntioxidative enzymes in the roots and leaves of rice (*Oryza sativa* L. Dongjin). Plants were exposed to Cd for 21 d. Both seedling growth and photosynthetic efficiency decreased gradually with increasing cadmium concentrations. Lipid peroxidation increased slowly in both roots and leaves, causing oxidative stress. However, each tissue type responded differently to Cd concentrations with regard to the induction/inhibition of antioxidative enzymes. The activity of superoxide dismutase (SOD) increased in both roots and leaves. Ascorbate peroxidase (APX) activity increased in leaves treated with up to 0.25 μM Cd, then decreased gradually at higher concentrations. In contrast, APX activity in roots increased and remained constant between 0.25 and 25 μM Cd. Enhanced peroxidase (POD) activity was recorded for treatments with up to 25 μM Cd, gradually decreasing at higher concentrations in the leaves but remaining unchanged in the roots. Catalase (CAT) activity increased in the roots, but decreased in the leaves, whereas the activity of glutathione reductase (GR) was enhanced in both roots and leaves, where it remained elevated at higher Cd concentrations. These results suggest that rice seedlings tend to cope with free radicals generated by Cd through coordinated, enhanced activities of the antioxidative enzymes involved in detoxification.

Keywords: antioxidant enzyme, cadmium, photosynthesis, rice

Organisms commonly generate free radicals when under environmental stress. Plants exposed to heavy metals will produce reactive oxygen species (ROS) in response to disturbances in both the chloroplasts and the mitochondria (Asada, 1994; Gille and Singler, 1995). These ROS -- hydroxyl radicals ($OH^{\cdot}$), superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), and the less reactive hydrogen peroxide (H_2O_2) – are very harmful to plant metabolism, and can affect future biological and physiological activities. A relationship exists between the generation of free radicals and their detoxification (Foyer et al., 1994; Luna et al., 1994). High concentrations of these free radicals can damage vital components of the membrane lipids, proteins, and other organelles (Somashekharaiah, 1992; Gille and Singler, 1995). This damage by heavy metals results in the formation of malondialdehyde (MDA), whose measured content is often used as a metabolite indicator of oxidative stress.

Heavy metals cause the peroxidation of membranes by generating free radicals. By catalyzing the Haberweise and Fenton-type reaction (Kappus, 1985), they are able to increase their rate of peroxidation. To defend against these toxic ROS, plants change or modify their enzymatic and non-enzymatic antioxidants, regulating them according to environmental factors (Foyer and Halliwell, 1976). An enzymatic antioxidant, such as superoxide dismutase (SOD), can convert superoxide ions to less reactive H_2O_2, later re-converting it to H_2O and O_2 by ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD). CAT is a haeme-containing enzyme that catalyzes the dismutation of H_2O_2 into H_2O and O_2. It is found in all aerobic cells, primarily in the peroxisomes. In contrast, POD uses guaiacol as a substrate to decompose H_2O_2 into H_2O and O_2. Glutathione reductase (GR) reduces oxidized glutathione (GSSG) to reduced glutathione by using the NADPH that is partially involved in the detoxification of H_2O_2 (Foyer et al., 1994).

Cadmium has a strong affinity to the sulfhydryl group that might induce and detoxify metal ions from the cell through phytochelatin synthesis (Sanita di Toppi and Gabbrielli, 1999). This very toxic metal reduces plant growth and affects photosynthesis (Atal et al., 1991; Maksymiec and Baszynski, 1996), perhaps by interfering at the oxidizing site of PS II (Dubey, 1997). Photosynthetic activity is affected at the manganese cluster of the water-splitting complex because of the enhanced production of free radicals under Cd stress (Wydrzynski et al., 1989).

*Corresponding author; fax +82-51-890-1529
e-mail cblee@dongeui.ac.kr

The objective in this study was to identify any cadmium-induced changes in antioxidative enzymes (i.e., SOD, APX, CAT, POD) as well as its effects on lipid peroxidation, shoot and root growth, and photosynthetic efficiency in rice. Plants generally need to maintain a balance between generating free radical oxygen species and their scavenging mechanisms to enhance growth and metabolism. Increased understanding of reactive oxygen free radicals would help to define their involvement in the defense mechanism of rice plants under Cd stress.

MATERIALS AND METHODS

Plant Material and Treatment Procedure

Seeds of rice (*Oryza sativa* L. Dongjin) were surface-sterilized for 30 min with a 1% Na hypochlorite solution, then washed with tap water several times to remove the reagent from the seed surface. They were then germinated for 3 d in the dark at room temperature on two sheets of filter paper moistened with distilled water. Afterward, the seeds were transferred to a 10% Hoagland solution containing different concentrations of cadmium (0.00, 0.10, 0.25, 0.50, 1.00, 25.00, 50.00, or 100.00 μM) for 8 h in the dark. About 50 seeds were later transferred to pots containing vermiculite. They were held for 21 d in a controlled environment growth chamber at 25°C, with 14/10 h (L/D) periods (200 μmol m^{-2} s^{-1}), and 70 to 80% humidity. The seedlings were supplemented daily with the specified amount of Cd. Leaves were collected after 21 d, with the roots being collected the following day. All tissue samples were then stored at −80°C.

Measurement of Chlorophyll Fluorescence

We used a Plant Efficiency Analyzer (PEA; Hansatech, UK) and a PAM Chlorophyll a Fluorometer (Walz; Effeltrich, Germany) to routinely monitor the emissions of chlorophyll a fluorescence from the upper surfaces of the leaves. The initial level (F_o) was elicited by a weak red light (655 nm, 1 μmol m^{-2} s^{-1} modulated at 1.6 KHz), and was measured at a wavelength >700 nm with a photodiode. Maximal fluorescence (F_m) was induced by a one-second pulse of white light (4000 μmol m^{-2} s^{-1}). The maximum variable fluorescence (F_v) was calculated as the difference between F_m and F_o at a specific time. All experiments were independently repeated at least three times.

Lipid Peroxidation

We used the method of Heath and Packer (1968) to determine the level of lipid peroxidation in both tissue types. About 0.5 g each of the leaf and root samples were homogenized in 0.1% trichloroacetic acid with a mortar and pestle. We then combined 1 mL of plant extract and 4 mL of 20% trichloroacetic acid containing 0.5% thiobarbituric acid. After being heated at 95°C for 30 min, the mixture was quickly cooled on ice and centrifuged at 1000*g* for 10 min. Absorbance of the supernatant was read at 532 nm, and was corrected for nonspecific turbidity by subtracting the absorbance measured at 600 nm. The blank comprised 1 mL of 0.1% trichloroacetic acid and 4 mL of 20% trichloroacetic acid containing 0.5% thiobarbituric acid. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Preparation of Enzyme Extract

To determine the activities of APX and GR, we first homogenized 0.4 g each of the leaf and root samples under liquid nitrogen in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. For APX, an additional 5 mM ascorbate was supplemented. SOD extracts were achieved by homogenizing 0.4 g each of the leaf and root samples under liquid nitrogen in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1% PVP, and 0.5% Triton X-100. The homogenates were filtered through four layers of cheesecloth and centrifuged at 35,000*g* for 15 min at 4°C. Supernatants were then re-centrifuged at 35,000*g* for 15 min at 4°C. For the determinations of CAT and POD contents, leaf and root samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0). This homogenate was filtered through four layers of cheesecloth and centrifuged at 28,000*g* for 15 min at 4°C. The supernatant was re-centrifuged at 28,000*g* for 15 min at 4°C. Protein content was measured according to the method of Lowry et al. (1951), using BSA as a standard.

Enzyme Assay

SOD (EC 1.15.1.1) activity was determined according to the method of Beyer and Fridovich (1987). The reaction mixture (30.25 mL) contained 100 mM potassium phosphate buffer (pH 7.8), 9.9×10^{-3} M methionine, 5.7×10^{-5} M nitro blue tetrazolium (NBT), 2.5×10^{-2}% Triton X-100 (w/v), and the required amount of

plant enzyme extract. This reaction was initiated by light illumination. One unit of SOD was defined as the amount of enzyme that caused a 50% decrease in SOD-inhibitable NBT reduction. One unit = V/v - 1, where V and v were the slopes of the changes in absorbance in the absence and presence of the extract, respectively. APX (EC 1.11.1.11) activity was assayed by monitoring the decrease in absorbance at 290 nm (extinction coefficient: 2.8 mM^{-1} cm^{-1}). The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 0.5mM ascorbate, 0.2mM H_2O_2, and the required amount of enzyme extract (Chen and Asada, 1989). GR (EC 1.6.4.2) activity was monitored by the oxidation of NADPH at 412 nm, using 5,5′ dithio-bis (2-nitrobenzoic acid) as described by Barata et al. (2000). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.5), 1 mM 5,5′ dithiobis (2-nitrobenzoic acid), 1 mM oxidized glutathione, and 0.1 mM NADPH. This reaction was initiated by the addition of the enzyme extract; the increase in absorbance was recorded for 2 min. CAT (EC 1.11.1.6) activity was monitored, according to the method of Aebi (1984), with the reaction mixture comprising 50 mM potassium phosphate buffer (pH 7.0), 30 mM H_2O_2, and the enzyme extract. The decomposition of H_2O_2 was measured as the decrease in absorbance at 240 nm. Activity was calculated using an extinction coefficient of 39.4 mM^{-1} cm^{-1}. Finally, POD (EC 1.11.1.7) activity was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, 0.3 mM guaiacol, and the enzyme extract. The reaction was started by the addition of 0.1 mM H_2O_2; an increase in absorbance at 470 nm was recorded due to the formation of tetraguaiacol. Activity was determined with an extinction coefficient of 6.39 mM^{-1} cm^{-1}, following the method of Pütter (1974).

RESULTS

Seedling Growth, Photosynthetic Efficiency, and Lipid Peroxidation

Lower levels of cadmium (0.1 to 0.25 μM) had little effect on seedling growth. However, the effect was severe at higher concentrations (Fig. 1). In the treated plants, growth decreased with increasing media concentrations compared with the control plants. Shoot and root growth rates were reduced by about 45% and 65%, respectively, at 100 μM Cd. The greater reduction in root development may have been caused by their direct contact with the cadmium solution. Performance for both tissue types also depended upon the length of the treatment period and the external Cd concentration.

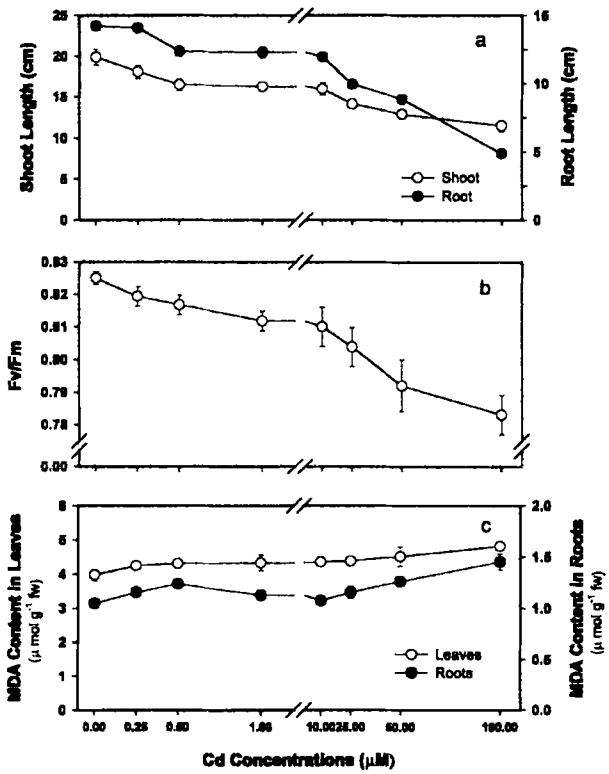

Figure 1. Effects of cadmium concentration on rice shoot and root lengths, F_v/F_m (leaves only), and malondialdehyde (MDA) contents. Plants were grown for 21 d in an environmental growth chamber at 25°C, 14/10 h (L/D) period, and 70 to 80% humidity. The level of lipid peroxidation was determined according to the methods of Heath and Packer (1968). All experiments were independently repeated at least three times.

Photosynthetic efficiency also decreased in proportion to the cadmium concentration in the treatment medium. This phenomenon was observed as a yellowing of the leaves, indicating the level of toxicity (Fig. 1).

Lipid Peroxidation

We used thiobarbituric acid to determine the extent of malondialdehyde formation in both roots and leaves. Lipid peroxidation gradually increased in both tissue types as the concentration of Cd increased (Fig. 1) This response was considered to be a general indicator of oxidative stress.

Antioxidative Enzymes

Both leaves and roots showed marked increases in

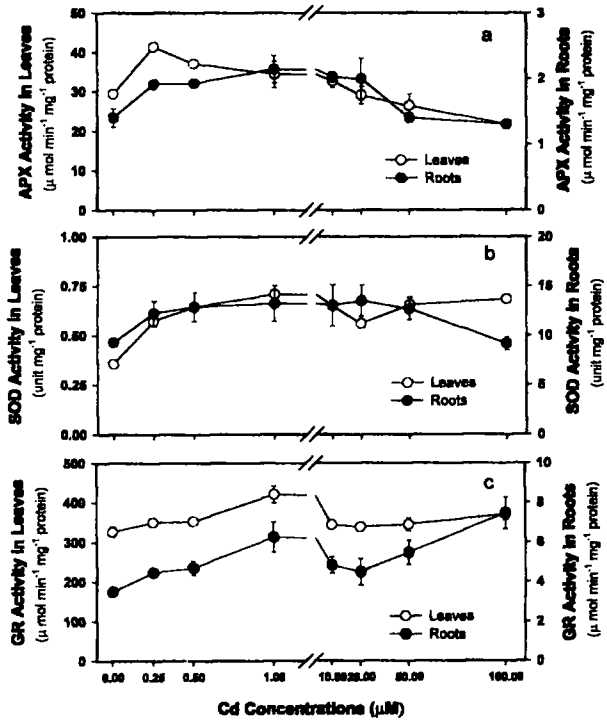

Figure 2. Effects of cadmium concentration on antioxidant enzymes -- superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) -- in rice leaves and roots. SOD activity was determined according to the method of Bayer and Fridovich (1987). APX activity was assayed by monitoring the decrease in absorbance at 290 nm (Chen and Asada, 1989). GR activity was monitored by following the oxidation of NADPH at 412 nm, using 5,5′ dithio-bis (2-nitrobenzoic acid) according to Barata et al. (2000).

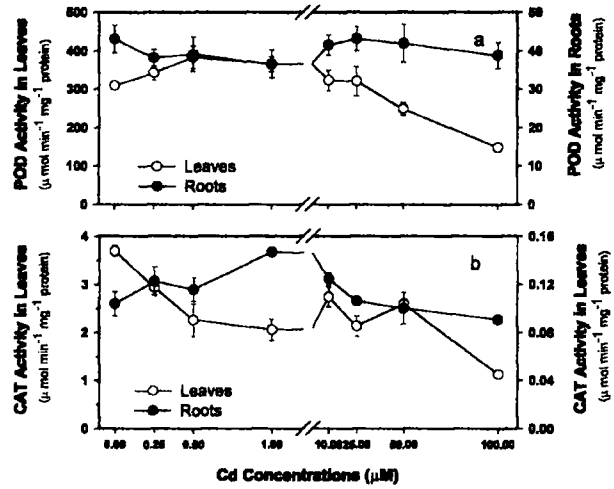

Figure 3. Effects of cadmium concentration on antioxidant enzymes -- guaiacol peroxidase (POD) and catalase (CAT) -- in rice leaves and roots. CAT (EC 1.11.1.6) activity was monitored according to the method of Aebi (1984); POD (EC 1.11.1.7) activity was determined using a coefficient of extinction of 6.39 mM^{-1} cm^{-1} following the method of Pütter (1974).

their APX activities following Cd exposure (Fig. 2). In the former, activity rose by up to 29% at 0.25 μM, then remained high up to 10 μM, compared with the control. APX activity in the leaves decreased gradually with higher Cd concentrations. In contrast, activity in the roots increased smoothly up to 25 μM Cd, then decreased at higher concentrations compared with the control. Similarly, SOD activity increased in both leaves and roots (Fig. 2). In the former, activity rose about 29% at 1 μM, and remained generally steady for all concentrations except 25 μM, where it decreased slightly compared with the control. In the roots, SOD activity increased gradually up to 50 μM Cd, but at higher concentrations it decreased slightly compared with the control. The activity of GR was strongly induced by Cd in both leaves and roots. In the former, it increased gradually, reaching a maximum at 1 μM and then decreasing. At other concentrations, activity remained higher than that measured in the control. A marked increase in GR activity was also observed in roots exposed to 1 μM Cd, decreasing gradually up to 25 μM. The maximum activity (53%) was found at 100 μM Cd (Fig. 2).

We also examined the H_2O_2 scavenger enzymes (CAT and POD) in rice leaves and roots (Fig. 3). In Cd-stressed plants, each enzyme behaved differently. In the leaves, POD activity markedly increased at 0.5 μM, then slowly declined, although it remained above the level measured in the control plants. In contrast, the roots showed an initial decrease in POD activity at low Cd concentrations, but at higher amounts, values were the same as those found in the control. Compared with the performance of POD, CAT activity in the leaves decreased for Cd concentrations up to 1 μM, then increased slightly between 10 and 50 μM. However, activity was severely less at 100 μM. In the roots, CAT activity showed an increase up to 1 μM Cd, followed by a gradual decrease.

DISCUSSION

In our study, the growth rate of the leaves and roots gradually declined with increasing Cd concentrations in the medium, a result consistent with that of Viloria et al. (2001). Aidid and Okamoto (1992, 1993) reported

that cadmium caused irreversible inhibition of the proton pump, thus reducing the rate of elongation growth by the cells, especially in the stems.

At higher Cd concentrations, the observed decrease in photosynthetic efficiency (F_v/F_m) could have been due to disturbance in the flow of electrons as well as changes in the amount of fluorescence (see also Atal et al., 1991). Such a reduction in efficiency may be caused by the impact of Cd on PS II (Malik et al., 1992). Other possible factors include action at the level of the manganese protein cluster of the water-splitting systems (van Duijvendijk-Matteoli and Desmet, 1975); or possible inhibition of the synthesis of δ-amino laevulinic acid and the formation of a photoreactive protocholophyllide reductase complex with its substrate (Gadallah, 1995).

Reduced photosynthesis is associated with degradation of the membrane lipid, as measured by the formation of malondialdehyde. Often used as an indicator of oxidative stress, lipid peroxidation in our research, increased gradually in both leaves and roots following Cd exposure. This result agrees with those from studies in which the MDA content in various plants increased under Cd stress (Ros et al. 1990, 1992; Hendry et al., 1992; Somashekharaiah et al., 1992; Chaoui et al., 1997). Lipid peroxidation depends upon the metal content, the duration of the treatment, and the concentration of thiolic groups already present or reduced by Cd treatment (Sanita di Toppi and Gabbrielli, 1999).

Cadmium can enhance the activities of such antioxidant enzymes as SOD, APX, GR, POD, and CAT in plants (Hegedus et al., 2001; Schützendübel et al., 2001; Viloria et al., 2001). Likewise, the oxidative stresses from chilling, salt, or senescence may lead to the production of free radicals (Lee and Lee, 2000; Lee et al., 2001). However, those free radicals generated under Cd stress may be eliminated by SOD in both leaves and roots, thereby resulting in the formation of H_2O_2. We found that the SOD isoenzymes seemed to be independently regulated according to the degree of oxidative stress experienced in the various cellular components exposed to Cd (data not shown). Bowler et al. (1992) have suggested that such functions may lead to lipid peroxidation that diffuses from the oxidative damage site to the nucleus, where they induce transcription of specific SOD genes. Increases in SOD activity following Cd application have also been reported in *Pisum sativum* (Dalhurzo et al., 1997), *Alyssum* sp. (Schickler and Caspi, 1999), and *Pisum* sp. (Dixit et al., 2001).

Hydrogen peroxide generated under Cd stress in the membrane is rapidly diffused in the cytosol. There it acts as both an oxidant and a reductant toxin (Gille and Singler, 1995). Our results indicated that increases in APX in both leaves and roots appeared to be due to changes in the APX isoforms under Cd stress (Fig. 2). APX plays an important role in the detoxification of H_2O_2 from the cells, and may provide tolerance for the plant against free radicals. Elevated APX activity resulting from Cd stress has also been reported by Shaw (1995) and Chaoui et al. (1997).

GR levels increased in both roots and leaves following Cd exposure (Fig. 2). Our results indicate that this induced activity provides sufficient protection from free radicals in cadmium-stressed rice plants. The enhanced response by GR was greater than for the other enzymes examined here. Significant increases in GR activity in both tissue types resulted from the synthesis of reduced glutathione (GSH), which can stabilize the membrane structure by removing the acylperoxides formed by lipid peroxidation (Price et al., 1990). Increased GR activity in plants under Cd stress has also been reported by Chaoui et al. (1997), Stroinski et al. (1999), Schützendubel et al. (2001), and Viloria et al. (2001), and may due to synthesis of the GR protein at the transcript level (Xiang and Oliver, 1998).

These increases in GR activity help to partially eliminate the toxic levels of H_2O_2 in the cell while converting oxidized glutathione (GSSH) into reduced glutathione (GSH). The latter may then be used as a precursor for phytochelatin synthesis. Phytochelatins act as heavy metal-binding peptides in plants, and play an important role in detoxifying free metal ions (Kneer and Zenk, 1992). Cadmium is a potent inducer of phytochelatin (Somashekharaiah et al., 1992; Sanita di Toppi and Gabbrielli, 1999). High thiol content may enable the metabolites to function in the detoxification of free radicals and ROS. Free radical species are reductively detoxified by concomitant oxidation of sulfhydryl groups to disulfides or sulfenic acid (Elstner, 1990). The antioxidant potential of the plant cell could be increased by the induction of phytochelatin synthesis. In the present study we also noted a positive relationship between APX and GR in both roots and leaves. This indicates that the glutathione/ascorbate cycle commonly plays an important role in detoxifying H_2O_2, a result that is supported in research by Smith et al. (1989), Schraudner et al. (1997), and Baccouch et al. (1998).

We found a significant decrease in CAT activity in our rice leaves, while the CAT content showed a small increase in the roots (Fig. 3). This reduced activity in the leaves might have been caused by either H_2O_2 inactivation or light absorption by the haeme group. Other researchers have found that stressors such as

salinity, heat shock, or chilling reduce the rate of protein synthesis and cause a depletion of catalase activity (Hurtwig et al., 1992; Lee et al., 2001). Both inhibition and induction of CAT activity has been reported in various plant species under Cd stress. For example, activity decreased in *Phaseolus vulgaris* (Somashekharaiah et al., 1992), *Phaseolus aureus* (Shaw, 1995), and *P. sativum* (Dalhurzo et al., 1997) when the growth media were supplemented with Cd. Although no change in activity was found in the stems of *P. vulgaris*, the roots and leaves did show enhanced activity (Chaoui et al., 1997). In a separate study, however, CAT activity did not vary considerably in the tissues of *Helianthus annuus* (Gallego et al., 1996).

In contrast to the activity of CAT, POD content in our rice leaves increased in treatments with up to 1 μM Cd, but decreased at higher concentrations (Fig. 3). However, no significant changes were observed in the roots. Increases in POD content under cadmium treatment have also been reported by Shaw (1995), Hegedus et al. (2001), and Schützendübel et al. (2001). This enhanced activity by POD might be responsible for the elimination of H_2O_2 from the cytosol, an action that contrasts with that of APX in eliminating H_2O_2 from the chloroplasts instead (Inze and van Montagu, 1995). Our study results are supported by those from other research on both the induction (Wecks and Clijsters, 1996; Baccouch et al., 1998) and the inhibition of POD and CAT activities (Somashekharaiah et al., 1992; Shaw, 1995; Gallego et al., 1996). We suggest that the contradictory responses by POD and CAT to Cd stress indicate that different mechanisms are involved in their operations against oxidative stress.

In summary, treatment of rice roots and leaves with cadmium induced increases in the activities of SOD, APX, and GR. In particular, the enhanced SOD content was implicated in the generation of H_2O_2. While POD activity increased in the leaves at higher Cd concentrations, roots showed negligible changes in its activity. Because these contrasting activities corresponded with the induction and inhibition of CAT in the roots and leaves, respectively, this again may indicate that the response to Cd is promoted by different mechanisms in each organ. Nevertheless, the magnitude of the response by these enzymes varied according to the concentration of Cd; at higher levels, enzymatic activities decreased due to either the high amount of metal ions or the presence of toxic ROS.

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