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Response of Antioxidant Enzymes in Rice (*Oryza sativa* L. cv. Dongjin) under Mercury Stress

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We studied the effects of different concentrations of mercury (0.0 to 100 μM) on growth and photosynthetic efficiency in rice plants treated for 21 d. In addition, we investigated how this metal affected the malondialdehyde (MDA) content as well as the activity of five antioxidant enzymes – superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (POD), and catalase (CAT). Photosynthetic efficiency (F_v/F_m) and seedling growth decreased as the concentration of Hg was increased in the growth media. Plants also responded to Hg-induced oxidative stress by changing the levels of their antioxidative enzymes. Enhanced lipid peroxidation was observed in both leaves and roots that had been exposed to oxidative stress, with leaves showing higher enzymatic activity. Both SOD and APX activities increased in treatments with up to 50 μM Hg, then decreased at higher concentrations. In the leaves, both CAT and POD activities increased gradually, with CAT levels decreasing at higher concentrations. In the roots, however, CAT activity remained unchanged while that of POD increased a bit more than did the control for concentrations of up to 10 μM Hg. At higher Hg levels, both CAT and POD activities decreased. GR activity increased in leaves exposed to no more than 0.25 μM Hg, then decreased gradually. In contrast, its activity was greatly inhibited in the roots. Based on these results, we suggest that when rice plants are exposed to different concentrations of mercury, their antioxidative enzymes become involved in defense mechanisms against the free radicals that are induced by this stress.

Keywords: antioxidant enzyme, mercury, photosynthesis, rice

Mercury (Hg) is a toxic metal. When released from its natural and anthropogenic activities, it enters the atmosphere primarily in vapor or elemental form, as inorganic, mono- or divalent salts; or as an organo-mercurial such as methyl mercury. Plants have the ability to accumulate Hg under higher concentrations, which reduces their photosynthetic pigments and adversely affects seedling growth, development, and metabolism (Ali et al., 2000; Cho and Park, 2000). Mercury produces reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\cdot}$), superoxide anions ($O_2^{\cdot-}$), and singlet oxygen ($^1O_2$). This is done via generation of hydrogen peroxide (H_2O_2), depletion of glutathione, and reactivity with the membrane-bound protein thiols, all of which may lead to lipid peroxidation (Luckey et al., 1975; Ribarov and Benov, 1981; Lund et al., 1991). These ROS react with the membrane lipids, resulting in the formation of malondialdehyde (MDA). The Hg^{2+} ion is able to attack the sulfhydryl group of the membrane (Passow et al., 1961; Stohs and Bagchi, 1995), which leads to membrane destabilization and enhanced peroxidation. Kappus (1985) and Mazhoudi et al. (1997) have reported that the occurrence of heavy metal-induced lipid peroxidation varies by species. This enhanced peroxidation is associated with reduced photosynthetic activity (Baszynski et al., 1988) because it affects the photosynthetic enzymes present in the membranes.

Plants detoxify reactive free radicals by modifying enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPX), and catalase (CAT). SOD, a metalloenzyme that converts superoxide ions to the less reactive H_2O_2, is found in three forms: 1) Cu/Zn-SOD in choloroplasts, 2) Fe-SOD in choloroplasts and cytoplasms, and 3) Mn-SOD in mitochondria. APX (whether cytosolic-, chloroplastic-, or thylakoid membrane-bound) uses ascorbate as an electron donor to convert H_2O_2 to H_2O. GR is responsible for the production of reduced glutathione (GSH) via oxidized glutathione (GSSG) and NADPH. Peroxidases, using guaiacol as a substrate, cause IAA degradation and detoxify H_2O_2 to H_2O. In contrast, though CAT does not require any substrate, it also plays an important role in the detoxification of H_2O_2 from plant cells, and may provide resistance against oxidative stress.

The aim of this study was to examine the activities of various enzymes in order to identify any biochemical and physiological changes in the leaves and roots

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of rice plants grown under mercury stress. We wished to monitor the effect of mercury on lipid peroxidation as well as plant growth and photosynthetic efficiency.

MATERIALS AND METHODS

Plant Material and Treatment Procedure

Seeds of rice (*Oryza sativa* L. Dongjin) were surface-sterilized with a 1% Na hypochlorite solution for 30 min, 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. After 3 d, the seeds were transferred to a 10% Hoagland solution containing different concentrations of mercury (0.00, 0.10, 0.25, 0.50, 1.00, 25.00, 50.00, or 100.00 μM). After 8 h in the dark, approximately 50 seeds were transferred to pots containing vermiculite. They were then held for three weeks in a controlled environmental growth chamber at 25°C, with 14/10 h (L/D) periods (200 mol m^{-2} s^{-1}) and 70 to 80% humidity. The seedlings were supplemented daily with the specified concentration of mercury. Leaves were gathered after 21 d of this Hg treatment; the roots were collected the following day. All plant samples were then stored at –80°C.

Measurement of Chlorophyll Fluorescence

The emission of chlorophyll a fluorescence from the upper surfaces of the leaves was routinely monitored under light by using a Plant Efficiency Analyzer (PEA; Hansatech, UK) and a PAM Chlorophyll Fluorometer (Walz; Effeltrich, Germany). The initial level (F_o) of fluorescence was elicited by a weak red light (655 nm, 1 μmol m^{-2} s^{-1} modulated at 1.6 KHz), and was measured with a photodiode at a wavelength >700 nm. The maximal fluorescence (F_m) was induced by a one-second pulse of white light (4,000 μmol m^{-2} s^{-1}). Maximum variable fluorescence (F_v) was calculated as the difference between F_m and F_o at a specific time. All the experiments were independently repeated at least three times

Lipid Peroxidation

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

Preparation of Enzyme Extract

To prepare for the APX and GR analyses, 0.4 g each of the leaves and roots was homogenized under liquid nitrogen in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA. For APX, 5 mM ascorbate was supplemented. To determine the amount of SOD activity, 0.4 g each of the leaf and root samples was homogenized under liquid nitrogen in 100 mM potassium phosphate buffer (pH 7.8) that contained 0.1 mM EDTA, 1% polyvinyl-pyrrolidone (PVP), and 0.5% Triton X-100. The homogenate was filtered through four layers of cheesecloth and centrifuged at 35,000*g* for 15 min at 4°C. The supernatant was then re-centrifuged at 35,000*g* for 15 min at 4°C. For the determination of CAT and POD activities, the leaf and root samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0). The homogenate was then filtered through four layers of cheesecloth and centrifuged at 28,000*g* for 15 min at 4°C. Afterward, 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 nitroblue tetrazolium (NBT), 2.5×10^{-2}% (w/v) Triton X-100, and the required amount of the plant enzyme extract. The reaction was initiated by 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 the absorbance in the absence and presence of the enzyme 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 50 mM potassium phosphate buffer (pH, 7.0), 0.5 mM ascorbate, 0.2 mM 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 prescribed by Barata et al. (2000). The reaction mixture consisted of 100mM potassium phosphate buffer (pH, 7.5), 1 mM 5,5′ dithio-bis (2-nitrobenzoic acid), 1 mM oxidized glutathione, and 0.2 mM NADPH. This reaction was initiated by adding the enzyme extract; the increase in absorption was recorded for 2 min. CAT (EC 1.11.1.6) activity was monitored according to the method of Aebi (1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH, 7.0), 30 mM H_2O_2, and the enzyme extract. Decomposition of H_2O_2 was measured as the decrease in absorbance at 240 nm. The activity was then 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, with an increase in absorbance recorded due to the formation of tetraguaiacol at 470 nm. Activity was determined using an extinction coefficient of 6.39 mM^{-1} cm^{-1}, following the method of Pütter (1974).

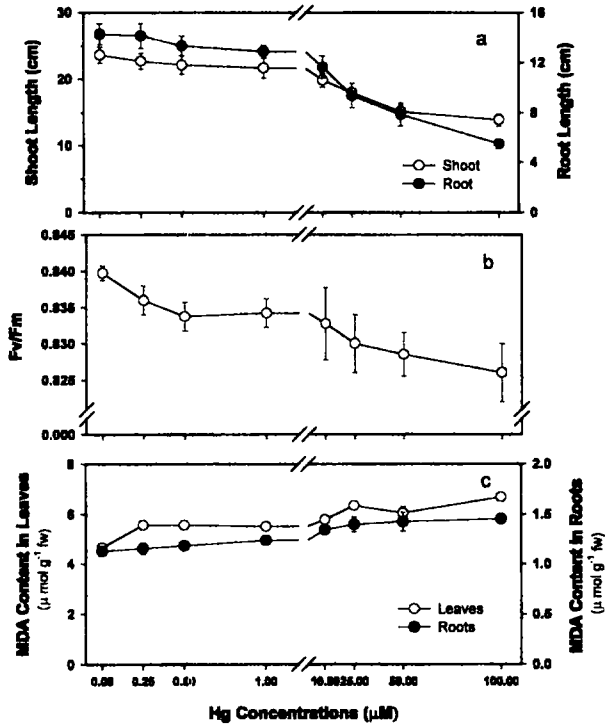

Figure 1. Effects of mercury concentration on seedling growth; F_v/F_m (shoots only); and malondialdehyde (MDA) content for plants grown for 21 d in an environmental growth chamber at 25°C, with 14/10 h (L/D) periods and 70 to 80% humidity. The level of lipid peroxidation was determined according to the methods of Heath and Packer (1968). All experiments were independently repeated at least three times.

RESULTS

Seedling Growth, Photosynthetic Efficiency, and Lipid Peroxidation

Under high concentrations of Hg (e.g., 100 μM), root and shoot growths were reduced up to 62% and 41%, respectively, compared with the control (Fig. 1). These decreases were affected by both the external concentration of mercury and the duration of the treatment. However, the effect of 100 μM Hg on photosynthetic efficiency (F_v/F_m) was negligible, about 1.5% less than that measured in the control (Fig. 1). The amount of MDA formation increased gradually at high Hg concentrations, rising by 25% and 23% in the leaves and roots, respectively, compared with the control.

Antioxidative Enzymes

Under mercury stress, APX activity was enhanced in both tissue types, with the maximum increases in value measured in the leaves (41%) at 25 μM Hg and in the roots (40%; 10 μM Hg), compared with the control (Fig. 2). In the leaves, the APX content increased slowly for treatments of up to 1 μM Hg, decreased at 10 μM Hg, rose again to its maximum at 25 μM, and then decreased gradually. Despite these fluctuations, however, the level of APX always remained higher (up to 50 μM Hg) than for the control. In contrast, activity in the roots increased for treatments of up to 10 μM Hg, and then started decreasing sharply compared with the control.

Maximum SOD activity (Fig. 2) was found at 50 μM in the leaves (45% increase over the control), whereas the value was higher in the roots (47% at 25 μM Hg). In fact, activity was always higher in the treated leaves and roots than in the control plants. Moreover, when defined by protein measurements, SOD activity was greater in the leaves than in the roots. Similarly, GR activity in the leaves initially increased at 0.25 μM Hg, then gradually decreased with higher concentrations (Fig. 2). In contrast, activity in the roots decreased gradually and consistently as the concentration of mercury

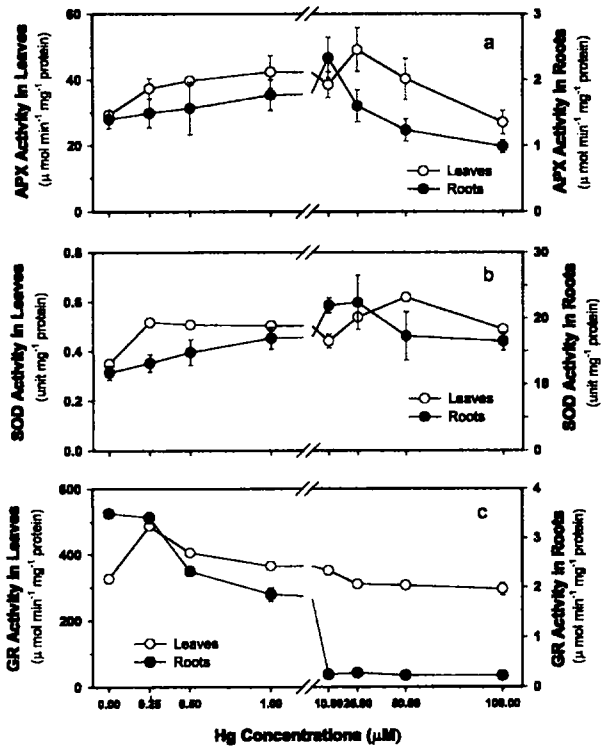

Figure 2. Effects of mercury concentration on antioxidant enzymes -- superoxide dismutase (SOD): ascorbate peroxidase (APX); and glutathione reductase (GR) -- in leaves and roots. SOD activity was determined according to the method of Bayer and Fridovich (1987); APX activity was assayed by monitoring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

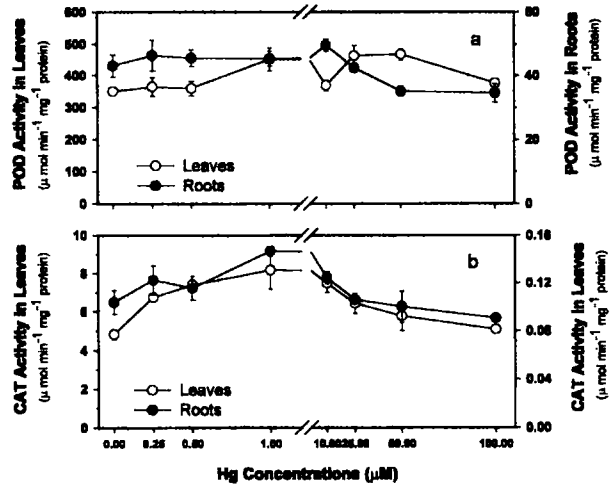

Figure 3. Effects of mercury concentration on antioxidant enzymes -- guaiacol peroxidase (POD) and catalase (CAT) -- in leaves and roots. CAT activity was monitored according to the method of Aebi (1984); POD activity was determined by using an extinction coefficient of 6.39 mM^{-1} cm^{-1}, according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

was raised.

No significant change was recorded in POD activity in the leaves when up to 0.50 μM Hg was supplemented; at higher concentrations, the level increased and remained higher than in the control (Fig. 3). However, a non-significant increase was observed in POD content in the roots for treatments of up to 10 μM Hg. Finally, CAT activity in the leaves increased initially up to 10 μM Hg, then gradually decreased but remained higher than for the control (Fig. 3). In contrast, CAT activity in the roots did not change for treatments of up to 1 μM Hg. At higher mercury levels, however, activity did decrease sharply.

DISCUSSION

Seedlings accumulated mercury and showed decreased growth, roots more than shoots, as the Hg concentration was increased This more severe effect on root development may have been due to their more direct contact with the metal ions. Cho and Park (2000) and Cuypers et al. (2001) have suggested that shoot growth may be reduced by mercury-induced breakdown of the photosynthetic pigments. In fact, we observed the yellowing of leaves at higher Hg concentrations. Burzynski (1987) has reported that other heavy metals, e.g., lead and cadmium, can induce a deficiency in nutrients by blocking their translocation. Because mercury has a strong tendency to react with sulfhydryl groups in plant membranes (Passow et al., 1961), this metal also may block the entry of nutrients such as Ca, Mg, and K, thereby reducing plant growth.

We found that photosynthetic efficiency was slightly decreased with higher Hg concentrations (Fig. 1). Krupa et al. (1993) and Maksymiec and Baszynski (1996) have reported similar results in tests using a variety of plant species and heavy metals. This particular reduction in photosynthetic efficiency by mercury may result when the photo-oxidizing site of PS II is inhibited, as has been demonstrated in barley chloroplasts (Chun et al., 1993).

Levels of MDA gradually increased in both leaves and roots at high Hg concentrations, a result that agrees with that of Ali et al. (2000) and Cho and Park (2000), who reported that Hg increased lipid peroxidation, thereby inhibiting photosynthesis and stunting seedling growth. In addition, Prasad and Prasad

(1987a, 1987b) have found that both lead and mercury inhibit biosynthesis of chlorophyll by interfering with the membrane-bound sulfhydryl (-SH) group of δ-amino laevulinic acid dehydratase (ALAD). Because the plant cell membrane is the primary site of metal activity, heavy metal-induced oxidative stress can be measured by MDA formation (Gallego et al., 1996). Lipid peroxidation, an indicator of this stress, increased gradually in our treated plants as the concentration of Hg rose. Kappus (1985) and Mazhoudi et al. (1997) also observed increased peroxidation in various species treated with different heavy metals.

To protect against toxic concentrations of mercury, plants have developed protective mechanisms by modifying the antioxidative enzymes that scavenge the resultant ROS. Although mercury does not generate ROS directly as do other heavy metals such as Cu, it does create oxidative stress by modifying the plant's antioxidative defense systems. Among the detoxifying enzymes that we analyzed, CAT initially showed increased activity in the leaves. In contrast, we observed no significant changes in POD activity at low Hg concentrations. However, POD levels later increased and remained higher than those measured in the control plants (Fig. 3).

Increased activities by CAT and POD in leaves are further evidence that mercury indirectly promotes the formation of ROS. Subhadra et al. (1999) have reported that activities of both enzymes are induced when aquatic plants are treated with mercury. Increased CAT contents in leaves in conjunction with low concentrations of Hg may indicate that free radicals are formed in response to exposure to light (Noctor and Foyer, 1998; Foyer and Noctor, 1999). The higher level of POD activity in treated versus untreated leaves suggests that this enzyme may have a role in detoxifying the H_2O_2 that results from metal toxicity. POD activity did not increase significantly in the treated roots, although relatively higher values were maintained for treatments of no more than 10 μM Hg (Fig. 3). At higher mercury concentrations, however, POD activity did decrease.

Our results indicate that both enzymes developed tolerance to Hg stress by modifying their activities, whether in the leaves or in the roots. Likewise, Teisseire and Guy (2000) have found that these two enzymes have enhanced activities under Cu stress in *Lemna minor*. Decreases in CAT activity at higher Hg concentrations may have been caused either by direct contact with proteins and high concentrations of free radicals, or by the inhibition of protein synthesis and other oxidase proteins (Luna et al., 1994). Nevertheless, some studies have shown no changes in CAT and POD activities when plants are treated with Cd, Zn (Bonnet et al., 2000), or Cu (Mazhoudi et al., 1997).

SOD activity increased considerably in both leaves and roots -- circumstantial evidence that point to the production of both free radical oxygen species and H_2O_2 as a result of Hg stress. Likewise, enhanced levels of SOD have been reported in several species treated with a variety of metals (del Rio et al., 1985; Mishra and Choudhury, 1996; Prasad et al., 1999). However, SOD activity can also be diminished under certain conditions, i.e., the inhibition of enzymatic reactions, disturbance of metabolic activities, or poisoning of SOD by cadmium ions (Wecks and Clijsters, 1997).

The increased APX activity in both the roots and leaves indicates detoxification of the free radicals and H_2O_2 generated by SOD. APX levels have also been enhanced in bean plants treated with Cu (Wecks and Clijsters, 1997), in *Zea mays* with Ni (Baccouch et al., 1998), and in rye grass exposed to Cu, Ni, Pb, or Zn (Bonnet et al., 2000). Those study results suggest that ascorbate plays an important role in the detoxification of free radicals through successive oxidation and reduction reactions, as well as by the action of certain enzymes (Rennenberg, 1982; Asada and Takahashi, 1987). In addition, earlier reports have shown that APX and CAT are critical to the detoxification of free radicals generated by SOD (Foyer et al., 1994; Wecks and Clijsters, 1996). For example, Kampfenkel et al. (1995) demonstrated that the activities of both APX and CAT increased two-fold in *Nicotiana plumbaginifolia* under Fe treatment.

In leaves, GR activity initially increased (up to 0.50 μM of Hg), then decreased (Fig. 2). In the roots, however, it decreased gradually, then severely at higher concentrations, probably because the roots were in direct contact with the mercury ions, which led to inhibited enzymatic activity. The enhanced level of GR found in leaves under Hg stress shows that this enzyme plays an important role in using NADPH to maintain glutathione (GSH) in a reduced state. Likewise, oxidized glutathione (GSSG) is partially involved in the detoxification of H_2O_2. Prasad et al. (1999) have also reported increased GR contents in Zn-treated *Brassica juncea*. In contrasting studies, however, the level of GR was reduced in plants exposed to toxic levels of Fe, Cu, or Cd (Gallego et al., 1996; Mazhoudi et al., 1997; Patra and Panda, 1998).

In conclusion, activities of the antioxidant enzymes SOD, APX, CAT, and POD increased when we treated rice plants with low concentrations of mercury. This indicates, therefore, that the Asada-Halliwell cycle is

involved in detoxifying the reactive oxygen species. GR levels also increased in leaves under treatment with up to 0.25 μM Hg, but then decreased gradually. In the roots, however, the activity of GR was greatly inhibited.

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