Nickel Toxicity of Rice Seedlings: The Inductive Responses of Antioxidant Enzymes by $NiSO_4$ in Rice Roots

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ABSTRACT

The effect of $NiSO_4$ on lipid peroxidtion, antioxidant enzyme activities and H_2O_2 content in roots of rice seedlings was investigested. $NiSO_4$ treatment resulted in increases in H_2O_2, malondialdehyde contents and superoxide dismutase and ascorbate peroxidase activities. However, $NiSO_4$ had no effect on catalase and glutathione reductase activities. Diphenyleneiodonium chloride, an inhibitor of NADPH oxidase, did not inhibit $NiSO_4$-induced H_2O_2 production, suggesting NADPH oxidase is not a source of $NiSO_4$-induced H_2O_2 production. $NiSO_4$ treatment enhanced diamine oxidase activity in rice roots. Results suggest that $NiSO_4$-induced H_2O_2 production is possibly mediated through diamine oxidase.

Key words: Lipid peroxidation, $NiSO_4$, *Oryza sativa* L., Oxidative stress.

水稻幼苗鎳之毒害：硫酸鎳誘導水稻根抗氧化酵素之反應

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摘要

本研究探討硫酸鎳對水稻幼苗根脂質過氧化作用、抗氧化酵素活性與過氧化氫含量之影響。硫酸鎳處理會增加脂質過氧化作用、過氧化氫含量與 superoxide dismutase 及 ascorbate peroxidase 活性。然而硫酸鎳不能影響 catalase 及 glutathione reductase 活性。NADPH oxidase 之抑制劑 diphenyleneiodonium chloride 不會抑制硫酸鎳所引起之過氧化氫產生，顯示 NADPH oxidase 不是硫酸鎳所引起過氧化氫產生之來源。硫酸鎳會促進 diamine oxidase 活性增加，顯示硫酸鎳所誘導過氧化氫含量增加可能是經由 diamine oxidase 作用所造成。

關鍵詞：脂質過氧化作用、硫酸鎳、水稻、氧化逆境。

INTRODUCTION

Nickel (Ni) is an essential element for plant growth (Brown *et al.* 1987). In general, there is much more concern about Ni toxicity in crop plants. Critical toxicity level in crop species are in the range of > 10 μg g^{-1} dry weight (DW) in sensitive, and 50 μg g^{-1} DW in moderately, tolerant species (Marschner 1995). At toxic concentrations Ni interferes with numerous physiological, anatomical and morphological processes (Mishra and Kar 1974).

Ni, a non-redox reactive metal, cannot generate active oxygen species directly by Fenton-type reaction.

Abbreviations： AOS, active oxygen species; APX, ascorbate peroxidase; CAT, catalase; DPI, diphenyleneiodonium chloride; DAO, diamine oxidase; DW, dry weight; GR, glutathione reductase; MDA, malondialdehyde; POX, peroxidase; SOD, superoxide dismutase.

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However, Ni can cause oxidative stress in plant tissues as indicated by lipid peroxidation (Baccouch *et al.* 1998, Boominathan and Doran 2002, Gonnelli *et al.* 2001, Rao and Sresty 2000, Wang *et al.* 2001). Exposure to Ni resulted in a severe depletion of reduced glutathione (GSH) (Rao and Sresty 2000), which is believed to be a critical step in Ni-induced active oxygen species (Schübenzübel and Polle 2002). H_2O_2 is a constituent of oxidative metabolism and is itself an active oxygen species (AOS). It has been shown that H_2O_2 content increased significantly with Ni treatment (Boominathan and Doran 2002, Wang *et al.* 2001).

Celluar damage caused by active oxygen species (AOS) might be reduced or prevented by antioxidant enzymes such as superoxide dismutase(SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Foyer *et al.* 1997). SOD catalyzes the dismutation of superoxide to produce H_2O_2. CAT catalyzes the decomposition of H_2O_2 to water and oxygen; alternatively, H_2O_2 can be eliminated via the ascorbate/glutathione reaction system involving APX and GR. It has been shown that antioxidant enzyme activities were either enhanced or reduced by Ni^{2+} in plants (Baccouch *et al.* 1998, Boominathan and Doran 2002, Gonnelli *et al.* 2001, Rao and Sresty 2000, Wang *et al.* 2001).

It is not known whether Ni induces oxidative stress in rice roots. In the present study, we investigated the effect of excess $NiSO_4$ on the changes in malondialdehhde (MDA) content, an indicator of lipid peroxidation, antioxidant enzyme activities, and H_2O_2 content in roots of rice seedlings.

MATERIAL AND METHODS

PLANT MATERIAL

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. In order to get more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pretreated at 37℃ for 1-day under dark condition. Uniformly germinated seeds were then selected and transferred to a Petri dishes (9.0 cm) containing two sheets of Whatman No. 1 filter paper moistened with 10 mL of distilled water or $NiSO_4$ at the desired concentration as specified in the individual experiments. Root growth of rice seedlings grown in distilled water is similar to that grown in medium containing inorganic salts, thus seedlings grown in distilled water were used as the controls. Each Petri dish contained 10 germinated seeds. Each treatment was replicated four times. The germinated seeds were allowed to grow at 27℃ in darkness.

DETERMINATION OF H_2O_2 AND LIPID PEROXIDATION

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing with phosphate buffer (50 mM, pH 6.8) including 1 mM hydroxylamine. The homogenate was centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 levels, extracted solution was mixed with 0.1% titanium chloride (Aldrich) in 20% (v/v) H_2SO_4 and mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of yellow color of supernatant was measure at 410 nm. H_2O_2 level was calculated using the extinction coefficient 0.28 $\mu mol^{-1}\ cm^{-1}$. MDA, routinely used as an indicator of lipid peroxidation, was extracted with 5% (v/v) trichloroacetic acid and determined according to Heath and Packer (1968). H_2O_2 and MDA contents were expressed on the basis of dry weight (DW).

ENZYME ASSAYS

The assays of antioxidant enzymes in detail have been described previously (Hurng and Kao 1994). CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Kato and Shimizu 1987). The decrease in H_2O_2 was followed as the decline in absorbance at 240 nm, and activity was calculated using the extinction coefficient [40 $mM^{-1}\ cm^{-1}$ at 240 nm] for H_2O_2 (Kato and Shimizu 1987). SOD was determined according to Paoletti *et al.* (1986). APOD was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as the decline in optical density at 290 nm and activity was calculated using the extinction coefficient [2.8 $mM^{-1}\ cm^{-1}$ at 290 nm] for ascorbate. GR was determined by the method of Foster and Hess (1980). One unit of activity for CAT, SOD, APOD, and GR was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per min, inhibited 50% the rate of NADH oxidation observed in control, degraded 1 μmol of ascorbate per min, and decreased 1 A_{340} per min, respectively. For extraction of diamine oxidase

(DAO), roots were homogenized with ice-cold phosphate buffer (50 mM, pH 7.8) using a pestle and mortar. The homogenate was centrifuged at 10,000 *g* for 20 min at 4℃. DAO activity was measured by the method of Naik *et al.* (1981). The detail procedure has been described previously (Lin and Kao 2002). One unit of DAO activity was defined as an increase of 1 A_{510} per h. Activities of all enzymes were expressed on the basis of DW.

STATISTICAL ANALYSIS

The results presented were the mean of four replicates. Means were compared by Duncan´s multiple range test at $P < 0.05$.

RESULTS

The effect of various concentrations of $NiSO_4$ on MDA content in roots of rice seedlings is shown in Fig. 1. Increasing concentrations of $NiSO_4$ from 20 to 60 µM progressively increased MDA content, indicating that $NiSO_4$ brings about lipid peroxidation.

Plant cells are equipped with several AOS detoxifying enzymes. Antioxidants enzymes include SOD, APX, GR, and CAT (Foyer *et al.* 1997). The striking increase in lipid peroxidation seen in roots treated with $NiSO_4$ may be a reflection of the changes of the activities of antioxidant enzymes. As shown in Fig. 2, activities of SOD and APX increased with the increasing of $NiSO_4$ concentrations. However, $NiSO_4$ had no effect on GR and CAT activities in rice roots (Fig. 2).

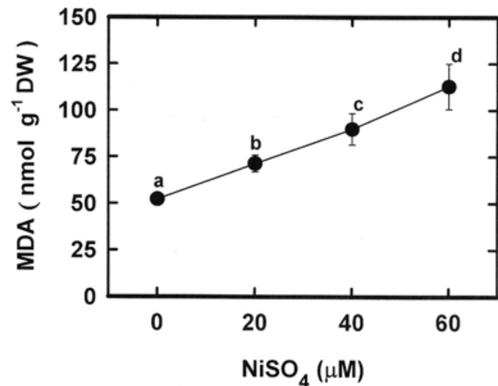

Fig. 1. Effect of $NiSO_4$ on MDA content in roots of rice seedlings. MDA content was determined 5 days after treatment. Values with the same letter are not significantly different at $P < 0.05$.

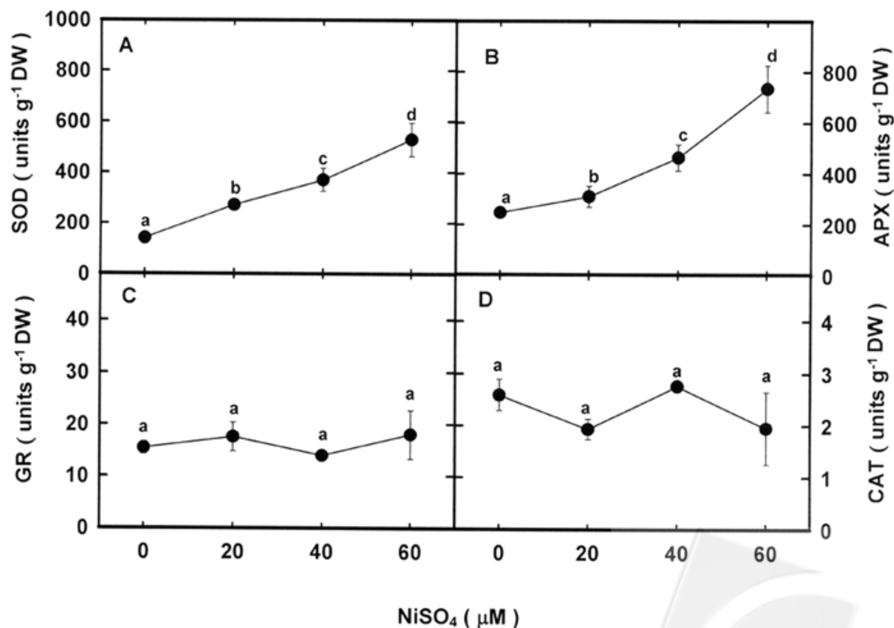

Fig. 2. Effect of $NiSO_4$ on the activities SOD (A), APX (B), GR (C), and CAT (D) in roots of rice seedlings. Enzymes were extracted and assayed 5 days after treatment. Values with the same letter are not significantly different at $P < 0.05$.

Lipid peroxidation is caused by AOS (Kellogg and Fridovicn 1975, Thompson *et al*. 1987). $NiSO_4$ at the concentrations of 40 and 60 μM caused an increase in H_2O_2 content (Fig. 3A). However, $NiSO_4$ at a concentration of 20 μM had no effect on H_2O_2 content (Fig.2). It is likely that when rice roots were treated with 20 μM $NiSO_4$, $O_2^{\cdot-}$ and / or hydroxyl radicals ($OH^{\cdot}$) rather than H_2O_2 were the active species responsible for lipid peroxidation.

In plants, polyamines are thought to play an important role in growth development and stress response (Bouchereau *et al.* 1999). DAO catalyzes the catabolism of diamine, especially putrescine, to their corresponding aldehyde, H_2O_2 and NH_4^+ (Bouchereau *et al.* 1999). Thus, DAO is likely to be affected by $NiSO_4$. As shown in Fig. 3B, it is indeed that $NiSO_4$ increases DAO activity in roots. The increase in DAO activity (Fig. 3B) by $NiSO_4$ is closely related to the increase in H_2O_2 content (Fig. 3A). Clearly, DAO is a source for H_2O_2 generation by $NiSO_4$.

AOS, originating from the plasma-membrane NADPH oxidase, which transfers electrons from cytoplasmic NADPH to O_2 to form O_2^-, followed by dismutation of $O_2^{\cdot-}$ to H_2O_2, has been a recent focus in AOS signaling. Recently, we have shown that abscisic acid- and NaCl-induced H_2O_2 accumulation in rice leaves (Hung and Kao, 2004) and rice roots (Tsai *et al.* 2005) are mediated by the activation of plasma-membrane NADPH oxidase. Diphenyl-eneiodonium chloride (DPI) has been used as an inhibitor of NADPH oxidase (Hung and Kao 2004, Tsai *et al.* 2005). When rice roots were treated with DPI, $NiSO_4$-induced accumulation of H_2O_2 in rice roots was not reduced (Table 1).

DISCUSSION

Superoxide ($O_2^{\cdot-}$) is a toxic by-product of oxidative metabolism. Thus, the dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 by SOD is an important step in protecting the cell (Foyer *et al.* 1997). Baccouch *et al.* (1998) observed that SOD activity was stimulated by Ni^{2+} in *Zea mays* shoot. It has been shown that Ni^{2+} had no effect on SOD activity in roots of *Alyssum bertolonii*, *Nicotiana tabacum*, and *Silene paradox*a (Boominathan and Doran 2002, Gonnelli *et al.* 2001). On the other hand, decrease in SOD activity by Ni^{2+} has been shown in rice leaves (Wang *et al.* 2001). In this study, we observed that $NiSO_4$ enchanced SOD activity in rice roots (Fig. 2A).

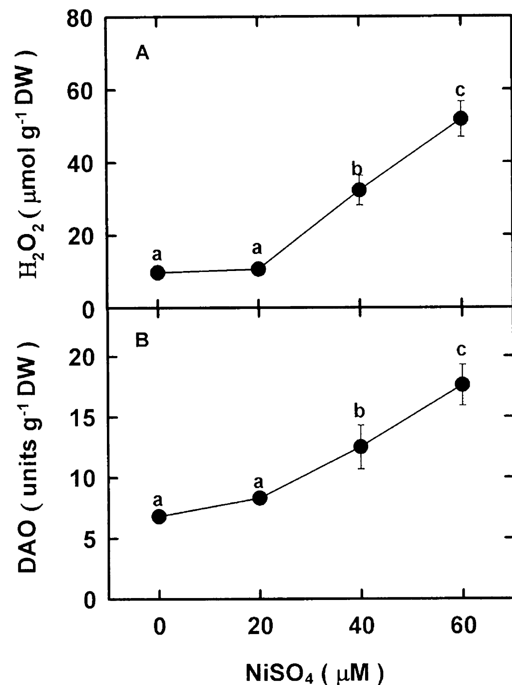

Fig. 3. Effect of $NiSO_4$ on H_2O_2 content (A) and DAO activity (B) in roots of rice seedlings. Measurement were made 5 days after treatment. Values with the same letter are not significantly different at *P* < 0.05.

Table 1. Effect of DPI on H_2O_2 content in roots of rice seedling in the presence and absence of $NiSO_4$. Rice seedlings (1-day-old) were treated with $NiSO_4$, DPI, or $NiSO_4$ + DPI for 2 day in the dark. Means ± S.E. (n = 4). Values with the same letter are not significantly different at *P* < 0.05.

Treatment	H_2O_2 (μ mol g^{-1} DW)
H_2O	12.2 ± 0.30^a
DPI (50 μ M)	13.0 ± 0.61^a
DPI (100 μ M)	15.3 ± 1.80^a
$NiSO_4$ (40 μ M)	24.7 ± 2.40^b
$NiSO_4$ (40 μ M) + DPI (50 μ M)	24.8 ± 0.74^b
$NiSO_4$ (40 μ M) + DPI (100 μ M)	29.9 ± 0.34^c

C.E.P.S.

The role of APX and GR in the H_2O_2 scavenging in plant cells has been well established in the ascorbate-glutathione cycle (Bowler *et al.* 1992). Both APX and GR activities were increased by Ni^{2+} in shoot of *Zea mays* (Baccouch *et al.* 1998) and in roots and shoot of sensitive population of *Silene paradox* (Gonnelli *et al.* 2001). Rao and Sresty (2000) also reported that Ni^{2+} increased GR activity in *Cajanus cajan*. Boominthan and Doran (2002) demonstrated that APX activity did not change in roots *Alyssum bertolonii* and *Nicotiana tabacum* during Ni^{2+} stress. Wang *et al.* (2001) found that APX activity was reduced in rice leaves. Here, we observed that $NiSO_4$ increased APX activity but had no effect on GR activity in roots of rice seedlings (Figs. 2B and 2C).

CAT is known to dismutate H_2O_2 into H_2O and O_2. It has been shown that Ni reduced CAT activity in leaves of rice and in roots and shoot of *Cajanus cajan* (Rao and Sresty 2000, Wang *et al.* 2001). On the contrary, Ni^{2+} did not affect CAT activity on roots of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominthan and Doran 2002). We also observed that Ni^{2+} had no effect on CAT activity (Fig. 2D).

Our results not only have shown that Ni^{2+} increased the activities of SOD and APX (Figs. 2A and 2B) and the content of H_2O_2 (Fig. 3A), but also demonstrated that Ni^{2+} caused an increase in lipid peroxidation (Fig. 1). These results suggest that Ni^{2+} cause an oxidative stress in roots of rice seedlings.

It has been shown that Ni^{2+} treatment resulted in an increase in H_2O_2 content in roots of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominthan and Doran 2002) and leaves of *Oryza sativa* (Wang *et al.* 2001). Here, we also observed that Ni^{2+} enhanced H_2O_2 production in roots of rice seedlings (Fig. 3A). To our knowledge, there is no information about the mechanism of Ni^{2+}-induced H_2O_2 accumulation. The fact that Ni^{2+}-induced H_2O_2 accumulation in rice roots cannot be inhibited by DPI seems to suggest that Ni^{2+}-dependent H_2O_2 generation is unlikely originated from plasma-membrane NADPH oxidase. NaCl-induced accumulation of H_2O_2 in rice leaves has been suggested to be due to NaCl-enhanced SOD activity (Lee *et al.* 2001). This seems to be the case in Ni^{2+}-treated roots of rice seedlings, because $NiSO_4$ significantly enhanced SOD activity (Fig. 2A). In the present study, we observed that Ni-increased H_2O_2 accumulation in rice roots (Fig. 3A) is closely correlated Ni^{2+}-increased diamine oxidase activity (Fig. 3B). Thus, diamine oxidase is most likely to be the source of Ni^{2+}-induced H_2O_2. An alternative source for H_2O_2 generation includes oxalate oxidase, an enzyme that degrades oxalate to CO_2 and H_2O_2 (Dumas *et al.* 1995). Oxlate oxidase gene expression is induced by salt stress, salicylate, and methyl jasmonate (Hurkman and Tanaka 1996). It is not known whether Ni^{2+} will activate oxalate oxidase in rice roots. Further work is necessary to clarify this possibility.

In previous work, we observed that exogenous H_2O_2 inhibited root growth of rice seedlings (Lin and Kao 2001). H_2O_2 has been shown to cause a rapid cross-linking of cell-wall polymers (Bradley *et al.* 1992, Schopfer 1996). H_2O_2 is a necessary substrate for a cell-wall stiffening process catalyzed by peroxidase (Schopfer 1994) and is also required for the biosynthesis of lignin (Rogers and Campbell 2004). Recently, we reported that cell-wall stiffening and lignification are the processes responsible for $NiSO_4$-inhibited root growth of rice seedlings (Lin and Kao 2005). Clearly, $NiSO_4$-induced H_2O_2 production in roots of rice seedlings may play a role in regulating $NiSO_4$-inhibited root growth.

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