Nickel Toxicity of Rice Seedlings: Cell Wall Peroxidase, Lignin, and $NiSO_4$-inhibited Root Growth

Yu-Ching Lin and Ching-Huei Kao*
Department of Agronomy, National Taiwan University, Taipei 106, Taiwan (ROC)

ABSTRACT

The effects of various concentrations of $NiSO_4$ (0-60 μM) on the activities of cell-wall peroxidase against ferulic acid (FPOX) or syringaldazine and phenylalanine ammonia-lyase (PAL) and the content of lignin in root of rice seedlings were investigated. $NiSO_4$ was found to inhibit root growth and this inhibition was pH independent. $NiSO_4$ was also found to increase the activities of FPOX and syringaldazine peroxidase and the content of lignin in roots. However, $NiSO_4$ did not affect the activity of PAL, the first enzyme of phenylpropanoid pathway. Results suggest that cell-wall stiffening and lignifications are the processes that are enhanced by $NiSO_4$ to reduce growth of rice roots.

Key words: Lignin, $NiSO_4$, *Oryza sativa* L., Peroxidase, Root growth.

水稻幼苗鎳之毒害：細胞壁過氧化酵素、木質素與硫酸鎳所抑制根之生長

林玉菁、高景輝*
國立臺灣大學農藝系

摘要

本研究主要探討不同濃度(0-60 μM)硫酸鎳處理對細胞壁過氧化酵素與 phenylalanine ammonia-lyase (PAL)活性與木質素含量的影響。硫酸鎳抑制水稻根之生長，此抑制作用與 pH 值無關。硫酸鎳增加細胞壁內以 ferulic acid 或以 syringaldazine 為受質的過氧化酵素活性及木質素的含量，但不影響 PAL 的活性。結果說明細胞壁硬化與木質化可能是控制硫酸鎳抑制水稻根生長的主要步驟。

關鍵詞：木質素、硫酸鎳、水稻、過氧化酵素、根生長。

INTRODUCTION

Nickel (Ni) is a micronutrient required at very low concentration by plants (Brown *et al.* 1987). Ni^{2+} at high concentrations is known to inhibit plant growth (Baccouch *et al.* 1998, Das *et al.* 1978, Gabbrielli *et al.* 1999, Gonnelli *et al.* 2001, Pandolfini *et al.* 1992, Rao and Sresty 2000, Wang *et al.* 2001). However, the mechanism underlying this inhibition is not yet clear. A key role of cell-wall peroxidases in the stiffening of the cell wall, and consequently, in the growth reduction of cell elongation has been postulated (Fry 1986). Thus, to test the hypothesis that an increase in cell-wall peroxidase activity is associated with growth inhibition of seedling roots of rice, data from peroxidase activity associated with cell walls is required. Guaiacol is usually used as a substrate to assay peroxidase activity. However, guaiacol is not the natural substrate for the peroxidase in the cell-wall stiffening process. Ferulic acid has been identified as being ester linked to arabinoxylans in mono-cotyledonous plants (Kato and Nevins 1985). A key role in the cell-wall stiffing of dimerization of ferulic acid (FPOX) catalyzed by cell-wall peroxidase has been reported (Sánchez *et al.* 1996). Thus, ferulic acid appears to be the suitable substrate to establish the relationship between cell-wall peroxidase activity and

Abbreviations: DW, dry weight; FPOX, peroxidase against ferulic acid; PAL, phenyl-alanine ammonia-lyase.

* 通信作者, kaoch@ccms.ntu.edu.tw
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189 Chung-Cheng Rd., Wufeng, Taichung Hsien 41301, Taiwan (ROC)

$NiSO_4$-inhibited root growth of rice seedlings. The present investigation was therefore designed to study the changes in peroxidase activity against FPOX associated with cell walls during growth reduction of rice seedling roots caused by $NiSO_4$.

It is known that peroxidases also influence plant growth through lignin synthesis (Lewis and Yamamoto 1990, Whetten and Sederoff 1995). Lignification is part of cell wall differentiation and irreversibly inhibits cell elongation (Sauter and Kende 1992). It has been shown that syringaldazine, a hydrogen donor, has a particularly high affinity for peroxidases associated with lignification (Goldberg *et al.* 1983). Syringaldazine peroxidase activity has been shown to be increased in Ni^{2+}-treated roots of *Pisum sativum* and *Triticum aestivum* (Gabbrielli *et al*. 1999, Pandolfini *et al.* 1992). Therefore, effect of $NiSO_4$ on lignin content and syringaldazine peroxidase activity in roots of rice seedlings was also studied in the present investigation.

MATERIALS AND METHODS

Rice (*Oryza sative* L. cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in a Petri dish (20 cm) containing distilled water at 37℃ under dark condition. After an 1-day incubation, uniformly germinated seeds were selected and transferred to Petri dishes (9 cm) containing two sheets of filter paper moistened with 10 mL of distilled water or test solution. Each Petri dish contained 10 germinated seeds. Each treatment was replicated 4 times. The germinated seeds were allowed to grow at 27℃ in darkness.

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 a cell wall fraction.

Peroxidase 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℃, and centrifuged at 1,000 *g*. The supernatant was used for FPOX assay according to Sánchez *et al.* (1996). The oxidation of ferulic acid was measured spectrophotometrically following the absorbance decrease at 310 nm in a reaction mixture containing 1.35 mL Na-phosphate buffer (0.2 mM, pH 5.8), 0.5 mL ferulic acid (240 μM), 0.5 mL H_2O_2 (3 mM) and 0.15 mL enzyme extract. One unit of FPOX was defined as a decrease of 1 A_{310} per min.

When syringaldazine was used as the substrate, the assay medium contained: 10 mM potassium phosphate buffer (pH 7.4), 0.018 mM syringaldazine and 0.5 mM H_2O_2 (Grison and Pilet 1985). The reaction was started by introducing 0.5 mL of enzyme preparation. The change in absorbance at 530 nm was monitored. One unit of peroxidase against syringaldazine was expressed as an increase of A_{530} per min.

Phenylalanine ammonia-lyase (PAL) was extracted and determined according to Hyodo and Fujinami (1989). The calculation was based on the extinction coefficient [9,500 M^{-1} cm^{-1}] for *trans*-cinnamic acid. One unit of activity for PAL was defined as the amounts of enzyme which caused the formation of 1 μmol *trans*-cinnamic acid per h. Activities of all enzymes were expressed on dry weight (DW) basis.

The lignin content in roots was measured by the Sasaki *et al*. (1996) method, a method originally described by Morrison (1972). Roots were homogenized with a pestle and mortar in 95% ethanol. The homogenate was centrifuged at 1,000 *g* for 5 min. The pellet was washed three times with 95% ethanol and twice with a mixture of ethanol and hexane (1:2, v/v). The material was allowed to air dry and its lignin level measured. The dried sample was washed one time with 2 mL acetyl bromide in acetic acid (1:3, v/v). Then 1 mL acetyl bromide in acetic acid (1:3, v/v) was added to the pellet and incubated at 70℃ for 30 min. After cooling of the mixture to room temperature, 0.9 mL of 2 N NaOH and 0.1 mL 7.5 M hydroxylamine hydrochloride were added, and the volume was made up to 10 mL with acetic acid. After centrifugation at 1,000 *g* for 5 min, the absorbance of the supernatant was measured at 280 nm (A_{280}). Lignin content was expressed on DW basis.

Statistical differences between measurements on different treatments were analyzed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Fig. 1A shows the effects of $NiSO_4$ on root and shoot growth of rice seedlings. Increasing concentrations of $NiSO_4$ from 0 to 60 μM progressively decreased root growth (Fig. 1A) and increased Ni content in roots (Fig. 1B). It was found that $NiSO_4$ had no effect on shoot growth and Ni content in shoot (Figs.

1A and 1B). These results were in contrast to those reported by other workers, who found that Ni^{2+} reduced both root and shoot growth (Das *et al*. 1978, Pandolfini *et al.* 1982, Rao and Sresty 2000). Our results seem to suggest that Ni^{2+} is mainly accumulated in roots of rice seedlings and to a minor extent in shoots and also indicate that the primary effect of Ni^{2+} is to take place in roots of rice seedlings.

It has been shown that soil pH values below 5.6 seem to favor the absorption of Ni^{2+} while values above 5.6 do not (Mishra and Kar 1974). When roots of rice seedlings were treated with $NiSO_4$ at different pH values (4-8), it was found that root growth was inhibited to a similar extent (Fig. 2). It seems that $NISO_4$-inhibited root growth of rice seedlings is pH independent.

In rice, a role for cell-wall peroxidase has been established in controlling anoxia- and ethylene-induced growth of coleoptiles (Lee and Lin 1995, 1996b) and abscisic acid-, methyl jasmonate-, cadmium-, and NaCl-inhibited growth of roots (Chen and Kao 1995, Lee and Lin 1996a, Lin and Kao 1999, 2001). Here, we addressed the question whether $NiSO_4$-inhibited root growth of rice seedlings is associated with an increase in cell wall peroxidase activity. The cell-wall peroxidase we determined in this study is FPOX. The reduction of root growth with increasing $NiSO_4$ concentrations was found to be associated with an increase in FPOX activity extracted from cell walls (Figs. 1A and 3A). Sánchez *et al.* (1996) demonstrated a negative relationship of FPOX and growth capacity in pine hypcotyl, suggesting a role for peroxidase in the cell-wall stiffening. Furthermore, the ability of peroxidase to catalyze wall cross-linking through ferulic acid esterified to polysaccharides has already been shown (Whitemore 1976). It seems that cell-wall stiffening catalyzed by FPOX may participate in the regulation of root growth reduction of rice seedlings under $NiSO_4$ conditions.

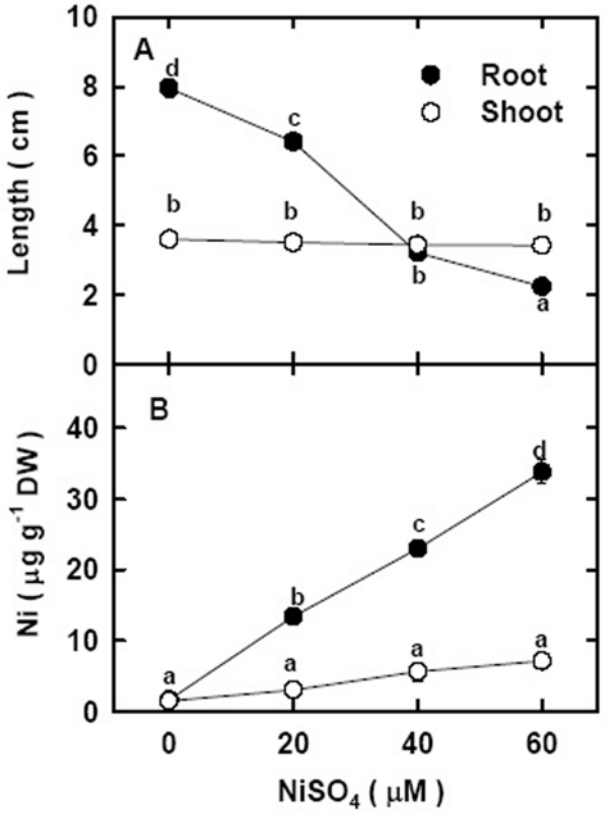

Fig. 1. Effect of $NiSO_4$ on root and shoot growth (A) and Ni content in roots and shoot (B) of rice seedlings. All measurements were made 5 days after treatment in the dark. Values with the same letter are not significantly different at $P < 0.05$.

Peroxidase was also found to be related to lignin synthesis (Lewis and Yamamoto 1990, Whetten and Sederoft 1995). Sauter and Kende (1992) demonstrated that lignin content in newly formed internodal tissue of rapidly growing deepwater rice was lower than in slowly growing tissue, indicating that lignification plays a role in regulating cell elongation. Thus, it is of great interest to know whether $NiSO_4$ has an effect on lignin content in roots of rice seedlings. As expected, increasing concentration of $NiSO_4$ from 0 to 40 µM progressively increased lignin content in roots of rice seedlings and no further increase in lignin content in roots was observed at 60 µM $NiSO_4$ (Fig. 3D). It has been shown syringaldazine peroxidase is associated with lignification (Golgberg *et al.* 1983). Here, we also observed that $NiSO_4$ increased the activity of syringaldazine peroxidase in rice roots (Fig. 3B), confirming that $NiSO_4$-inhibited root growth is linked to lignification in roots of rice seedlings. Gabbrielli *et al.* (1999) and Pandolfini *et al.* (1992) also reported the similar results. Phenylalanine ammonia-lyase (PAL) is a highly regulated enzyme that plays a central role in the phenylpropanoid pathway. Its reaction product *trans*-cinnamic acid, is a precursor in the synthesis of many secondly plant products, including lignin (Rogers and Campbell 2004). $NiSO_4$ did not affect PAL activity in rice roots (Fig. 3C). This indicates that the activity of PAL is not correlated with $NiSO_4$-induced lignification in rice roots. This finding is not surprising, considering the many different metabolic pathways in which *trans*-cinnamic acid is an intermediate. In conclusion, the results of this work suggest that cell-wall stiffening and lignification are the processes that are enhanced by $NiSO_4$ to permit growth reduction of rice roots.

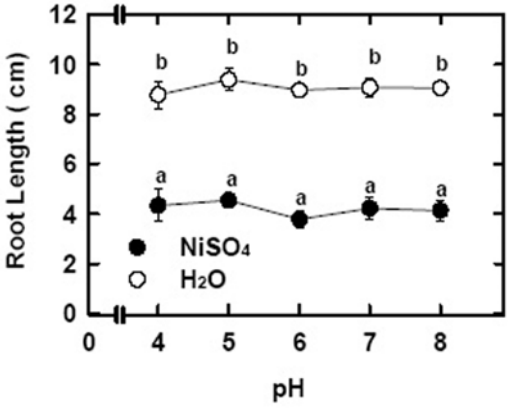

Fig. 2. Effect of pH on root growth of rice seedlings in the presence or absence of $NiSO_4$. Rice seedlings were treated with distilled water or $NiSO_4$ (40 µM) for 5 days in the dark. Values with the same letter are not significantly different at $P < 0.05$.

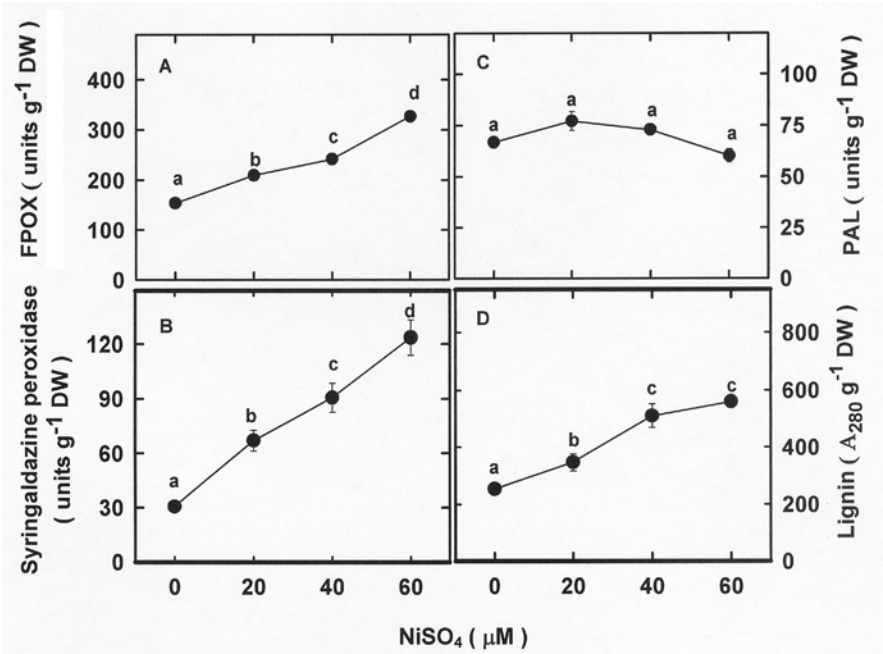

Fig. 3. Effect of $NiSO_4$ on the activities of FPOX (A), syringaldazine peroxidase (B), and PAL (C) and the content of lignin (D) in the roots of rice seedlings. All measurements were made 5 days after treatment. Values with the same letter are not significantly different at $P < 0.05$.

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