Journal of Animal & Plant Sciences, 2010. Vol. 7, Issue 2: 810- 821.
Publication date: 29/06/2010, *http://www.biosciences.elewa.org/JAPS;* **ISSN 2071 - 7024**

Responses of growth and antioxidant metabolism to nickel toxicity in *Luffa cylindrica* seedlings

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Key words: *Luffa cylindrical*, defensive mechanism, nickel toxicity, *in vitro* culture

1 SUMMARY

To assess nickel-induced toxicity in plants, an experiment was performed focusing on the metabolic adaptation of *Luffa cylindrica* seedlings to nickel-induced oxidative stress. *Luffa cylindrica* (sponge gourd) seeds were germinated and cultured in the Murashige and Skoog (MS) media with nickel concentrations of 50-800 μM. Nickel concentrations were negatively correlated with the biomass of cotyledons, stems and roots in Luffa cylindrica seedlings. Significant increases in superoxide dismutase (SOD) activity of cotyledons, stems and roots over that of control were observed at all tested nickel concentrations. The elevated guiacol peroxidase (GPX) activity was observed in the cotyledons, stems and roots of *Luffa cylindrica* seedlings exposed to different levels nickel. Catalase (CAT) activity was significantly enhanced by tested nickel concentrations except for in the roots at 800 μM. Phenylalanine ammonia-lyase (PAL) activity in the cotyledons, stems and roots was significantly induced and was positively correlated to increasing nickel concentrations except for in the roots under 800 μM stresses. The present results suggested that treatment with different levels of nickel may enhance the antioxidant activities in the cotyledons, stems and roots of *Luffa cylindrica* seedlings, thus alleviating Ni-induced oxidative damage and enhancing Ni tolerance.

2 INTRODUCTION

Heavy metal pollution is a worldwide problem with serious environmental consequences. Amongst heavy metals, nickel (Ni) is an essential micronutrient for plant growth. Trace elements are necessary for normal metabolic functions in plants, but higher concentrations of these metals are toxic and may severely interfere with many physiological and biochemical processes of plants (Seregin and Kozhevnikova 2006, Chen et al. 2009). Earlier studies have shown that excessive Ni can inhibit seed germination, plant growth, induce chlorophyll degradation, and interfere with photo-system activity (Léon et al. 2005, Ahmad

et al. 2009, Ali et al. 2009). At the molecular level, Ni may also induce the generation of reactive oxygen species (ROS), and these ROS cause oxidative damage of lipids, proteins, and nucleic acids in plant cells (Gajewska et al. 2006, Gajewska and Skłodowska 2007). However, plants are able to cope with the effects of overproduction of partly reduced ROS during oxidative stress by activating numerous protective mechanisms, including the regulation of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). This is done by replenishing the cellular levels of natural antioxidants such as the reduced form of glutathione, or by turning on and off the expression of multiple genes encoding various antioxidant proteins, such as thioredoxin and related molecules (Mittler 2002). Thus, they have developed a broad range of strategies, collectively known as defense or stress responses, to protect themselves against biotic and abiotic stresses.

Luffa cylindrica L., which is known as sponge gourd, are a daily vegetable cultivated in the tropical and subtropical Asian regions. Usually, the skin of the gourd is peeled off when it is used as vegetable. Seeds and sponge of the old fruits are also used in Traditional Chinese Medicine as anti-helmintic, stomachic, and antipyretic phytomedicinal drugs (Oboh and Aluyor, 2009). Previous investigations have demonstrated that the effects of Ni on the antioxidant enzyme systems have already been studied in some plant species (Gajewska et al. 2006, Gajewska and Skłodowska 2007, Yan et al. 2008). However, few reports are available concerning the responses of growth antioxidant metabolism in *Luffa cylindrica* plants to toxic nickel levels. In this study, the effects of nickel levels on the biomass and antioxidant enzymes including POD, SOD and CAT as well as PAL in *Luffa cylindrica* seedlings were evaluated after 7 days exposure. Thus, the aim of this work was to perform a comprehensive investigation on the impacts of different levels nickel on the responses of growth and antioxidant metabolism.

3 MATERIALS AND METHODS

3.1 Plant materials and chemicals: Mature *Luffa cylindrica* seeds were purchased from a Traditional Chinese medicine market, in Chengdu, China. Nitro blue tetrazolium (NBT) were purchased from Sigma (St. Louis, MO, USA). Others reagents, such as l-phenylalanine, methionine, ethylenediaminetetraacetic acid (EDTA) and guaiacol, were of grade or higher.

3.2 Seed germination and seedling growth: The basal medium consisted of full strength MS medium (Murashige and Skoog 1962) and supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8 ± 0.1 prior to the addition of 0.6% (w/v) agar. To melt the agar, the medium were heated in the oven and then distributed into 100 ml wide-neck bottles and autoclaved for 20min at 121± 2 °C. After autoclaving, the medium were allowed to cool at room temperature. All cultures were grown under 12/12 hours light/dark photoperiod at a temperature of 25± 2 °C. The seed coats were removed and embryos were surface sterilized with 70% ethanol for 30-60 seconds and 0.1% mercuric chloride solution for 6-8 min. Then, these embryos were washed 3-4 times with sterilized distilled water before culturing. These embryos were grouped into six lots having four pots each. Embryos in lot 1 were cultured with basal medium (control), for lots 2-6, an excess

supply of 50, 100, 200, 400 and 800 µM nickel, respectively, was superimposed on the basal medium. Ni was supplied as Ni_2SO4. The pots were arranged in a completely randomized design with three replicates per treatment. After 7 days, seedlings were harvested and the cotyledons, stems and roots were separated.

3.3 Protein extraction and estimation: All biochemical analyses were performed at 4 °C. The fresh cotyledons, stems and roots of seedlings (0.5) were homogenized using a chilled pestle and mortar under liquid nitrogen, and then were extracted in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) including 1.0 mM EDTA and 150 mM NaCl. The crude extract was centrifuged at 12 000 rpm for 5min at 4 °C and the supernatant was used for assaying of protein contents and enzyme activities. The protein content was determined according to Lowry's method (1951) using bovine serum albumin as standard.

3.4 Assay of superoxide dismutase (SOD) activity: Superoxide dismutase (SOD) assay was performed according to McCord and Fridovich (1969) with some slight modifications. A 3 ml reaction mixture contained 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin and 0.1 mM EDTA and 50 µl of enzyme extract. Riboflavin was added at the end and the microplates containing reaction mixtures were incubated at 25 °C for 5 min in the dark and later on for 15 min in light to start of the reaction. Absorbance was read at 560 nm using a UV/vis spectrophotometer (TU-1901 UV-Vis Spectrophotometer, Purkinje General, Beijing, China). Non-illuminated plates without enzyme extract were the control. The enzyme volume corresponding to 50% inhibition of the reaction (one unit) was calculated. The activity was expressed in U/g fresh weight.

3.5 Assay of catalase (CAT) activity: The activity of CAT was measured following the method of Montavon et al (2007). One unit of CAT activity is defined as the amount that decomposes 1 µM of H_2O_2 in 1 min. The activity was expressed in U/g fresh weight.

3.6 Assay of guaiacol peroxidase (GPX) activity: GPX activity was performed by measuring the increase in absorbance at 470 nm due to the formation of tetraguaiacol (Sakharov and Aridilla 1999). The reaction mixture (3 ml final volume) consisted of: 2.8 ml 3% guaiacol in 50 mM Tris-HCl (pH 7.0) and 0.1 ml 2% H_2O_2. The reaction was started by adding the 0.1 ml enzyme extract and the absorbance increase at 470 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which produces 1 absorbance change at 470 nm per min in the above assay conditions. The activity was expressed in U/g fresh weight.

3.7 Enzyme extraction and phenylalanine ammonia-lyase (PAL) activity assay: For PAL assay, cotyledons, stems and roots tissues were ground in ice-cold 0.1 M Tris-HCl buffer pH 8.8 containing 1% polyvinylpolypyrrolidone and 1 mM EDTA. The homogenate was centrifuged at 12,000 rpm, at 4 °C for 10 min and was tested for PAL activity. PAL activity was determined by monitoring the reaction product *trans*-cinnamate at 290 nm (Hahlbrock and Ragg 1975). The reaction mixture contained 50 mM Tris-HCl, pH 8.8, 20 mM L-phenylalanine, and enzyme in a total volume of 3 mL. The reaction was allowed to proceed for 30 min at 30°C and was

stopped by the addition of 0.5 mL of 10% trichloroacetic acid. One unit of enzyme activity was defined as the amount of enzyme that increased the absorbance by 0.01/min under assay conditions. The activity was expressed in U/g fresh weight.

3.8 Statistical analysis: Data are reported as the mean ± SD. Three independent experiments for each condition were performed. Statistical significance was evaluated with Student's t-test, and considered to be significant when the *P* value was less than 0.05.

4 RESULTS

Changes of the fresh weight in cotyledons, stems and roots obtained from all the experimental seedlings were shown in Fig.1. According to Figure 1, there was significant decrease in the fresh weight of cotyledons exposed to nickel treatments compared to the control, and the greatest inhibition of 75.6% was observed at 800 μM. Decreases of the fresh weight were also found in the stems and roots treatment group with increasing nickel concentrations, and the maximum values of inhibition represented 73.2% and 81.7% at highest tested Ni concentrations, respectively.

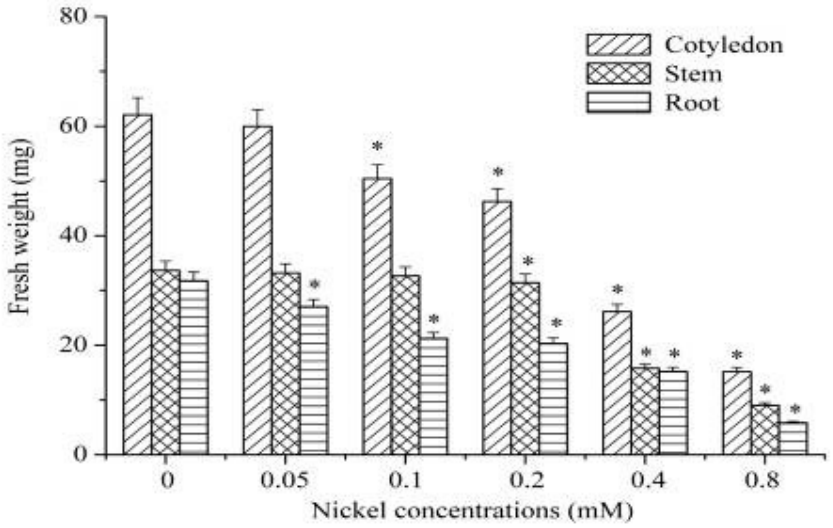

Figure 1: Effects of different concentrations of nickel on the fresh weight of cotyledon, stems, and roots of *Luffa cylindrical* seedlings. Embryos were germinated and grown under different levels of nickel for 7 days. Data points and error bars represent means ± S.D. (n = 3). Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

SOD activities in the cotyledons, stems and roots tissues homogenates obtained from all the experimental seedlings were shown in Fig.2. As shown in Figure 2, SOD activity was significantly affected by nickel treatment compared with the control values. The activities in the cotyledons increased gradually up to nickel concentrations of 800μM, and increasing by 124.7% compared to the control. At all

tested nickel levels, the activities in the stems were significantly induced, and were 74.8%, 113.3%, 153.1%, 210.9% and 242.1% higher than that of the controls, respectively. Similarly, SOD activities in the roots were higher of 6.97%, 19.3%, 49.3%, 57.7% and 45% at 50-800μM levels than corresponding to the control, respectively.

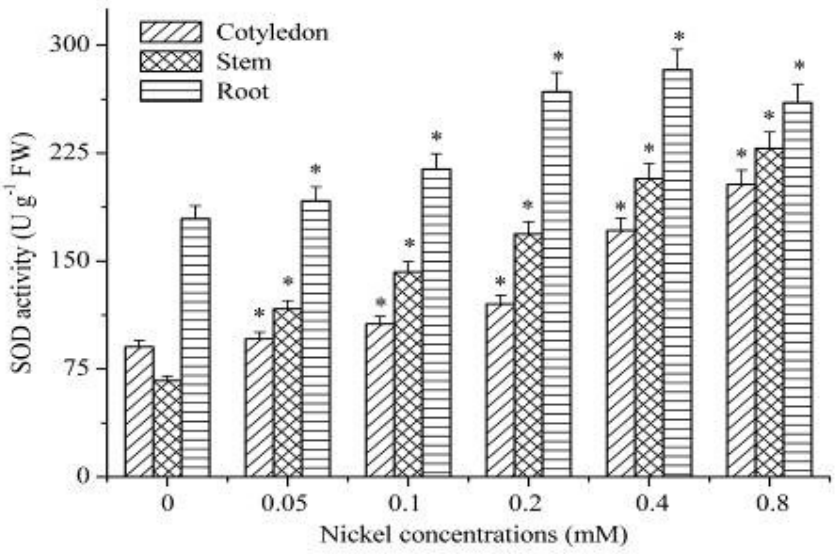

Figure 2: Effects of different concentrations of nickel on SOD activity of cotyledons, stems, and roots of *Luffa cylindrical* seedlings. Embryos were germinated and grown under different levels of nickel for 7 days. Data points and error bars represent means ± S.D. (n = 3). Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

GPX activities in the cotyledons, stems and roots tissues homogenates obtained from all the experimental seedlings were shown in Figure 3. According to Figure 3, there was a significant increase in the cotyledons of nickel treatments compared to the control (57 ± 2.16 U/g fresh weight), and the highest value was observed at 200 μM Ni treatment group (96.6 ± 3.87 U/g fresh weight). Elevations of GPX activity were found in the stems of treatment group, and the levels of GPX activity were 47.4 ± 1.78, 54.1 ± 2.39, 66.3 ± 3.06, 73.2 ± 2.95 and 40.1 ± 1.85 U/g fresh weight compared to that of the control (36.7 ± 1.54 U/g fresh weight), respectively. The levels of GPX activity was also elevated in the roots treated with different nickel concentrations, and increased by 11.7%, 29.2%, 33.1 %, 37.9 % and 74.6 % compared to the controls, respectively.

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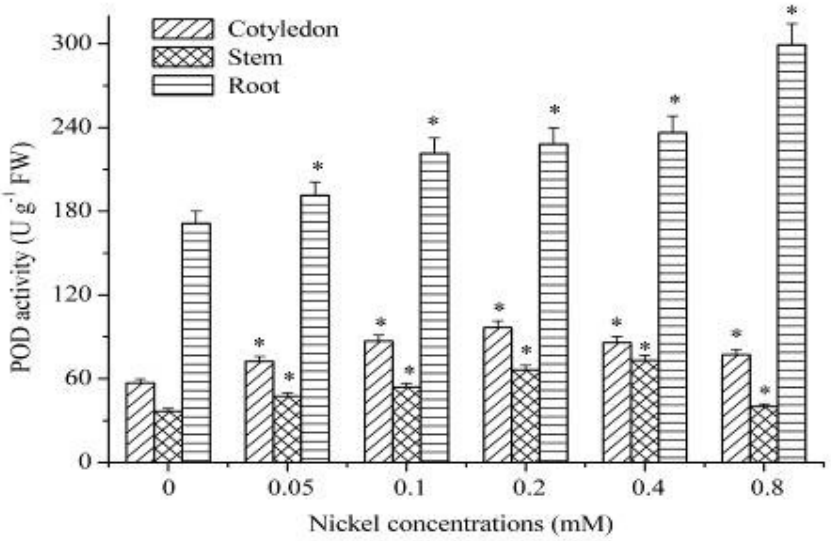

Figure 3: Effects of different concentrations of nickel on GPX activity of cotyledons, stems, and roots of *Luffa cylindrical* seedlings. Embryos were germinated and grown under different levels of nickel for 7 days. Data points and error bars represent means ± S.D. (n = 3). Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

CAT activities in the cotyledons, stems and roots tissues homogenates obtained from all the experimental seedlings were shown in Figure 4. From Figure 4, CAT activities in the cotyledons were significantly induced in different nickel levels up to 200 µM, and then followed a decline. The levels of CAT activities were increased by 14%, 192.4%, 230.1%, 184.9% and 158.8 % compared to the control, respectively. In the stems, CAT activity was significantly higher than that in the control under nickel stresses of different concentrations, and increased by 12.1%, 21%, 35.1%, 17.7% and 8.81%, respectively. When subjected to nickel stress, CAT activity in the roots was higher of 14%, 89.9%, 17.3% and 6.14% at nickel concentrations of 50, 100, 200 and 400µM than corresponding to the control, respectively. However, a further increase in nickel concentrations led to a lightly decrease in the CAT activity compared to the control.

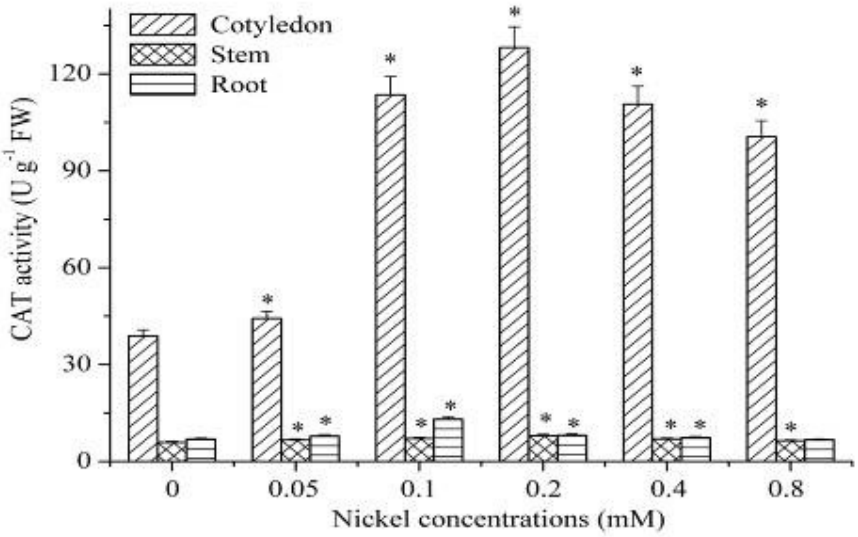

Figure 4: Effects of different concentrations of nickel on the CAT activity of cotyledons, stems, and roots of *Luffa cylindrical* seedlings. Embryos were germinated and grown under different levels of nickel for 7 days. Data points and error bars represent means ± S.D. (n = 3). Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

PAL activities in the cotyledons, stems and roots tissues homogenates obtained from all the experimental seedlings were shown in Figure 5. As shown in Figure 5, the activities of PAL in the cotyledons increased progressively with the increasing nickel concentrations. They increased by 24.4%, 37%, 80.7%, 209.4% and 342.7% at nickel concentrations of 50, 100, 200, 400 and 800µM compared to the control, respectively. The activities in the stems showed a similar trend when subjected to different nickel concentrations, and increased by 40.6%, 60.2%, 62.1%, 109.7% and 191.6% with increasing nickel concentrations compared to the control, respectively. In the roots, the activities rose gradually up to 77.1% higher than that of the control at nickel concentrations of 400 µM, whereas it decreased take place at 800 µM nickel exposure. Therefore, the top value in the roots was found at 400µM Ni exposure.

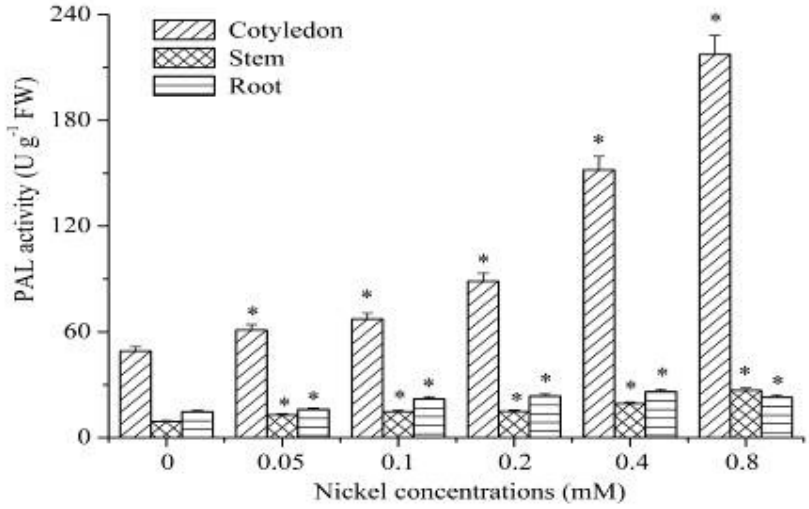

Figure 5: Effects of different concentrations of nickel on the PAL activity of cotyledon, stems, and roots of *Luffa cylindrical* seedlings. Embryos were germinated and grown under different levels of nickel for 7 days. Data points and error bars represent means ± S.D. (n = 3). Asterisk indicates that mean values are significantly different between the treatment and control ($P < 0.05$).

5 DISCUSSION

Nickel is one of the heavy metals widely used in modern industry that has been recognized as highly toxic and carcinogenic. Although it has also been recognized as an essential element for most living systems at trace levels, its negative effects on plant development and growth have been frequently observed in previous studies. Symptoms of Ni phytotoxicity include decrease of seed germination, reduction of root growth, induction of leaf chlorosis and reduction of biomass (Seregin and Kozhevnikova 2006, Chen et al. 2009). In the present study, the tested Ni concentrations resulted in the decrease in fresh weight of cotyledons, stems and roots compared to the control. The results showed that a negative effect on seedling growth at tested Ni concentration was observed. Similar decreases in the biomass have been reported in cabbage, wheat and *Jatropha curcas* plants (Pandey and Sharma 2002, Gajewska et al. 2006, Yan et al. 2008). Thus, the present study lends further support to previous findings.

Oxidative stress can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids. This leads to change in selective permeability of bio-membranes and thereby membrane leakage and change in the activity of enzymes bound to membrane occurs (Mittler 2002). Thus, it is important to understand the behavior of those enzymes in the protection against nickel toxicity. SODs catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. A number of environmental stresses can lead to enhanced production of ROS within plant tissues, and plants are believed to rely on the enzyme SOD to detoxify those ROS (Alscher et al. 2002). Thus, SOD has been proposed to be important in maintaining normal physiological conditions and coping with stress. In the present study,

there was significant increase in SOD activities of cotyledons, stems and roots exposed to different nickel concentrations compared to the control. Many studies suggest that nickel exerts its adverse actions by promoting or exacerbating oxidative stress. In several plants, the Ni-induced changes in the activities of SOD have been detected (Gajewska and Skłodowska 2007, Sun et al. 2009, Chen et al. 2009). There is substantial interest in the ability to increase SOD activity in plant cell so as to provide for these plants which have increased tolerance to biotic and abiotic stresses. Thus, increased SOD activity in our studies might be either due to increased production of ROS or be a protective mechanism by *Luffa cylindrica* plants against excessive nickel stress.

POD belongs to a large family of plant secretory enzymes catalyzing oxidoreduction between a variety of phenolic substrates and hydrogen peroxide. Various experiments of POD have been shown to be involved in hormone regulation, defence mechanisms, indoleacetic acid degradation during maturation and senescence of fruits and vegetables and lignin biosynthesis. In addition, POD may be considerer useful markers for environmental stresses since their activity is induced by heavy metals, salts and drought (Passardi et al. 2005, Yan et al. 2008). Thus, POD is believed to be one of the most important parameter of defense mechanism of plants against biotic and abiotic stresses. In the present study, it appears that tested nickel concentration significantly induces GPX activities in the cotyledons, stems and roots of *Luffa cylindrica* seedlings. In a similar way, Schickler and Caspi (1999) as well as Gajewska and Sklodowska (2005) reported that excessive nickel may significantly induced POD activities in order to enhance the activation of other antioxidant defenses and hence lead to the removal (or scavenging) of ROS. Therefore, we may propose that, in *Luffa cylindrica* seedlings, the increase in GPX activities of *Luffa cylindrica* plants under nickel stress are circumstantial evidence for tolerance mechanisms developed by this plant. Expression of POD genes is complicated since they are induced at different times, tissues and places by various kinds of biotic and abiotic stresses (Yoshida et al., 2003). Further studies should be investigated on molecular cloning and localizing specific GPX isoenzymes in order to understand gene regulation mechanism of this enzyme.

CAT, which catalyses conversion of hydrogen peroxide into water and oxygen, is the major H_2O_2-scavenging enzyme in all aerobic organisms (Willekens et al. 1995). Effects of Ni toxicity on CAT activity in different plant species and tissues have been reports. Yan et al. (2008) reported that Ni treatment resulted in a significant increase in CAT activities of plant, while the results of Madhava Rao and Sresty (2000) showed that the activity decreased significantly in pigeon pea seedlings grown at higher Ni levels. These results suggested that CAT activities in plant tissues are correlated with the tested Ni concentrations. The present results suggested that the CAT activities are remarkably increased in the cotyledons, stems and roots under excessive Ni stress except for in the roots under nickel concentrations of 800 µM, and these results are in agreement with the previous results.

PAL catalyses the first step of the phenylpropanoid pathway, leading to the synthesis of a wide variety of secondary

metabolites including flavonoids, coumarins, hydroxycinnamoyl esters and lignin. Due to the nature and defense related functions of these metabolites, the activation of PAL against abiotic and biotic stresses have been considered a part of defensive mechanism of plant (MacDonald and D'Cunha 2007, Kováčik et al. 2008). PAL, in the present study, is also induced by excessive nickel in different tissues of *Luffa cylindrica* seedlings. PAL has been shown to play an important role in the plant resistance. Studies with several different species of plants have shown that the activity of PAL is increased by excessive heavy metals stress (Kováčik and Bačkor 2007, Yan et al. 2008, Kováčik et al. 2008). The induction of PAL activity in plants is made more complex by the existence in many species of multiple PAL-encoding genes, and the levels vary depending on the stress and species of plant (MacDonald and D'Cunha 2007). The present findings suggested that the enhancement of PAL activity could be related to excessive nickel stress.

6 CONCLUSION

In conclusion, the results of this study suggested that there were significant differences in terms of responses of growth and antioxidant enzymes in *Luffa cylindrica* seedlings when exposed to Ni toxicity. Increased antioxidant enzyme activities (SOD, GPX and CAT) and PAL might be involved as part of the defenses against oxidative stress. Such a test will also be of interest to elicit what is responsible for the decreasing or increasing level of antioxidant defense systems.

7 ACKNOWLEDGEMENTS

We gratefully acknowledge the contribution and enthusiasm of our coworkers in the present studies.

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