



## Responses of growth and antioxidative enzymes to various concentrations of nickel in *Zea mays* leaves and roots

Fatemeh Ghasemi\*, Reza Heidari, Rashid Jameii and Latifeh Purakbar

Department of Biology, Faculty of Science, Urmia University, Urmia, Iran

### Abstract

To assess nickel-induced toxicity in plants, *Zea mays* seeds were germinated and cultured on nutrient solution with nickel concentrations of 50-200  $\mu\text{M}$  for a period of two weeks. Observed biological makers included biomass, soluble and total protein contents, and the activities of guaiacol peroxidase (GPX), ascorbate peroxidase (APX), catalase (CAT), and phenylalanine ammonia-lyase (PAL) in the leaves and roots of maize. The fresh and dry weight of leaves and roots increased in 50  $\mu\text{M}$  nickel but decreased in 100 and 200  $\mu\text{M}$ . Soluble and total protein contents were significantly increased by increasing nickel concentrations up to 200  $\mu\text{M}$  nickel in both roots and leaves of maize. Significant increases of ascorbate peroxidase (the highest activity at 200  $\mu\text{M}$  nickel), catalase (the highest activity at 50  $\mu\text{M}$  nickel), and phenylalanine ammonia-lyase (the highest activity at 100  $\mu\text{M}$  nickel) were observed in the leaves and roots of *Zea mays* seedlings at all tested nickel concentrations. Guaiacol peroxidase activity was decreased in the leaves and roots of *Zea mays* seedlings exposed to different levels of nickel. The present results suggested that treatment with different levels of nickel may enhance the antioxidant activities in the leaves and roots of *Zea mays* seedlings, thus alleviate Ni-induced oxidative damage and enhance Ni tolerance.

**Keywords:** *Zea mays*; defensive mechanism; nickel toxicity; antioxidant activity

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### Introduction

Nickel (Ni) is an essential micronutrient for plants since it is the active center of the urease enzyme required for nitrogen metabolism in higher plants (Yan et al., 2008). However, excess Ni is known to be toxic and many studies have been conducted concerning Ni toxicity of various plant species (Yan et al., 2008). The most common symptoms of nickel toxicity in plants are

inhibition of growth, photosynthesis, mineral nutrition, sugar transport and water relations (Seregin and Kozhevnikova, 2006).  $\text{Ni}^{2+}$  is a transition metal that, except in ultramafic or serpentinitic soils, is found in natural soils at trace concentrations (Welch, 1981). However, the  $\text{Ni}^{2+}$  concentration is increased in certain areas by human activities such as mining works, emission of smelters, burning of coal and oil, sewage, phosphate fertilizers and pesticides (Rahman Khan and Mahmud Khan, 2010). During the period of metal treatment, plants develop different resistance mechanisms to avoid or tolerate metal stress, including the changes of lipid composition, the profiles of isozymes and

\*Corresponding author

E-mail address: f.ghasemi@urmia.ac.ir

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enzyme activity, sugar or amino acid contents, and the level of soluble proteins and gene expressions. These adaptations entail qualitative and/or quantitative metabolic changes that often provide a competitive advantage, and affect plant survival (Schützendübel and Polle, 2002). Therefore, plant cells contain protective and repair systems that, under normal circumstances, minimize the occurrence of oxidative damage. It is known that excessive heavy metal exposure may increase the generation of reactive oxygen species (ROS) in plants, and oxidative stress would arise if the balance between ROS generation and removal were broken (Foyer et al., 1997). Oxidative stress is a part of general stress that arises when an organism experiences different external or internal factors changing its homeostasis. (Mittler, 2002). Plants possess a number of antioxidant molecules and enzymes that protect them against oxidative damage. Superoxide dismutase (SOD), the first enzyme in the detoxifying process, converts  $O_2^-$  radicals to  $H_2O_2$  at a very fast rate (Polle and Rennenberg, 1994; Schickler and Caspi, 1999; Gajewska and Skłodowska, 2007). In the ascorbate-glutathione cycle, the enzymatic action of ascorbate peroxidase (APX) reduces  $H_2O_2$  using ascorbate as an electron donor. Oxidized ascorbate is then reduced by reduced glutathione (GSH), generated from oxidized glutathione (GSSG) by glutathione reductase (GR) at the expense of NADPH. GR also plays an essential role in protecting against oxidative damage by maintaining a high GSH:GSSG ratio (Foyer et al., 1997). Therefore, it is imperative to compare the ROS-scavenging enzyme activity among plant genotypes with different Ni tolerance, as it will tell us the intrinsic role of antioxidant enzymes against Ni tolerance.

*Zea mays* is cultivated as an oil plant in many Middle Eastern countries. There is little information concerning the antioxidant defense responses of maize root and shoot to Ni treatment. Thus, the aim of this work was to perform a comprehensive investigation on the impacts of different levels of nickel on the responses of growth and antioxidative enzymes, with emphasis on the changes of CAT, APX, GPX and PAL.

## Materials and Methods

Maize (*Zea mays* cv. 704) seeds were sterilized with 10% 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 27 °C under dark condition. Three days following germination, individual seedlings transferred to 600-ml beakers containing 500 ml of aerated half-strength Hoagland solution (Hoagland and Arnon, 1950). Full concentration nutrient solution with different concentration of nickel chloride (0, 50, 100 and 200  $\mu$ M) were applied after establishing the seedlings. The experiments were arranged in a completely randomized design with three replicates and each replicate contained four seedlings. The pH was adjusted to 5.8 and solutions changed three times a week. Plants were maintained in controlled-environment chambers with a 16/8 hr light cycle, a day/night temperature cycle of 26/22 °C, and 50% RH. Fifteen days after treatment, seedlings were harvested and the shoot and roots were separated and stored at -80 °C until used.

## Growth parameters

Length and fresh weight (FW) of shoot and root were measured immediately after harvesting of the maize plants. For the determination of their dry weight, they were wrapped in paper bags and dried in an oven at 70 °C to a constant dry weight.

## Determination of soluble and total protein

Fresh leaves and roots of seedlings (0.5 g) were homogenized using a chilled pestle and mortar, and then were extracted in 50 mM sodium phosphate buffer (pH 7.0) including 1 mM EDTA and 150 mM NaCl. The crude extract was centrifuged at 12,000 rpm for 5 min and the supernatant was used to determine protein content. Protein content was measured by the Lowry method (Lowry et al., 1951).

## Enzyme extraction and estimation

All biochemical analyses were performed at 4 °C. The fresh leaves and roots of seedlings (0.5 g) were homogenized using a chilled pestle and mortar, and then were extracted in 3 ml of 0/05 M Tris - HCl buffer (pH 7.5) including 1 mM EDTA and 3 mM MgCl<sub>2</sub>. Extraction buffer for assaying ascorbate peroxidase activity contained 0.2 mM ascorbate. The crude extract was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was used for assaying of enzyme activities.

### Assay of catalase (CAT) activity

The activity of CAT was measured following the modified method of Montavon et al. (2007). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 30 mM H<sub>2</sub>O<sub>2</sub> and enzyme extract. The decomposition of H<sub>2</sub>O<sub>2</sub> was followed by measuring the decrease in absorbance at 240 nm. The activity was expressed in U/mg protein.

### 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<sub>2</sub>O<sub>2</sub>. 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/mg protein.

### Assay of ascorbate peroxidase activity

APX activity was assayed following the oxidation of ascorbate to dehydroascorbate at 290 nm by the modified method of Nakano and Asada (1981). The assay mixture consisted of 50 mM sodium phosphate buffer pH 7.0 containing 1 mM EDTA, 1 mM sodium ascorbate, 10 mM H<sub>2</sub>O<sub>2</sub> and enzyme extract. Addition of H<sub>2</sub>O<sub>2</sub> started the

reaction. Rates were corrected for the non-enzymatic oxidation of ascorbate by the inclusion of reaction mixture without enzyme extract. The activity was expressed in U/mg protein.

### Enzyme extraction and phenylalanine ammonia-lyase (PAL) activity assay

For PAL assay, shoot and roots tissues were ground in ice-cold 0.1 M Tris-HCl buffer pH 8.8 containing 1% polyvinylpyrrolidone 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/mg protein.

### Statistical analysis

Data are reported as the mean ± SD. Three independent experiments for each condition were performed. Student's t-test was used as the statistical tools for the study and the difference between means were considered to be significant when the P value was less than 0.05.

### Results

Figs. I, II, and III show changes of the length, fresh and dry weight of the shoot and roots obtained from all the experimental seedlings, respectively. As Fig. I suggests, there was significant decrease in the length of the shoot and roots exposed to nickel treatments compared to the control.

There was significant increase in the fresh weight of the shoot and roots exposed to 50 and 100 µM nickel, but it was decreased in 200 µM nickel compared to the control (Fig. II). As Fig. III shows, there was a significant decrease in the dry weight of the shoot and roots exposed to nickel

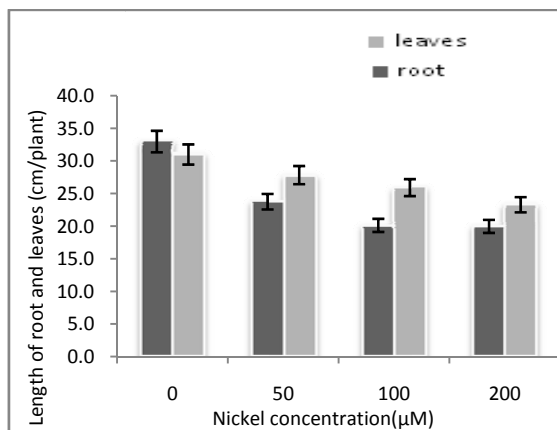


Fig. I. Effects of different concentrations of nickel on the length of the shoots and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

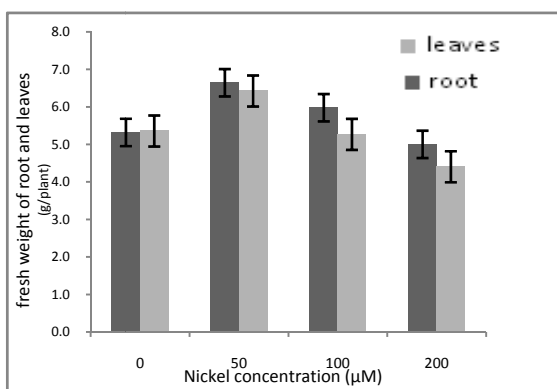


Fig. II. Effects of different concentrations of nickel on the fresh weight of the shoot and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

treatments compared to the control, especially in 200 μM nickel.

The soluble protein contents of the leaves and roots of *Zea mays* seedlings increased with increasing nickel concentrations up to 100 μM nickel and then decreased in 200 μM nickel (Fig. IV). The total protein contents of the leaves and roots of *Zea mays* seedlings increased constantly with increasing nickel concentrations (Fig. V).

As shown in Figs. VI, VII and IX, the activities of CAT, APX and PAL in the leaves and roots increased with increasing nickel concentrations, but GPX activity decreased (Fig. VIII).

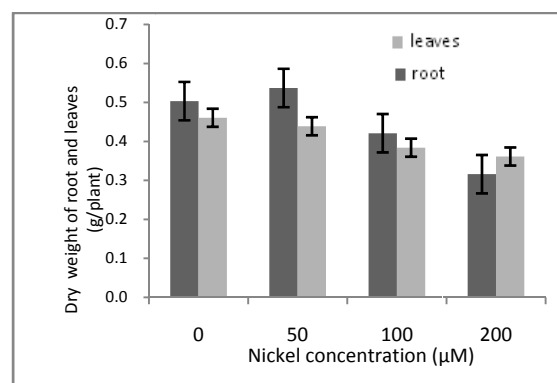


Fig. III: Effects of different concentrations of nickel on the dry weight of the shoots and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

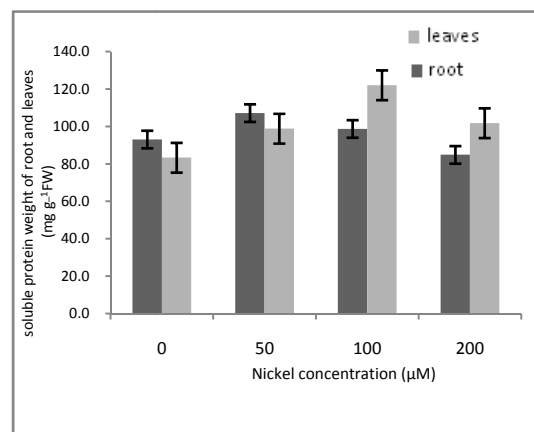


Fig. IV. Effects of different concentrations of nickel on the soluble protein content of the leaves and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

## 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 our experiment decreases in length, fresh and dry weight of the leaves and roots was found after treatment of maize seedlings with Ni. Reduction in elongation

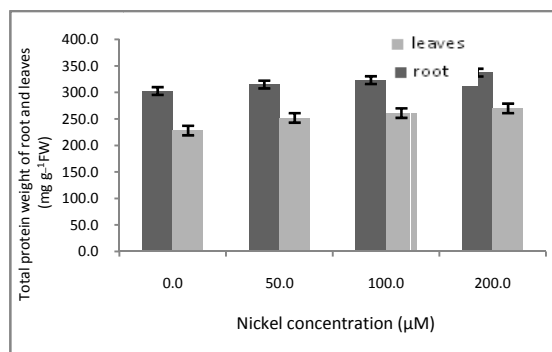


Fig. V. Effects of different concentrations of nickel on the total protein content of the leaves and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

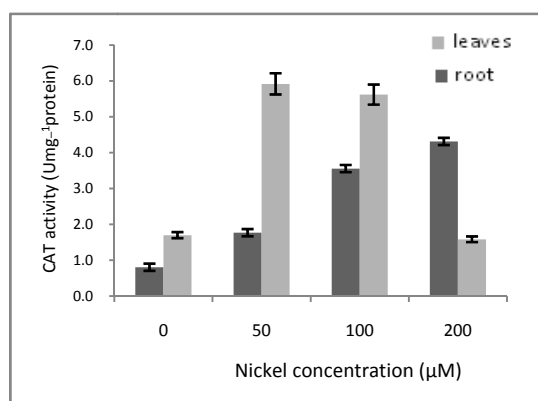


Fig. VI. Effects of different concentrations of nickel on CAT activity of the leaves and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

may be associated with the intensification of cell wall strengthening by lignification, which was reported in heavy metal-stressed plants (Díaz et al., 2001). Decrease in fresh weight (excepted increase in 50  $\mu$ M nickel because of increasing water content to cope with primarily Ni toxicity) may be partly due to the metal-induced decline in tissue water content (Gajewska et al., 2006). Apart from restriction of growth Ni application led to the appearance of chlorosis and necrosis on the wheat leaves. These symptoms were observed mainly on the first leaves and only occasionally occurred on the newly developed ones. This may indicate that in the above ground part of the seedling Ni was accumulated mostly in the oldest leaves, which may function as metal sinks and therefore protect younger leaves against toxic effect of Ni. However, analysis of Ni content separately in the first and second leaves

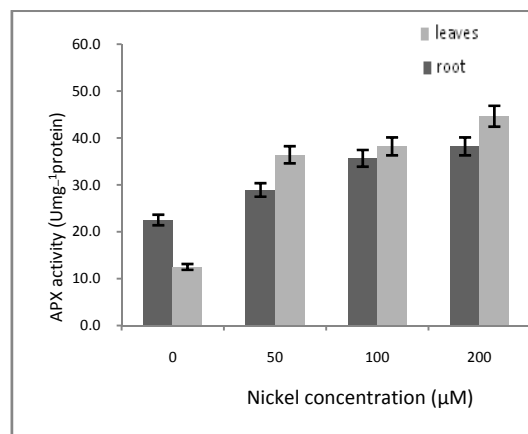


Fig. VII. Effects of different concentrations of nickel on APX activity of the leaves and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

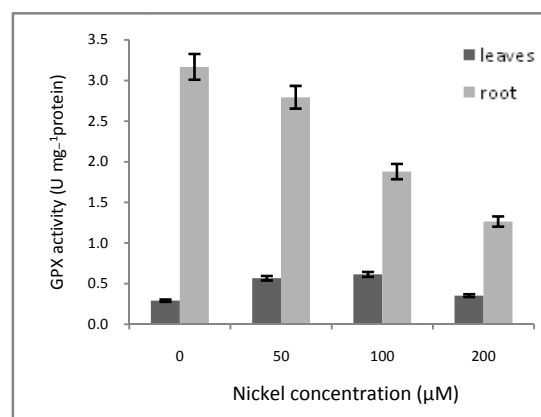


Fig. VIII. Effects of different concentrations of nickel on GPX activity of the leaves and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

is necessary to prove this suggestion. Similar mechanism has been described for plants subjected to salt stress (Hasegawa et al., 2000).

The results showed that a negative effect on seedling growth at the tested Ni concentration was observed. Similar decrease in the biomass has 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.

Soluble proteins were significantly increased by increasing nickel concentrations in both roots and leaves of maize. Biotic stress may induce the synthesis of some proteins and inhibit others (Ericson and Alfinito, 1984). The decrease

in protein content in 200  $\mu\text{M}$  nickel may be caused by enhanced protein degradation as a result of increased protease activity under stress conditions (Palma et al., 2002). Also, these heavy metals may have induced lipid peroxidation and fragmentation of proteins due to the toxic effects of reactive oxygen species leading to a reduction in protein content (Davies et al., 1987). Such inhibitory effects of high levels of Ni have been reported to be the result of inhibition of protein synthesis and changes in carbohydrate metabolism (Bishnoi et al., 1993; Lin and Kao, 2006; Maheshwari and Dubey, 2007).

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. Reactive oxygen species (ROS) such as  $\text{O}^{-2}$  and  $\text{H}_2\text{O}_2$  are continuously generated in plant tissues as by-products of several metabolic processes. To cope with ROS plant cells possess an antioxidative system consisting of both enzymatic and nonenzymatic antioxidants. Superoxide dismutase (SOD) catalyzes disproportionation of  $\text{O}^{-2}$  to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$ . Influencing the concentrations of these two Haber–Weiss reaction substrates, SOD is considered to be the first line of defense against ROS. Catalase (CAT) and ascorbate peroxidase (APX) are responsible for the scavenging of  $\text{H}_2\text{O}_2$ . CAT converts  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$  and APX catalyzes the reduction of  $\text{H}_2\text{O}_2$  using ascorbate as an electron donor. Other peroxidases, including guaiacol peroxidase (GPX) are also involved in  $\text{H}_2\text{O}_2$  elimination. (Gajewska et al. 2006).

In the present study, it appears that tested nickel concentration significantly induces GPX activities in the leaves of *Zea mays* seedlings. In a similar way, Schickler and Caspi (1999) as well as Gajewska and Sklodowska (2005) reported that excessive nickel may significantly induced peroxidases 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

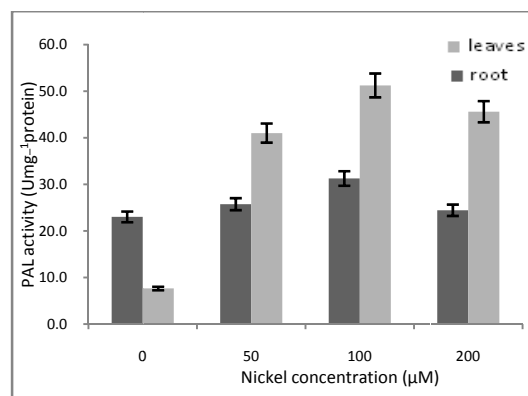


Fig. IX. Effects of different concentrations of nickel on PAL activity of the leaves and roots of *Zea mays* seedlings. Data points and error bars represent means  $\pm$  S.D.

that, in *Zea mays* seedlings, the increase in GPX activities under nickel stress are circumstantial evidence for tolerance mechanisms developed by this plant. Expression of peroxidases 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  $\text{H}_2\text{O}_2$ -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 shoot and roots under excessive Ni stress except for in the roots under nickel concentrations of 200  $\mu\text{M}$ , 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). PAL, in the present study, is also induced by excessive nickel in different tissues of *Zea mays* 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 (Yan 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.

In the present work the highest value of APX activity detected in leavers and roots of maize, which suggests that APX might play an important role in the removal of H<sub>2</sub>O<sub>2</sub> from cells in the leaves and roots of Ni-stressed maize. Contrary to APX and CAT, GPX activity in the maize roots decreased in response to Ni treatment, which is in accordance with the findings for the leaves of cabbage (Pandey and Sharma, 2002) and pigeonpea (Madhava Rao and Sresty, 2000). Nevertheless, induction of this enzyme activity has been also reported in Ni-exposed plants (Baccouch et al., 1998). Discrepancies between data concerning the response of plant antioxidative enzymes to Ni stress may be explained by differences in plant tolerance to this metal toxicity, varying experimental conditions as well as by various Ni concentrations used in experiments. It has been suggested that non redox active metals, including Ni, may induce oxidative stress indirectly, by decreasing the efficiency of antioxidative system (Helmy Latif, 2010).

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