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Effect of nickel on ROS content and antioxidative enzyme activities in wheat leaves

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Abstract

Influence of 100 μM Ni on growth, Ni accumulation, $O_2^{\cdot-}$, H_2O_2 and lipid peroxides contents as well as the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD) and glutathione peroxidase (GSH-Px) were studied in the leaves of wheat plants on the 3rd, 6th and 9th days after treatment. Exposure of the plants to Ni for only 3 days led to almost 200-fold increase in this metal concentration in the leaf tissue but later the rate of Ni accumulation was much slower. Length and fresh weight of the leaves were substantially reduced, up to 25% and 39%, respectively at the end of experiment. Visible symptoms of Ni toxicity: chlorosis and necrosis were observed following the 3rd day. Treatment with Ni resulted in the increase in $O_2^{\cdot-}$ and H_2O_2 contents in the leaves. Both showed their highest values, approximately 250% of those of the control, on the 3rd day and then their levels decreased but still markedly exceeded the control values. SOD and CAT activities decreased significantly in response to Ni treatment, however a several-fold increase in APX and POD activities was found. No significant changes in lipid peroxides content were observed in the leaves after Ni application. The activity of GSH-Px showed a 29% induction on the 3rd day. Our results indicated that despite prolonged increases in $O_2^{\cdot-}$ and H_2O_2 levels, oxidative damage, measured as the level of lipid peroxidation, did not occur in the leaves of Ni-treated wheat.

Abbreviations: APX – ascorbate peroxidase; CAT – catalase; GSH – reduced glutathione; GSH-Px – glutathione peroxidase; MBTH – 3-methyl-2-benzothiazolinone hydrazone; NBT – nitro blue tetrazolium; POD – guaiacol peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase; TBARS – thiobarbituric acid reacting substances

Introduction

Excess concentrations of nickel are known to have a detrimental impact on plant growth. It has been reported that Ni is able to disturb several physiological processes such as photosynthesis, transport of photoassimilates, mineral nutrition and tissue water status (Samarakoon & Rauser 1979; Krupa *et al.* 1993; Pandey & Sharma 2002; Parida *et al.* 2003). Toxic effects of Ni are manifested by various symptoms of injury, including inhibition of growth, chlorosis, necrosis and wilting (Madhava Rao & Sresty 2000; Pandey & Sharma 2002). Although influence of heavy metals on plants has been extensively studied, the mechanisms of toxic action of Ni still remain poorly understood. However, a growing body of evidence indicates that Ni phytotoxicity may be attributed, at least in part, to oxidative stress (Baccouch *et al.* 2001; Gonnelli *et al.* 2001).

Reactive oxygen species (ROS) such as $O_2^{\cdot-}$ and H_2O_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 non-enzymatic antioxidants. Superoxide dismutase (SOD, EC 1.15.1.1) catalyzes disproportionation of $O_2^{\cdot-}$ to H_2O_2 and 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, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) are responsible for the scavenging of H_2O_2. CAT converts H_2O_2 to H_2O and O_2 and APX catalyzes the reduction of H_2O_2 using ascorbate as an electron donor. Other peroxidases, including guaiacol peroxidase (POD, EC 1.11.1.7) are also involved in H_2O_2 elimination. POD catalyzes oxidation of many phenolic compounds at the expense of H_2O_2 and is considered to be a key enzyme in biosynthesis of lignin (Gaspar *et al*. 1999).

Under stress conditions, including exposure to excess concentrations of heavy metals, an imbalance between generation and removal of ROS can arise in plant tissues (Gratão *et al*. 2005). This may subsequently lead to oxidative injuries of many important macromolecules like lipids, proteins and nucleic acids (Kehrer 2000). The most common indicator of oxidative stress is lipid peroxidation resulting in disturbances in the membrane integrity and consequently in its enhanced permeability. Peroxidation of lipids is believed to be an important factor of growth inhibition in plants subjected to heavy metals, including Ni (Baccouch *et al*. 2001).

Glutathione peroxidase (GSH-Px, EC 1.11.1.9) catalyzes the reduction of organic hydroperoxides and H_2O_2, to the corresponding alcohols and H_2O, respectively, using reduced glutathione (GSH) as an electron donor. GSH-Px is considered to play an important role in the elimination of lipid peroxides (Edwards 1996). Changes in this enzyme protein or activity were found in plants subjected to both biotic and abiotic stresses (Faltin *et al*. 1998; Roeckel-Drevet *et al*. 1998) but there is relatively little information about the participation of GSH-Px in the response of plants to heavy metal toxicity.

In the previous studies concerning Ni-induced oxidative stress attention was paid mainly to the antioxidative system and lipid peroxidation. The ratio of ROS production has usually been concluded indirectly, on the basis of the changes in the activity of antioxidative enzymes. Literature data concerning influence of Ni on the activity of antioxidative enzymes in leaves are contradictory, since both their induction and inhibition has been found. To our knowledge there are no reports on the effect of Ni on ROS level in aboveground parts of plants.

The purpose of the present work was to contribute to a better understanding of the phytotoxic effects of Ni in relation to oxidative stress. Our study was focused on the possible ability of Ni to enhance generation of ROS and to induce oxidative damage in plants. Our investigation was undertaken also to examine the influence of Ni on the activities of antioxidative enzymes. Contents of $O_2^{\cdot-}$ and H_2O_2, activities of SOD, CAT, APX, POD and GSH-Px as well as lipid peroxidation were studied in the leaves of wheat plants subjected to Ni stress. In parallel, Ni uptake and its effect on growth parameters were monitored.

Materials and methods

Plant material

Wheat (*Triticum aestivum* L. cv. 'Zyta') seeds (supplied by Hodowla Roślin Strzelce Sp. z o.o., Poland) were sown (20 per pot) in polyethylene pots filled with perlite (volume 300 cm^3). The seedlings were grown in a controlled climate room at 24 °C with 16/8 h light/dark photoperiod and light intensity of 175 $\mu mol\ m^{-2}\ s^{-1}$. They were regularly watered with half-strength Hoagland's solution (Hoagland & Arnon 1950). Four days after sowing 30 cm^3 of a $NiSO_4$ solution was applied to the perlite at the final concentration of 100 μM. Plants treated with distilled water were referred to as control. Samples of the leaves were taken on 3rd, 6th and 9th days after treatment and used for analyses.

Growth parameters, Ni and protein concentrations

Growth parameters: length and fresh weight (FW) of the leaves were measured immediately after harvesting of the wheat plants on 3rd, 6th and 9th days after Ni application. Nickel concentration in the leaves was determined by atomic absorption

spectrometry following wet digestion of oven dried tissue in HNO_3:$HClO_4$ (4:1, v/v) solution at 140 °C. Ni content in the tissue was expressed in μgrams per gram dry weight (DW). Total soluble protein in the leaves was determined by the method of Bradford (1976). Leaves were homogenized (1:10 w/v) in an ice cold mortar using the same buffer as for enzyme extraction (50 mM sodium phosphate buffer pH 7.0 containing 0.5 M NaCl, 1 mM EDTA and 1 mM sodium ascorbate). After centrifugation (20,000 × *g*, 20 min) the supernatant was used for the estimation of protein content. 0.05 cm^3 of supernatant was added to 2.5 cm^3 of Bradford's reagent and after 5 min the absorbance was measured at 595 nm. Protein content in the sample was calculated by referring to a standard curve for bovine serum albumin and was expressed in milligrams per gram fresh weight.

$O_2^{\cdot-}$ and H_2O_2 contents

The level of $O_2^{\cdot-}$ was assayed spectrophotometrically by measuring the reduction of exogenously supplied nitroblue tetrazolium (NBT) according to Doke (1983). Ten leaf discs (diameter 3 mm) were immersed in 2 cm^3 of the mixture containing 0.01 M sodium phosphate buffer pH 7.8, 0.05% NBT and 10 mM NaN_3 in a beaker (volume of 25 cm^3). After 60 min of incubation 1.5 cm^3 of the reaction solution was transferred into a test tube and heated at 85 °C for 15 min. Then the solution was cooled and its absorbance at 580 nm was measured. NBT reducing activity (indicating $O_2^{\cdot-}$ generation) was expressed as the increase in A_{580} per hour per gram fresh weight.

For determination of H_2O_2 the leaves (500 mg) were homogenized in an ice cold mortar with 2.5 cm^3 of 5% TCA and 50 mg of activated charcoal (Aroca *et al*. 2003). The homogenate was filtered and centrifuged (20,000 × *g*, 20 min) and the obtained supernatant was adjusted to pH 3.5 with 4 N KOH. H_2O_2 concentration was measured according to the method of Capaldi and Taylor (1983) based on the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with its formaldehyde azine by H_2O_2 in the presence of horseradish peroxidase which results in the formation of a tetraazapentamethine dye. The sample (0.2 cm^3) was added to 0.1 cm^3 of reagent solution containing 3.4 mM MBTH and 3.32 mM formaldehyde. The reaction was initiated by adding 0.5 cm^3 of the solution of horseradish peroxidase (0.5 U) in 0.2 M sodium acetate buffer pH 3.5 and after 2 min was quenched with 1.4 cm^3 of 1 N HCl. The absorbance at 630 nm was measured 15 min after quenching. H_2O_2 content was estimated by referring to a standard curve drawn with known concentrations of H_2O_2 and was expressed in nmoles per gram fresh weight.

Enzyme extraction and assays

The leaves were homogenized (1:10 w/v) in an ice cold mortar using 50 mM sodium phosphate buffer pH 7.0 containing 0.5 M NaCl, 1 mM EDTA and 1 mM sodium ascorbate. After centrifugation (20,000 × *g*, 20 min) the supernatant was used for the determination of SOD, CAT, APX, POD and GSH-Px activities.

Total SOD activity was assayed according to the method of Minami and Yoshikawa (1979) based on pyrogallol autooxidation. The reaction mixture consisted of 50 mM Tris–cacodylic buffer (sodium salt) pH 8.2, 0.1 mM EDTA, 1.4% Triton X-100, 0.055 μM NBT, enzyme extract (approximately 20 μg protein) and 16 μM pyrogallol, which started the reaction. The reaction mixture was incubated in 37 °C, for 5 min. The reaction was stopped by addition of 3.5 cm^3 of the mixture consisting of 0.35 M formic buffer pH 3.5, 0.6% Triton X-100 and 3.5% formaldehyde and absorbance was measured at 540 nm. According to McCord and Fridovich (1969) one unit of SOD activity was defined as the amount of enzyme that causes inhibition of NBT reduction by 50%. The enzyme activity was expressed in units per minute per mg protein. APX activity was assayed following the oxidation of ascorbate to dehydroascorbate at 265 nm (ε = 13.7 $mM^{-1}cm^{-1}$) 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, 0.25 mM sodium ascorbate, 25 μM H_2O_2 and enzyme extract (10–30 μg protein). Addition of H_2O_2 started the reaction. Rates were corrected for the non-enzymatic oxidation of ascorbate by the inclusion of reaction mixture without enzyme extract. The enzyme activity was expressed in units, each representing 1 μmole of ascorbate oxidized per minute per mg protein. CAT activity was measured according to Dhindsa *et al*. (1981). The assay mixture contained 50 mM sodium

phosphate buffer pH 7.0, 15 mM H_2O_2 and enzyme extract (approximately 5 μg protein). Decomposition of H_2O_2 (ε = 45.2 M^{-1} cm^{-1}) was measured at 240 nm. The enzyme activity was expressed in units, each representing 1 μmole of H_2O_2 decomposed per minute per mg protein. POD activity was measured by the method of Maehly and Chance (1954). The assay mixture contained 50 mM sodium acetate buffer pH 5.6, 5.4 mM guaiacol, 15 mM H_2O_2 and enzyme extract (approximately 5 μg protein). The increase in absorbance due to the oxidation of guaiacol to tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}) was monitored at 470 nm. The enzyme activity was expressed in units, each representing 1 μmole of tetraguaiacol formed per minute per mg protein. GSH-Px activity was measured by the method of Hopkins and Tudhope (1973) based on the continuous regeneration of oxidized glutathione produced by the action of GSH-Px. This regeneration is catalyzed by glutathione reductase at the expense of NADPH, both added to the assay mixture. The assay mixture contained 50 mM potassium phosphate buffer pH 7.0, 2 mM EDTA, 150 mM GSH, 4.2 mM NADPH, 0.5 unit of glutatione reductase (from baker's yeast) and 2.2 mM *t*-butyl peroxide, which started the reaction. The oxidation of NADPH was followed spectrophotometrically by measuring the decrease in absorbance at 340 nm (ε = 6.22 mM^{-1} cm^{-1}). The enzyme activity was expressed in units, each representing 1 nmole of NADPH oxidized per minute per mg protein.

Lipid peroxidation

The quantity of lipid peroxidation products in the samples was expressed as thiobarbituric acid-reacting substances (TBARS) according to the modified method of Yagi (1976). The leaves were homogenized (1:10 w/v) in a mortar with 50 mM sodium phosphate buffer pH 7.0. The obtained homogenate (1 cm^3) was mixed with 1 cm^3 of TBA solution (29 mM TBA in 8.75 M acetic acid) and heated at 95 °C for 1 h. After cooling 3.5 cm^3 of *n*-butanol was added and the tubes were vigorously shaken. After centrifugation (10,000 × *g*, 10 min) the fluorescence of the resulting organic layer was measured at 531 nm (excitation) and 553 nm (emission). The concentration of TBARS was estimated by referring to a standard 1,1,3,3-tetraetoxypropane. The level of lipid peroxides was expressed in nmoles of TBARS per g fresh weight.

Statistical analysis

The results presented are the means of five independent experiments. For biochemical parameters a single sample was analyzed per treatment in each experiment. In each experiment sample for analysis was taken from 15 seedlings. From each seedling all developed leaves were harvested: one leaf on the 3rd day and two leaves on days 6th and 9th. For growth parameters four seedlings were analyzed per treatment in each experiment, and therefore, the data represent the means of 20 observations. Sample variability is given as the standard deviation of the mean. The significance of differences between control and treatment mean values was determined by Student's *t*-test. Differences at $p < 0.05$ were considered significant.

Results

Ni accumulation, growth parameters and protein content

Treatment of wheat seedlings with Ni resulted in a significant accumulation of this metal in the leaves (Table 1). Already 3 days after exposure Ni concentration in the leaf tissue was almost 200 times higher ($p < 0.001$) in comparison with the control and it only slightly increased with time.

Biomass production and elongation of the wheat leaves were substantially reduced in response to Ni application (Table 1). As early as 3 days after treatment length and fresh weight were 13% ($p < 0.01$) and 26% ($p < 0.001$) lower than in the control, respectively. At the end of experiment the above parameters were reduced by 25% ($p < 0.001$) and 39% ($p < 0.001$), respectively.

A slight increase in total protein content in the leaves of Ni-stressed seedlings was found, however it was not statistically significant (Table 1).

Besides decrease in growth exposure of the wheat seedlings to Ni resulted in the appearance of characteristic symptoms of this metal toxicity. Following the 3rd day after Ni treatment chlorosis and necrosis manifested themselves as colorless

Table 1. Effect of Ni treatment on leaf Ni accumulation, protein content, length and fresh weight of wheat leaves

Time after treatment (days)	Treatment	Ni content (μg g^{-1} DW)	Shoot length (mm)	Shoot fresh weight (mg)	Protein content (mg g^{-1} FW)
3	Control	0.432 ± 0.052	111.4 ± 10.3	96.4 ± 15.2	14.00 ± 1.24
	100 μM Ni	85.75 ± 12.01***	99.6 ± 11.95**	71.4 ± 9.3***	15.15 ± 1.65^{ns}
6	Control	0.437 ± 0.048	171.3 ± 16.4	149.1 ± 21.1	11.93 ± 2.58
	100 μM Ni	99.46 ± 11.89***	136.9 ± 17.4***	98.4 ± 9.7***	13.69 ± 2.43^{ns}
9	Control	0.502 ± 0.070	200.3 ± 16.6	185.2 ± 23.8	9.76 ± 1.25
	100 μM Ni	126.83 ± 18.52***	150.2 ± 18.7***	112.9 ± 14.2***	11.51 ± 1.64^{ns}

Data are means of five independent experiments ± SD. For Ni and protein contents a single sample was analyzed per each experiment ($n = 5$). For growth parameters four seedlings were analyzed per each experiment ($n = 20$). **, *** = significantly different from the control at $p < 0.01$ and $p < 0.001$, respectively and ns = significantly not different from the control at $p < 0.05$.

longitudinal stripes observed mainly on the first leaves.

$O_2^{\cdot-}$ and H_2O_2 contents

Exposure of the wheat seedlings to Ni caused a significant induction of $O_2^{\cdot-}$ generation in the leaf tissue (Fig. 1A). After 3 days the ratio of $O_2^{\cdot-}$ production measured by means of NBT reduction was 2.5 times higher ($p < 0.001$) than in the control. Following the 6th day $O_2^{\cdot-}$ level in the leaves of treated plants was lower, but it still substantially exceeded the control value. On the 6th and 9th days it was increased by 80% ($p < 0.01$) and 94% ($p < 0.01$), respectively. H_2O_2 concentration also increased over 2.5-fold ($p < 0.001$) as early as 3 days after Ni treatment (Fig. 2B). Later, the content of H_2O_2 in the leaves of Ni-stressed wheat decreased and was only 63% ($p < 0.001$) and 46% ($p < 0.01$) higher in comparison with the control, after 6 and 9 days, respectively.

Enzyme activities and lipid peroxidation

SOD activity in the leaves was reduced in response to Ni application (Fig. 2A). Inhibition of this enzyme activity, approximately 25% ($p < 0.05$) below the control level was observed throughout the experiment. The activity of CAT also decreased in the leaves of Ni-treated wheat (Fig. 2B). Significant reduction in this enzyme activity, by 16% ($p < 0.05$) and 20% ($p < 0.01$) was noticed on the 3rd and 6th days, respectively (Fig. 2B). In contrast, treatment of wheat plants with Ni led to the enhancement of APX activity, beginning from the 3rd day, when its almost 2.7-fold ($p < 0.001$) increase was observed (Fig. 3A). The most marked, 4.4-fold induction of this enzyme activity was found on the 6th day. At the end of experiment APX activity in the leaves of Ni-exposed seedlings still remained 2 times ($p < 0.001$) higher than in the control. The most pronounced induction in response of wheat leaves to Ni stress was observed in the case of POD (Fig. 3B), whose activity increased by 447% ($p < 0.001$), 458% ($p < 0.001$) and 261% ($p < 0.001$) on the 3rd, 6th and 9th days, respectively. No significant changes in TBARS content were found in the wheat leaves after Ni treatment (Fig. 4A). However, the metal application resulted in a slight enhancement of GSH-Px activity (Fig. 4B). A statistically significant increase in this enzyme activity, 29% ($p < 0.05$) over the control level, was noticed on the 3rd day.

Discussion

Exposure of the wheat seedlings to 100 μM Ni caused a rapid increase in this metal content in the leaves. Most of Ni detected in the leaf tissue accumulated up to the 3rd day after application, which is in line with the reports on the fast translocation of Ni ions from roots to the aboveground parts (Zeller & Feller 1999). In our experiment a decrease in both length and fresh weight of the leaves was found after treatment of wheat seedlings with Ni. Reduction in elongation 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 may be partly due to the metal-induced decline in tissue water content, found in our previous experiment (Gajewska

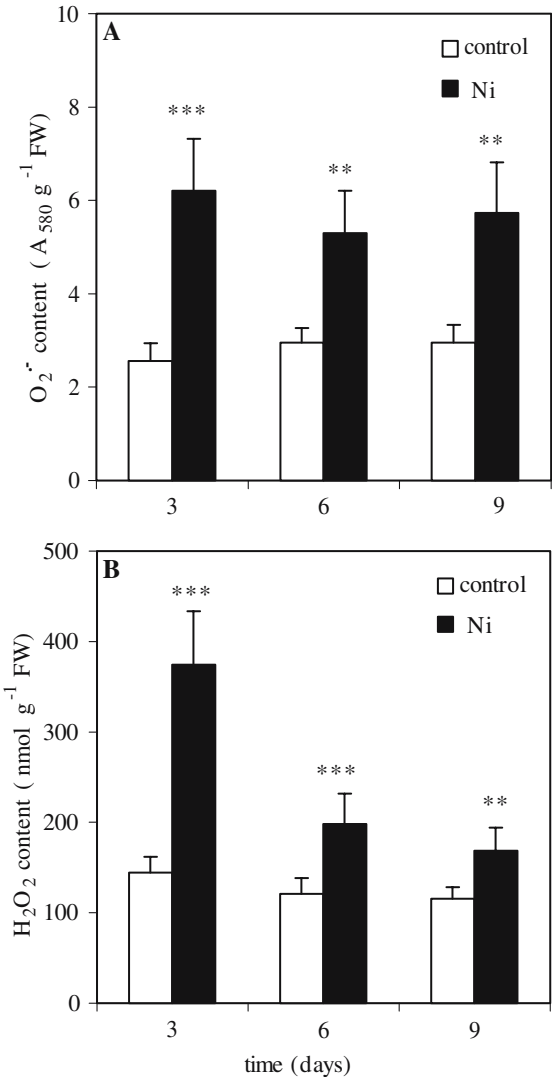

Figure 1. Superoxide anion (A) and hydrogen peroxide (B) contents in leaves of wheat plants treated with Ni. A single sample was analyzed per each of five independent experiments (n = 5). Bars represent SD of means. **, *** = significantly different from the control at $p < 0.01$ and $p < 0.001$, respectively.

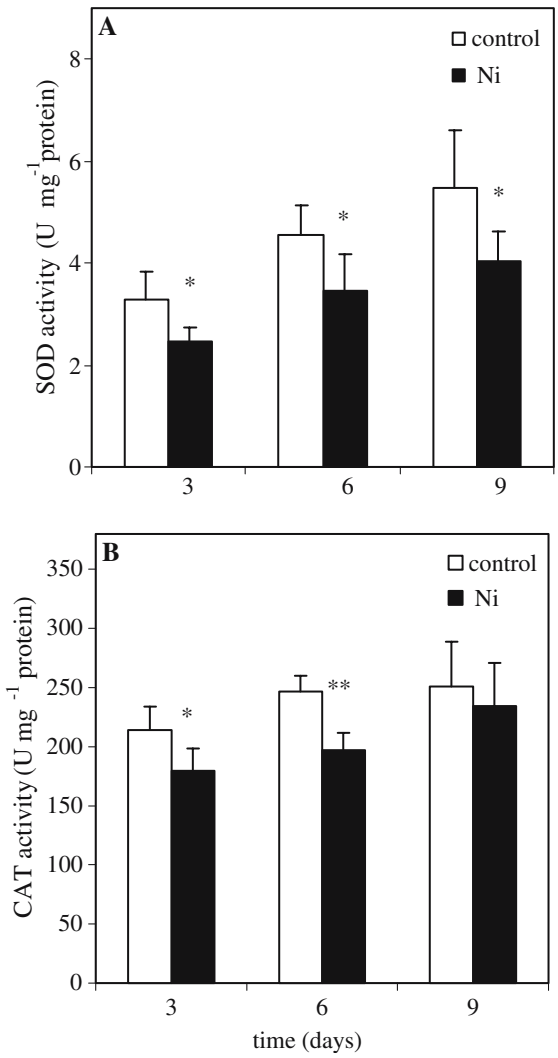

Figure 2. Superoxide dismutase (A) and catalase (B) activities in leaves of wheat plants treated with Ni. A single sample was analyzed per each of five independent experiments (n = 5). Bars represent SD of means. *, ** = significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively.

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

Heavy metals, similarly to other stress factors, have been reported to induce overproduction of ROS in plant tissues (Gratão *et al.* 2005). Increased generation of ROS has been found in plants in response to treatment with both redox active (Chen *et al.* 2000; Drążkiewicz *et al.* 2004) and non redox active metals (Weckx & Clijsters 1997; Shah *et al.* 2001; Pandey *et al.* 2005). There

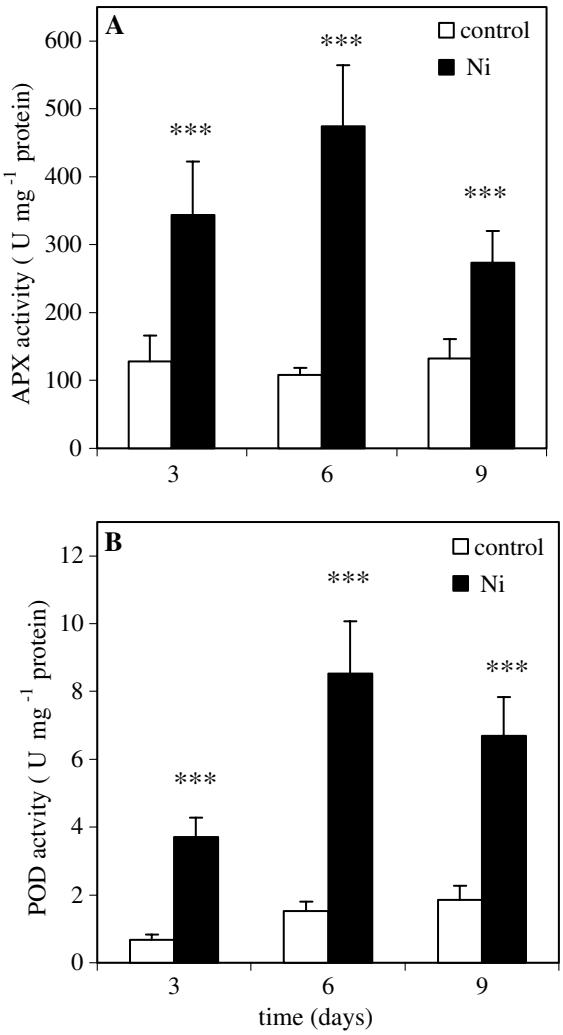

Figure 3. Ascorbate peroxidase (A) and guaiacol peroxidase (B) activities in leaves of wheat plants treated with Ni. A single sample was analyzed per each of five independent experiments (n = 5). Bars represent SD of means. *** = significantly different from the control at $p < 0.001$.

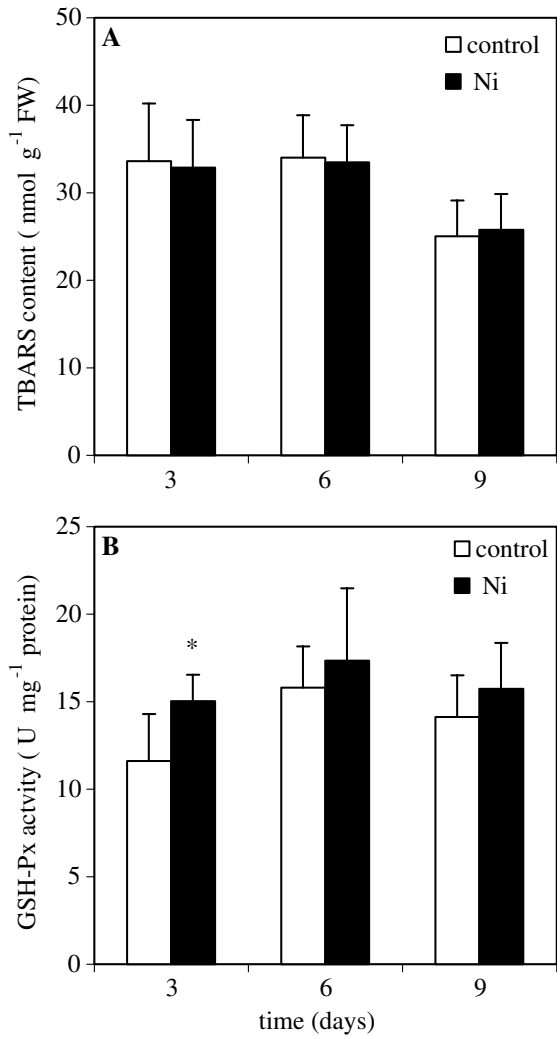

Figure 4. TBARS content (A) and glutathione peroxidase activity (B) in leaves of wheat plants treated with Ni. A single sample was analyzed per each of five independent experiments (n = 5). Bars represent SD of means. * = significantly different from the control at $p < 0.05$.

are only few reports on the induction of ROS production by Ni, representing the second group of metals (Boominathan & Doran 2002; Hao *et al.* 2006). In our experiment Ni exposure led to a significant increase in $O_2^{\cdot-}$ level, which was accompanied by a decline in SOD activity. Extent of the decrease in this enzyme activity was much lower than that of $O_2^{\cdot-}$ accumulation, so it seems that the enhancement in $O_2^{\cdot-}$ level in the wheat leaves only partly resulted from reduced SOD capacity and was probably mainly due to increased generation of this ROS. To the best of our knowledge in literature there is only one report on accumulation of $O_2^{\cdot-}$ under Ni stress. Hao *et al.* (2006) found enhanced production of $O_2^{\cdot-}$ in the roots of wheat seedlings treated with $NiCl_2$. They showed that plasma membrane NADPH oxidase was involved in generation of this ROS. Decrease in SOD activity in the leaves of Ni-treated wheat contradicts the earlier observations for maize (Baccouch *et al.* 1998) and pigeonpea (Madhava Rao & Sresty 2000), however is in agreement with those for hairy roots of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominathan & Doran 2002). SODs are metalloenzymes containing Fe, Cu/Zn

or Mn in their prosthetic groups, depending on the enzyme type. Since high concentrations of Ni have been shown to decrease Fe (Pandey & Sharma 2002), Cu and Zn (Parida *et al*. 2003) contents in plant tissues it can be speculated that reduction in SOD activity in the leaves of wheat plants subjected to excess Ni may result from deficiency of metals essential for catalytic action of this enzyme. Decrease in this enzyme activity could be also due to its inactivation by ROS, including H_2O_2 (Hodgson & Fridovich 1975).

Exposure of wheat plants to Ni led to a significant enhancement of H_2O_2 content in the leaves. Accumulation of H_2O_2 in these organs was previously observed in response of plants to Cd (Kuo & Kao 2004), Cu (Drążkiewicz *et al*. 2004), Cr (Pandey *et al*. 2005) and Hg (Cho & Park 2000). However, Ni-induced increase in this ROS content was reported only for roots of wheat (Hao *et al*. 2006) and hairy roots of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominathan & Doran 2002). Since Ni stress caused a decline in SOD activity, accumulation of H_2O_2 in wheat leaves could not have resulted from the enzymatic dismutation of $O_2^{\cdot -}$. It might possibly originate from non-enzymatic disproportionation of $O_2^{\cdot -}$ or from reactions catalyzed by some oxidases. In the roots of Al-treated wheat plants oxalate oxidase was found to be responsible for H_2O_2 production (Delisle *et al*. 2001). It has been suggested that H_2O_2 plays an important role in the inhibition of growth of heavy metal-stressed plants (Chen *et al*. 2000). Being a substrate for peroxidases it participates in the process of cell wall stiffening, which results in the restriction of cell elongation (Díaz *et al*. 2001).

Concentration of H_2O_2 in plant tissues is controlled by H_2O_2-scavenging enzymes as well as by non-enzymatic antioxidants. In the wheat leaves Ni stress led to a marked increase in APX activity, which is in agreement with the findings for maize shoots (Baccouch *et al*. 1998). In contrast, inhibition of this enzyme activity was reported for Ni-treated hairy roots of *Alyssum bertolonii* and *Nicotiana tabacum* (Boominathan & Doran 2002). In the present work the highest value of APX activity detected on the 6th day coincided with the decrease in H_2O_2 content, which suggests that APX might play an important role in the removal of this ROS from cells in the leaves of Ni-stressed wheat. A several-fold induction of POD activity was also found in the wheat leaves in response to Ni exposure. It is considered that the main function of POD under stress conditions is participation in lignin biosynthesis and consequently in the decrease in plasticity of cell walls (Gaspar *et al*. 1991). It is possible that a considerable increase in POD activity may be at least in part responsible for the reduction of wheat leaf growth. However, involvement of POD in lignification does not exclude its role as H_2O_2 scavenger. The activity of this enzyme increased earlier and to a greater extent in comparison with that of APX. Contrary to APX and POD, CAT activity in the wheat leaves decreased in response to Ni treatment, which is in accordance with the findings for the leaves of cabbage (Pandey & Sharma 2002) and pigeonpea (Madhava Rao & 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 (Boominathan & Doran 2002; Cho and Seo 2005; Smeets *et al*. 2005). Treatment of the wheat seedlings with Ni caused a significant reduction of SOD and CAT activities. Despite a substantial induction of APX and POD activities increased concentration of H_2O_2 in the wheat leaves persisted throughout the experiment. Prolonged maintenance of the elevated levels of $O_2^{\cdot -}$ and H_2O_2 in the leaf tissue could have led to oxidative damage of the cells. To check this possibility we determined the level of lipid peroxidation in the wheat leaves. Our experiment showed that after Ni application lipid peroxide content in the leaf tissue remained unaltered, which contradicts the findings for maize (Baccouch *et al*. 1998) and pea (Madhava Rao & Sresty 2000) shoots. However, we used lower concentration of Ni than in the studies mentioned above. To verify whether the unchanged level of lipid peroxides could have resulted from their removal we measured the activity of lipid peroxide-eliminating enzyme, GSH-Px. Previous studies showed that plants

may respond to heavy metal toxicity with both induction and inhibition of this enzyme activity (Edwards 1996; Dixit *et al*. 2001; Iannelli *et al*. 2002). We found that Ni stress resulted in a slight but significant increase in GSH-Px activity at the beginning of experiment. Therefore, induction of lipid peroxides production and their fast and effective removal by GSH-Px can not be ruled out. Lipid peroxidation may be also restricted by non-enzymatic antioxidants, mainly tocopherol, which possesses the ability to scavenge lipid peroxy radicals (Munné-Bosch and Alegre 2002). Lipid peroxides may also serve as substrates for glutathione transferase, whose activity has been reported to increase in response to Ni stress (Gajewska & Skłodowska 2005).

In conclusion, the results of the present work showed that exposure of the wheat seedlings to 100 μM Ni led to the accumulation of $O_2^{\cdot-}$ and H_2O_2 in the leaf tissue. Increase in these ROS content was accompanied by inhibition of SOD and CAT activities. Enhanced concentration of H_2O_2 must have originated from the sources other than SOD-catalyzed disproportionation of $O_2^{\cdot-}$. Both APX and POD but not CAT seem to be involved in H_2O_2 scavenging in the leaves of Ni-treated wheat. Despite the fact that $O_2^{\cdot-}$ and H_2O_2 were not effectively removed by the antioxidative system, peroxidation of lipids did not occur in the wheat leaves.

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