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NICKEL ALTERS ANTIOXIDATIVE DEFENSE AND WATER STATUS IN GREEN GRAM

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SUMMARY

Green gram [*Vigna radiata* (L.) wikzek] plants exposed to excess nickel (10, 100 and 200 µM) under glass-house conditions were quantified for selected parameters of oxidative stress and water deficit. Supply of excess nickel for 72 h induced chlorosis of young leaves, the effect being most pronounced at 200 µM Ni supply. Excess supply of nickel showed enhanced accumulation of antioxidants ascorbate and dehydroascorbate and decrease in the activity of catalase in the leaves, leading to accumulation of hydrogen peroxide. The activity of ascorbate peroxidase, peroxidase, glutathione reductase and SOD showed an increase. The effect on the enzyme activities varied with the level and duration of Ni supply. Leaves of plants supplied excess Ni showed decrease in water potential (Ψ_L) and relative water content (RWC) and an increase in proline. Results observed indicate that excess supply of nickel interferes with plant water relations and induces oxidative stress.

***Key words*: Green gram, Ni toxicity, oxidative stress, water deficit**

INTRODUCTION

Nickel has been claimed, though not unequivocally established as essential for higher plants (Gerendas *et al*. 1999). At the same time, there is convincing evidence of excess supply of Ni producing phytotoxic effects. The problem of Ni toxicity acquires a serious concern because of agricultural use of sewage sludge that is usually rich in Ni (Juste and Mench 1992) and the industrial use of Ni in production of Ni-Cd batteries which leads to discharge of Ni rich effluents.

Plants subjected to excess supply of Ni accelerates generation of toxic oxygen species leading to oxidative stress (Baccouch *et al.* 1998, Schicker and Caspi 1999) and induces physiological water stress (Poschenrieder and Barceló 1999, Pandey and Sharma 2002). The relation between these responses is, however, not well understood and it remains to be established if the physiological water stress induced in response to Ni toxicity is a condition for the development of oxidative stress in plants. The work was therefore, carried out to investigate the cellular responses to Ni toxicity and to quantify the phytotoxic responses of Ni excess at different stages of development of stress in terms of absorption-accumulation of Ni, water and oxidative stress parameters.

MATERIALS AND METHODS

Plants of green gram [*Vigna radiata* (L) wikzek] were raised from seeds in purified sand and after germination, plants were supplied with complete nutrient solution containing 4 mM $Ca(NO_3)_2$, 4 mM KNO_3, 2 mM $MgSO_4$, 1.33 mM NaH_2PO_4, 0.1 mM NaCl, 0.1 mM Fe-EDTA, 10 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, 33 µM H_3BO_3, 0.2 µM Na_2MoO_4, 0.1 µM $CoSO_4$ and 0.1 µM $NiCl_2$. After healthy growth for 30 d, plants were separated into four lots. While one lot was maintained with complete nutrient supply (0.1 µM $NiCl_2$), the other

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three lots were supplied Ni as $NiCl_2$ at three levels – 10,100 and 200 µM Ni, superimposed over the complete nutrient solution. At d 4 and d 12 after initiation of excess Ni supply, observations were made. Tissue concentration of Ni was determined in oven dried samples of leaves, stem and roots, digested in a mixture of nitric and perchloric acids (10:1), by atomic absorption spectrometry. Chlorophyll (*a* and *b*) and carotenoids were extracted in 80% acetone and estimated spectrophotometrically (Lichtenthaler 1987). Ascorbate was determined by extracting fresh leaf tissue in TCA (Law *et al*. 1978). Total ascorbate was determined after reduction of dehydroascorbate by dithiothretitol and assayed by the formation of the complex between Fe^{2+} and α, α, bipyridyl, with absorption max at 525 nm. Lipid peroxidation was measured in terms of malondialdehyde formation by method of Heath and Packer (1968).

Proline (Pro) was estimated colorimetrically as ninhydrin complex in toluene (Bates *et al.* 1973). Leaf water potential (Ψ_L) was measured hygrometrically on five leaf discs (11 mm diameter) cut from the interveinal areas using Wescor (Logan UT) microvoltmeter (model HR 33T) and C-52 leaf chambers. Relative water content (RWC) was determined according to Barrs and Weatherley (1962), by measuring fresh, hydrated (3 h at 11°C in the dark on deionised water) and oven dry weights of 45 discs from the same leaves used for Ψ_L measurements. Hydrogen peroxide was determined by titanium assay method of Brennan and Frenkel (1977). The H_2O_2 was determined in acetone extracts of fresh leaves. The peroxides which form a specific complex with titanium (Ti^{4+}) were measured colorimetrically at 415 nm.

For assay of superoxide dismutase (SOD) ascorbate peroxidase (APX) and glutathione reductase GR, fresh leaf samples were homogenized with 150 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 2% PVP. For APX assay 1 mM ascorbate was also included in the extracting medium. The homogenates were centrifuged at 15,000 g for 10 min. and the supernatant was used for assaying enzyme activities. Catalase (CAT) and peroxidase (POD) were assayed in fresh leaf tissue extracts prepared by homogenizing samples in ice-cold glass distilled water (1:10) with a cold mortar and pestle at 4°C. Enzyme activities use expressed on the basis of soluble protein in the enzyme preparation (Bradford 1976). The activity of CAT was assayed as described by Eüler and Josephson (1927) and POD according to Lück (1963). Activity of APX was determined by the method of Nakano and Asada (1981). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.5mM ascorbate and 0.1 mM hydrogen peroxide. Oxidation of ascorbate was followed as fall in absorbance per min. at 290 nm. Assay for GR was carried out in a reaction mixture containing 100 mM phosphate buffer pH 7.0, 1 mM oxidized glutathione (GSSG), 1 mM EDTA, 0.1 mM NADPH and 25 to 50 µl of the enzyme extract. The oxidation of NADPH was followed by monitoring the decrease in absorbance per min at 340 nm (Jablonski and Anderson 1978).

The activity of SOD was determined by measuring the ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) in 3 ml reaction mixture containing 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, 0.1 mM EDTA and 0.50 ml enzyme extract. Riboflavin was added last and tubes were illuminated for 10 min. Blanks were not illuminated. One unit of SOD represented the amount that inhibited the NBT reduction by 50% (Beauchamp and Fridovich 1971). Nickel toxicity effect on SOD isoforms was studied by PAGE. The enzyme extracts were loaded to 10% acrylamide gels at a concentration of 75 µg protein. Activity staining was performed by soaking gels in a staining solution consisting 50 mM potassium phosphate buffer, pH 7.8, 0.03 mM riboflavin, 0.326% (v/v) N-N-N'–N'- tetraethylmethylethylene diamine (TEMED) and 1.25 mM NBT in dark for 30 min. On removal from the staining solution, the gels were washed and immersed in a solution containing potassium phosphate buffer (pH 7.8) and 1 mM EDTA and exposed to a light source (15 W fluorescent lamp). The SOD isoenzymes were visualized as white bands against blue background. Identification of Mn SOD and Cu/Zn SOD isoenzymes was done by soaking the gels in 3 mM KCN prior to activity staining. The Fe+Mn SOD shows resistance to KCN and Cu/Zn SOD was sensitive to KCN (Fridovich 1986).

All measurements were made on samples drawn in triplicate and data presented are mean of three replicates ±SD. Effect of Ni on most parameters has been presented in terms of percentage as relative concentration/activity of control values.

RESULTS AND DISCUSSION

Excess supply of Ni (10, 100 and 200 µM Ni) caused reduction in growth and decrease in dry matter yield. The severity of the Ni toxicity effect on dry matter yield was more in roots than in shoots (stem and leaves) and increased with duration of Ni supply (Table 1). This was in consonance with the higher accumulation of Ni in the roots of green gram than in the leaves and stem. The appearance of visible effects of Ni toxicity was also related to the Ni concentration in the leaves. The visual toxicity effects of Ni were observed in leaves containing more than 25 µg Ni g^{-1} dry matter. The leaves of plants supplied excess Ni were smaller in size as compared to leaves of control plants (0.1 µM Ni). After 4 d, plants supplied 100 and 200 µM Ni developed chlorosis in the leaflets and after 12 d of Ni supply, these leaves developed black necrotic spots on either side of the midrib. These visual toxicity effects of Ni were observed in leaves containing more than 80 µg Ni g^{-1} dry matter (Table 1).

Plants subjected to Ni toxicity showed decrease in the concentration of chlorophyll and carotenoids (Fig 1). While concentration of both Chl a and Chl b was reduced in response to Ni supply, the decrease in Chl a was more marked than Chl b. The decrease in these plant pigments may be due to the cellular disorganization under Ni toxicity which causes agglutination of chloroplasts (Molas 2002).

Excess supply of Ni caused lipid peroxidation which was pronounced at 12 d and in plants receiving 100 and 200 µM Ni. (Fig 1). The decrease in carotenoids and accumulation of MDA and H_2O_2 more so at levels >10 µM Ni and which became more pronounced at 12d (Figs. 1 & 2), are indicative of oxidative damage. The cellular membranes have been found to be early targets for Ni toxicity (Baccouch *et al.* 1998, Schicker and Caspi 1999, Rao and Sresty 2000). Several redox and non-redox

Table 1. Effect of excess Ni supply on the dry matter yield and tissue Ni concentration in green gram

	Ni supply (µM)							
	0.1		10		100		200	
Plant part	**4 d**	**12 d**	**4 d**	**12 d**	**4 d**	**12 d**	**4 d**	**12 d**
	Dry matter yield (g plant $^{-1}$)							
Leaf	0.407 ±0.01	0.546 ±0.01	0.385 ±0.04	0.465 ±0.03	0.378 ±0.01	0.420 ±0.07	0.352 ±0.01	0.369 ±0.02
Stem	0.327 ±0.03	0.560 ±.0.10	0.294 ±0.07	0.351 ±0.03	0.286 ±0.01	0.331 ±0.02	0.277 ±0.01	0.296 ±0.09
Root	0.091 ±0.03	0.107 ±0.04	0.064 ±0.06	0.086 ±0.01	0.064 0.01	0.069 ±0.01	0.063 ±0.02	0.069 ±0.01
	Tissue Ni (µg g $^{-1}$ dw)							
Leaf	5.20 ±0.36	6.50 ±0.34	29.26 ±0.11	57.91 ±0.64	84.50 ±0.66	84.56 ±0.46	106.30 ±10.70	161.00 ±6.609
Stem	4.41 ±0.23	5.32 ±0.645	22.92 ±0.87	33.15 ±0.15	34.53 ±0.63	73.03 ±2.50	73.00 ±1.87	92.16 ±7.27
Root	8.15 ±0.41	8.53 ±0.32	110.36 ±5.421	129.71 ±4.14	134.06 ±6.74	175.18 ±8.49	188.74 ±10.60	239.03 ±12.59

Values are means (n=3) ± SD.

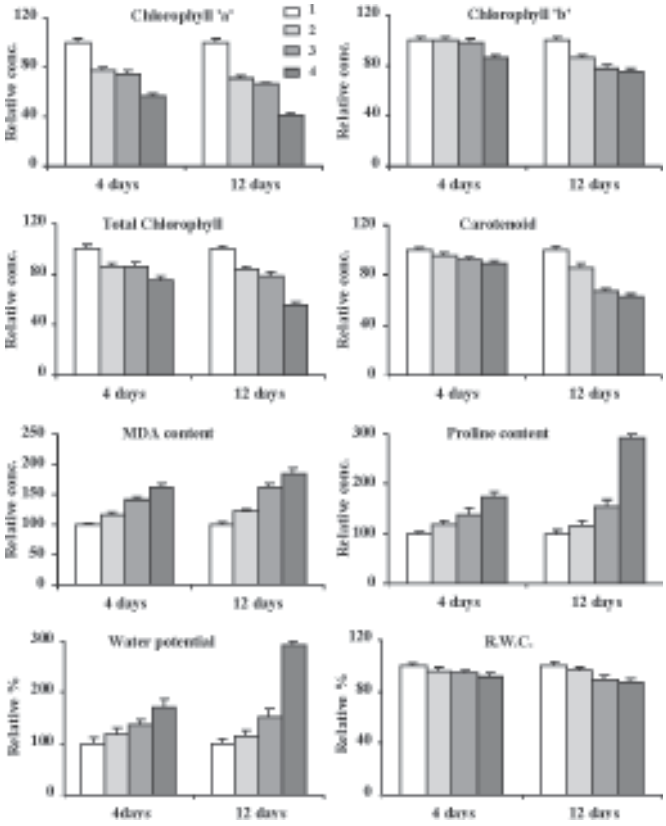

Fig. 1. The relative concentrations of chlorophylls, carotenoids, malondialdehyde, proline, water potential and relative water content (RWC) in the leaves of green gram supplied with 0.1 (1), 10 (2), 100 (3) and 200 (4) µM Ni.

metals are known to cause oxidative stress as indicated by lipid peroxidation and H_2O_2 accumulation in the cells (Schutzendubel and Polle 2002). Development of oxidative stress in plants exposed to heavy metals (De Vos *et al.* 1992, Weckz and Clijsters 1996) is largely ascribed to heavy metal induced disbalance between the generation of toxic oxygen radicals and their scavenging through the antioxidative defense mechanism. The latter provides an efficient system for detoxification and scavenging of the toxic oxygen species through an adaptive mechanism involving upregulation of antioxidative enzymes such as SOD, CAT, POD, APX and GR (Foyer *et al.* 1994) and enhanced accumulation of the cellular antioxidants such as ascorbate and glutathione (Noctor and Foyer 1998) through the ascorbate–glutathione cycle. These reactions also down regulate the conversion of the superoxide ion ($O_2^{\cdot -}$) to the highly reactive and genotoxic hydroxyl ($^{\cdot}OH$) ions.

In the present study, excess supply of Ni led to increase in activity of SOD and APX in leaves of green gram at 4 d as compared to the control plants (Fig. 2). However, after 12 d the relative activities of these enzymes decreased especially in the plants supplied 100 and 200 µM Ni but remained higher than the control. A similar trend was also reflected in the 3 isoforms of SOD

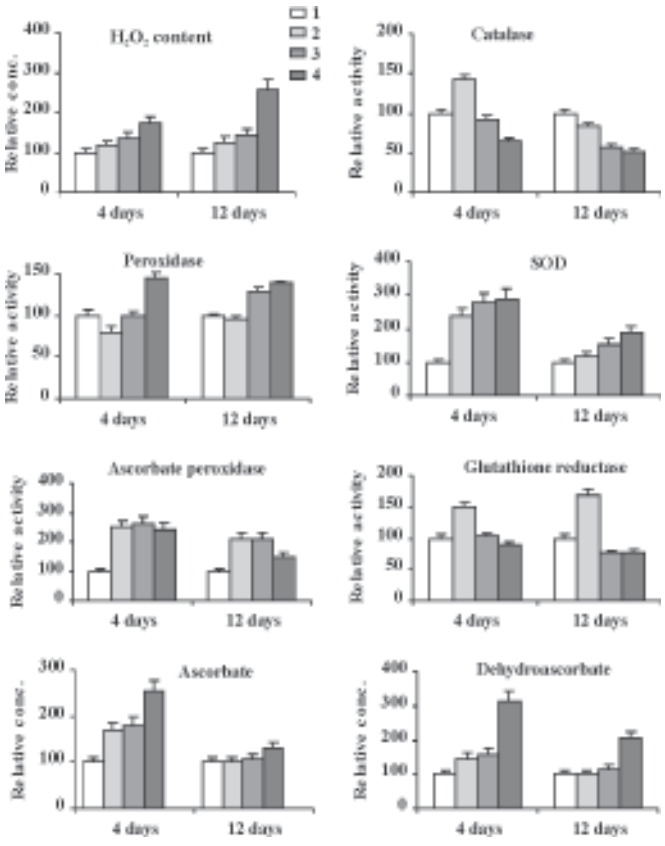

Fig. 2. The relative concentration of hydrogen peroxide, ascorbate, dehydroascorbate and activities of catalase, peroxidase, superoxide dismutase (SOD), ascorbate peroxidase and glutathione reductase in leaves of green gram supplied with 0.1 (1),10 (2),100 (3) and 200 (4) µM Ni.

(one of Fe+Mn SOD and two of Cu/Zn SOD) after PAGE separation and activity staining (Fig. 3 a, b). Glutathione reductase activity increased at d 4 and d 12 in plants supplied 10 and 100 µM Ni but decreased in plants supplied 200 µM Ni (Fig 2). The activity of catalase at d 4, was stimulated by 10 µM Ni, but 100 and 200 µM Ni inhibited its activity. The activity of peroxidase was inhibited by 10 µM Ni but increased in response to 100 and 200 µM Ni supply (Fig 2). The increase in the H_2O_2 concentration seemed to be well

correlated with the decrease in catalase and increase in activity of SOD but it did not correlate with the increase in the activity of APX. The marked increase in activity of the antioxidative enzymes SOD, APX and GR as well as an increase in the concentration of antioxidants, ascorbate and dehydroascorbate at d 4 indicated induction of the antioxidative system at initial stage following supply of Ni at 10 and 100 µM Ni. However, with continued supply of Ni, especially in plants supplied 100 and 200 µM, the toxicity effects were enhanced and were reflected in the breakdown of the antioxidative defenses of plants. This was manifest as appearance of the visible symptoms, increase in lipid peroxidation and H_2O_2 concentration and a relative decrease in the activity of antioxidative enzymes.

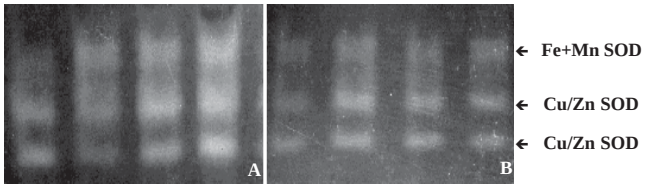

Fig. 3. Native gels stained for activity of SOD in leaves of green gram supplied 0.01 (1), 10 (2), 100 (3) and 200 (4) µM Ni at d 4 (A) and d 12 (B).

Nickel supply at 4 d showed insignificant decrease in RWC of leaves especially at 10 µM Ni but after 4 d (d 12) a marked decrease could be observed in the leaf water content especially at 100 and 200 µM Ni. Irrespective of the level of the supply, excess Ni lowered marginally the Ψ_L at d 4 but showed an appreciable decrease at 12 d. Plant exposure to excess Ni caused increased accumulation of proline at both the stages (Fig. 1). Accumulation of proline has been frequently used as a biochemical marker for water stress in plants (Alia and Saradhi 1991, Schat *et al*. 1997). The enhancement in proline concentration in green gram at higher levels of Ni supply especially at d 12 appears to be a signal of increase in intensity of water stress suffered by these plants. Induction of water stress in the Ni toxic plants has been reported earlier (Iturbe-Ormaetze *et al*. 1998, Pandey and Sharma 2002). We observed that the water deficit in leaves was manifested only in plants exposed to >10 µM Ni and that to when exposed for a long duration (>4 d). These observations discount the possibility of induction of oxidative stress (H_2O_2 production) and peroxidative damage (MDA production) as a consequence of water deficit.

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