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Research Article

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Nickel Stress Induced Antioxidant Defence System in Sponge Gourd (*Luffa Cylindrical*)

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Abstract

Nickel is an essential nutrient for plants, and it is required in very low amounts for normal growth of plants. However, since Ni pollution in the environment is increasing, it is essential to understand the functional roles and toxic effects of Ni in plants. To evaluate the influence of excess nickel (Ni) on sponge gourd (*Luffa cylindrical* L.) cv. Nirmal 28, plants were grown in refined sand with complete nutrition for 40 days (d). On the 41st day, pots with plants were separated into five lots, out of which one set was treated as a control, and rest of the four lots were given nickel as sulphate at 0.05, 0.1, 0.2 and 0.4 mM Ni, respectively. At d 46 (6 days after metal treatment-DMT), excess Ni induced interveinal chlorosis in young leaves, along with marked growth depression. With persistent excess Ni, chlorosis turned to bleached ivory. The chlorosis was comparatively delayed in plants grown at low Ni level. With an increase in Ni level, successive reduction in height and leaf area was observed. In sponge gourd, exposure of plants to excess Ni>0.05 mM decreased biomass, concentration of photosynthetic pigments, concentration of Fe, and catalase activity. The concentration of Ni in plants parts, proline and activities of peroxidase, superoxide dismutase, ascorbate peroxidation, acid phosphatase and ribonuclease increased with increasing levels of nickel. Oxidative damage measured as level of lipid peroxidation was observed in leaves of sponge gourd plants.

Keywords

Enzymes; Nickel; Oxidative stress; Sponge gourd (*Luffa cylindrical* L.)

Introduction

Nickel is an essential nutrient for plants. However, the amount of Ni required for normal growth of plants is very low. Hence, with the level of Ni pollution in the environment increasing, it is essential to understand the functional roles and toxic effects of Ni in plants [1]. Ni is also released into the environment from anthropogenic activities, such as metal mining, smelting, fossil fuel burning, vehicle emissions, disposal of household, municipal and industrial wastes, fertilizer application, and organic manure [2].

Biological and ecological consequences of heavy metals, including nickel (Ni), depend upon, among others, the chemical form in which the metal occurs in the environment. In plants, Ni is complexed by amino acids. Nickel, in high concentration, cause deleterious effects to plants, including inhibition of seed germination, reduction in plant growth [3], oxidative damage [4,5], enhanced lipid peroxidation, proline accumulation [6], DNA damage, and oxidation of proteins and reductants in cells [7], by altering biochemical reactions [8].

Heavy metals such as Cd, Cu, Ni, Pb, and Zn are major environmental pollutants. Known symptoms of their toxic effects on plants include reduced growth and production. Excessive Ni leads to significant increases in the concentration of hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide [5,9]. Since Ni is not a redox-active metal, it cannot directly generate these reactive oxygen species (ROS). However, it interferes indirectly with a number of antioxidant enzymes.

Consumption of vegetables is one of the most important pathways by which heavy metals enter the food chain. Sponge gourd is a vegetable used in regional food preparation. In India, sponge gourd can be irrigated with industrial effluent water which contains most of the heavy metals. Systematic studies on the role of Ni in oxidative metabolism and physiological and biochemical responses on spongegourd under stress is lacking. This investigation was undertaken to examine changes in growth, metabolism and oxidative damage induced by Ni in sponge gourd.

Materials and Methods

Sponge gourd (*Luffa cylindrical* L.) cv. Nirmal 28 seeds were sown in refined sand with a pH of 6.8-7.0, at 15-32°C and 60-70% humidity in a glasshouse [10], at the Department of Botany, Lucknow University, Lucknow, India. Plants were grown in 10-L polyethylene pots. Each pot was provided with a central drainage hole covered with an inverted watch glass, lined with glass wool under the rim, to allow free drainage of nutrient solution.

The composition of the nutrient solution was the same as detailed by Hewitt [11], in which it was indicated that Ni supplied at this level was beneficial to plant metabolism. The composition of the basal nutrient solution supplied to plants was: 4 mM KNO_3; 4 mM $Ca(NO_3)_2$; 2 mM $MgSO_4$; 1.5 mM NaH_2PO_4; 100 µM Fe-EDTA; 10 µM $MnSO_4$; 30 µM H_3BO_3; 1 µM $CuSO_4$; 1 µM $ZnSO_4$; 0.2 µM Na_2MoO_4; 0.1 µM $CoSO_4$; 0.1 µM $NiSO_4$ and 0.1 mM NaCl, based on a modification of the method of sand culture for Indian conditions [10].

Up to day 40, plants were supplied with the complete nutrient solution. On day 41, pots with two plants in each pot were divided into six groups, with six replicates in each treatment. The experiment was arranged in a randomized complete block design. One treatment, the control, continued to receive the full nutrient solution containing 0.0001 mM Ni. In the remaining five treatments, nickel sulphate at 0.05, 0.1, 0.2 and 0.4 mM was added in the basal nutrient solution. Treatments were supplied daily.

At 51 days (11 days after treatment), the concentration of chlorophylls and caroteinoids in leaves were determined in 80% acetone extract of young leaves, using the method of Lichtenthaler

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[12]. Absorbance of the cleared extract was measured at 663.2, 646.8, and 470 nm for chlorophyll a, chlorophyll b, and total carotenoids, respectively. Proline was estimated calorimetrically as ninhydrin complex in toluene [13].

Five hundred mg plant material (leaves) was extracted with 5 mL of 5% trichloroacetic acid (TCA), the homogenate was centrifuged at 1,000 rpm for 10 min at 20°C, 1 mL of supernatant was added to 4 mL of 0.5% TBA in 20% TCA. This solution was boiled for 30 min, and then quickly cooled in an ice bath for 5 min. After centrifugation at 1,000 rpm for 10 min at 20°C, the absorbance was read at 532 nm. Correction of nonspecific turbidity was made by subtracting the absorbance value taken at 600 nm. The MDA content was expressed as nmol g^{-1} FW, using an extinction coefficient of 155 Mm^{-1} cm^{-1}.

The reaction mix was for catalase (CAT) (E.C. 1.11.1.6), 500 µmoles H_2O_2 in 10 mL 100 mM phosphate buffer, pH 7.0, and 0.1 mL tissue extract. The H_2O_2 decomposed after 5 min was assayed by titrating the reaction mixture with 0.5 M $KMnO_4$ [14].

For peroxidase (POX) (E.C. 1.11.1.7), the reaction mixture was 5 mL of 100 mM phosphate buffer (pH 6.5), 1.0 mL of 0.5% p-phenylanadiamine, 1.0 mL of 0.01% (v/v) H_2O_2, and 0.05 mL of tissue extract. Changes in absorbance after 5 min were measured at 485 nm [15]. A change in OD of 0.01·min^{-1} represents one unit of peroxidase activity.

Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was assayed according to Beauchamp C, Fridovich I [16]. The reaction mixture contained 0.24 mM riboflavin, 2.1 mM methione, 1.72 mM nitro blue tetrazolium chloride (NBT) in 50 mM sodium phosphate buffer, pH 7.8. One unit of SOD is the amount of enzyme required to cause 50% inhibition in the rate of NBT photo reduction.

Ascorbate peroxidation (APX) (E.C. 1.11.1.11) was assayed in 3 mL of 50 mmol·L^{-1} phosphate buffer, pH 7.0, 0.5 mmol·L^{-1}AsA, 0.1 mmol·L^{-1} H_2O_2, 0.1 mmol·L^{-1} EDTA, and a suitable quantity of enzyme extract. No tissue extract and no H_2O_2 blanks were run, and changes in absorbance after every 15 s were recorded at 290 nm [17].

Ribonuclease (RNAse) was assayed by the method of Tuve and Anfinsen [18]. The reaction mixture contained 0.5 mL enzyme extract with 0.1 M citrate buffer (pH 6.0), and 2.5 mg RNA in 2 mL. The reaction was run at 30°C for 30 min, and stopped by addition of 0.5 mL 0.75% (w/v) uranyl acetate in 25% perchloric acid. After centrifugation, O.D. of the supernatant was measured at 260 nm. The change in O.D. of 0.01·min^{-1} corresponds to a unit of ribonuclease activity.

Means were separated with least significant difference (LSD) analysis [19].

Results and Discussion

In sponge gourd, after 6 days of metal supply, excess Ni induced adverse effects, noted as visible symptoms. The visible effects were observed on young leaves of plants as interveinal chlorosis, along with marked depression in growth within 3 days. These effects intensified over time, and while observed at lower levels of excess Ni, they were delayed and mild. In severely affected plants, chlorosis changed to bleaching. At maturity, all the young leaves turned bleached ivory and growth was checked. The height and leaf area were markedly decreased with excess Ni. These symptoms of excess Ni in both plants is similar to that described for other plant species [9].

The depression in biomass (Table 1) in plants with excess Ni might be due to reduced photosynthesis, disturbing carbohydrate and nitrogen metabolisms. This consequently lowered the protein content, which is responsible for lowering of biomass, and also due to water deficit in vascular system [20,21].

The concentration of both chlorophylls a and b decreased (Figure 1) significantly in the leaves with excess Ni. These results are in agreement with the observations on *Camellia sinensis* (L.) cv. Kuntze [22]. The decrease in chlorophyll content might be due to inhibition of the photosynthetic electron transport system. Nickel is known to inactivate photosystem II activity, without affecting PSI mediated electron flow. The lowered concentration in chlorophyll leaves at excess Ni is similar to several other reports [23]. It also appears that the decrease in chlorophyll content in Ni stressed leaves was mostly due to its enhanced degradation [9] in leaves with excess Ni. It has been suggested that the uptake of toxic amounts of Ni by plants leads to quantitative changes in the structure of the photosynthetic apparatus of plants, as well as to an inhibitor of the light and dark phase of photosynthesis [24]. The decrease in photosynthesis is also responsible for reduced biomass of crop plants in such conditions.

Lipid peroxidation was measured in terms of malonadehyde (MDA), a peroxidation product. MDA is the decomposition product of polyunsaturated fatty acid of biomembrane induced by ROS, and an indication of oxidative stress in plants. Ni-induced lipid peroxidation as demonstrated by changes in malondialdehyde concentration (Figure 1), was similar to that found by Baccouch et al. [25]. The increase in Ni increased activities of oxidative stress responsive enzymes, and lipid peroxidation could be attributed to

Table 1: Effect of Ni stress on biomass, iron and nickel content in different plant parts of sponge gourd.

Plant part		mM Ni				LSD p=0.05
	Control	0.05	0.1	0.2	0.4	
	DAMS 16	**d 56 Dry weight: g plant^{-1}**				
	6.78	5.44	4.80	4.59	3.83	0.52
	DAMS 29	**d 69**				
	9.31	8.15	7.48	6.52	5.23	1.01
	DAMS 16	**d 56: Iron concentration: µg g^{-1} dry matter**				
Y.L.	133	108	100	78	74	10.77
M.L.	117	95	80	77	75	7.43
O.L.	128	89	81	75	70	8.83
Y.S.	94	76	77	68	60	5.41
M.S.	91	78	72	68	62	7.07
O.S.	79	67	67	60	58	6.88
Root	137	103	87	73	68	7.72
	DAMS 16	**d 56: Nickel concentration: µg g^{-1} dry matter**				
Y.L.	4	31	39	59	79	6.71
M.L.	4	25	36	47	61	8.91
O.L.	2	23	29	40	50	7.53
Y.S.	3	27	35	50	66	7.16
M.S.	3	21	31	40	58	6.56
O.S.	2	21	28	38	56	7.09
Root	5	41	57	89	111	6.11

DAMS-Days after metal supply
Y.L.-Young Leaf; M.L.-Middle Leaf; O.L.-Old Leaf; Y.S.–Young Stem; M.S.-Middle Stem; O.S.- Old Stem

unavailability of functional Fe due to sequestration in ferritin. As excess supply of Ni is known to decrease tissue Fe level, this may possibly result in increased MDA in such plants, and therefore, increased lipid peroxidation in leaf tissue. An increased level of H_2O_2 also generates $OH^{\cdot}$ radicals in presence of transition metals (Fe^{2+}/Cu^{2+}), that can further accentuate the extent of lipid peroxidation. Therefore, lipid peroxidation is closely related to higher-level ROS under stress conditions [26]. The concentration of soluble proteins in these leaf tissues decreased. Decrease in protein concentration might be due to proteins damage by different ROS.

Accumulation of free amino acids is important for plant protection and survival under stress. Accumulation of proline in leaves (Figure 1) of spong gourd [27] indicates its involvement in antioxidant responses to elevated levels of Ni, and has been used as a biochemical marker of water stress in plants [6]. The increase in proline concentration indicates a change in the osmotic gradient, assuring water flow to the plant and increasing tolerance to Ni.

The activity of catalase decreased (Figure 2), and that of peroxidase increased at 0.4 mM Ni, perhaps because Ni in traces is an essential nutrient for growth and development of higher plants. But at higher levels, a significant reverse in their activity was observed. The decrease in catalase activity may have increased the H_2O_2 concentration, creating oxidative stress, enhancing the inactivation of catalase preventing synthesis of new enzyme [28]. This might also favour accumulation of reactive oxygen species in the leaf tissue and consequently, lead to oxidative damage of lipids [9]. This might also due to inhibitory role of excess heavy metals [3], inducing oxidative stress as has been observed in sunflower [29].

The stimulation in peroxidase activity (Figure 2) in high Ni might be due to accumulation of phenols in excess metal treated leaves. This was apparent in distortion and necrosis of young leaves, which exhibited severe Ni toxicity. This enhanced activity of peroxidase in excess Ni-treated leaves might result either in peroxidative damage of the thylakoid membrane of lower auxin and protein content, or high phenols in tissues inhibiting the growth of plants [30].

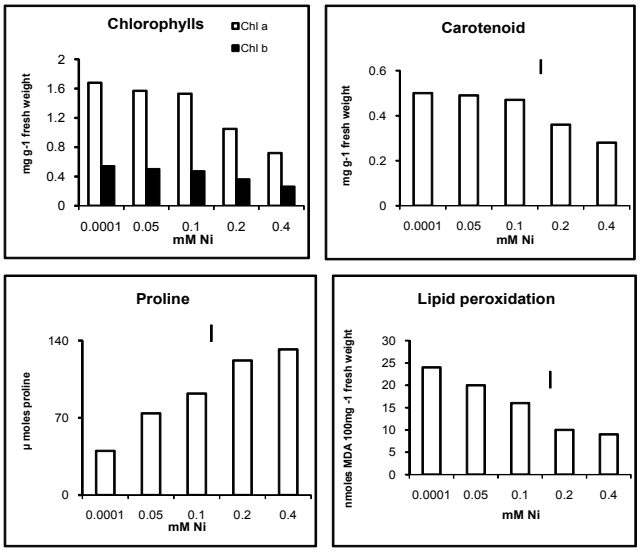

Figure 1: Effect of Ni concentration in sand culture on chlorophyll a, chlorophyll b, carotenoids, proline and lipid peroxidation in sponge gourd.leaves. Vertical lines represent LSD (*P*=0.05).

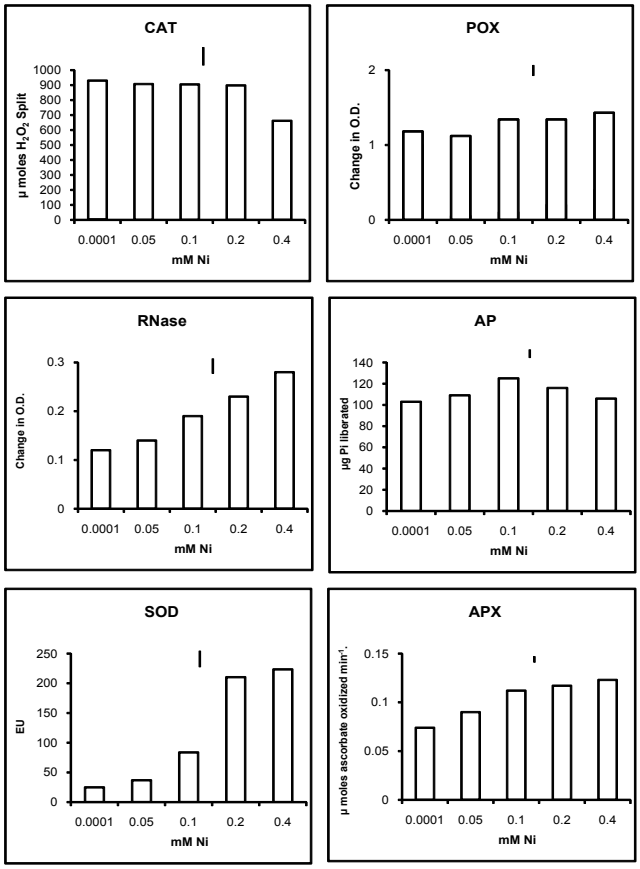

Figure 2: Effect of Ni concentration on specific activities of catalase (CAT), peroxidase (POX), acid phosphatase (AP), ribonuclease (RNAse), ascorbate peroxidase(APX), and superoxide dismutase (SOD), in leaves of sponggourd. Vertical lines represent LSD (*P*=0.05).

In sponge gourd, an increase in APX activity (Figure 2) was caused by an excess of Ni, suggesting its role in detoxification of H_2O_2, which was reported earlier by Schickler and Caspi [31]. Activity of SOD, enzyme responsible for dismutation of ($O_2^{\cdot}$) radical into H_2O_2, increased significantly in Ni treated plants. Increase in the activity of SOD is in agreement with the observations made by Halliwell and Gutteridge [32]. Isoforms of SOD in plants with excess Ni supply showed greater intensity, suggesting enhanced SOD activity. Increased SOD activity has been suggested resulting from higher $O_2^{\cdot}$ accumulation in Ni excess plants. H_2O_2, a product of SOD activity is a signal molecule, and is known to induce a number of genes and proteins involved in stress defences. The decrease in acid phosphatase activity in high nickel leaves of sponge gourd might be due to disturbed phosphorus metabolism. This is in agreement with earlier reports [33].

The increase in ribonuclease activity (Figure 2) in the leaves of both plants is a consequence of degradation of ribonucleic acid and mobilization of the hydrolysed products to the growing tissues. The increase in RNAse activity in nickel excess might also reflect enhanced synthesis of RNA in such conditions.

Table 2: Effect of Ni stress on sugars, starch and phenols in sponge gourd leaves.

			mM Ni		LSD p=0.05
Control	0.05	0.1	0.2	0.4	
DAMS 18	**d 58 Sugars: % in fresh weight**				
	Reducing:				
0.25	0.23	0.21	0.18	0.15	0.03
	Non-reducing:				
0.38	0.28	0.27	0.27	0.26	0.05
	Total:				
0.63	0.51	0.48	0.45	0.41	0.08
	Starch: % in fresh weight				
2.99	1.61	1.56	1.34	1.06	0.18
	Phenols: % in fresh weight				
0.01	0.011	0.017	0.020	0.025	0.002

DAMS-Days After Metal Supply

The decrease in carbohydrate fractions in leaves of sponge gourd (Table 2) might be due to low availability of photosynthates during their synthesis [34]. The disturbances in sugars and starch in Ni treated plants might be due to consequence of either impaired carbohydrate metabolism at the site of production (the source), or of low demands at the sink site, and this is responsible for inhibited growth.

Excess Ni adversely affected the iron metabolism by decreasing the activity of catalase and chlorophyll content, along with reduced concentration of iron in leaves. Interference of heavy metals, including nickel with iron in plant metabolism, is known to induce the disturbances, creating physiological iron deficiency and decrease in chlorophyll synthesis [3].The decrease in fruit formation in excess Ni treated plants is similar to the observations on tomato [20], where the fresh weight of fruits decreased due to excess Ni.

The disturbances in iron content due to excess Ni (Table 1) affect the plant carbohydrate and nitrogen metabolism, and might be responsible for depressed growth and lowered biomass with excess Ni. Similar observations of lower P content in high Ni conditions have been reported by Miller and Cumming [35] in Virginia pine (*Pinus virginiana* Mill).

The concentration of Ni in sponge gourd increased in all parts. The accumulation of Ni was highest in roots [36]. Excess Ni significantly restricted the translocation of iron from roots to tops (Table 1). This suggests that Ni, like other heavy metals, can displace several ions from physiologically important binding sites, and can thus decrease the uptake and mode of other heavy metals, including iron. This induced Fe deficiency effect has been indicated earlier [37].

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Top

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