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Nickel Induced Toxic Effects and Bioaccumulation in the Submerged Plant, *Hydrilla verticillata* (L.F.) Royle Under Repeated Metal Exposure

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Nickel has recently been defined as an essential micronutrient due to its involvement in enzymatic activity in legumes (Welch 1995), but it is toxic at supra-optimal concentrations in plants (Schickler and Caspi 1999; Rao and Sresty 2000; Pandey and Sharma 2002). Increased rates of free radical reactions have often been suggested to contribute to the toxicity of high doses of metal ions, including nickel (Ni^{++}), by affecting functioning of the membrane system due to both sulphydryl reaction and lipid peroxidation (Halliwell and Gutteridge 1993). Accumulation of some metals in the plants also retards growth and elicits various physiological and biochemical changes due to high affinity of metals for sulphydryl groups. Some of the changes, such as loss of chlorophyll, are specific to the toxic metal ions (Pandey and Sharma 2002). However, plants possess antioxidants and antioxidative enzymes to protect against the damage caused by free radicals. In healthy plants, antioxidants are sufficient to prevent the biological damage caused by toxic oxygen species by keeping the deleterious reactions to a minimum.

Nickel is a common pollutant resulting from various industrial activities like mining and refining of Ni ore, electroplating, production of Ni-Cd batteries, waste incineration etc., domestic wastewater, and to a lesser extent from natural weathering. The role of aquatic plants, particularly submerged species, in the removal of heavy metals (Cu, Cr, Cd, Fe, Mn, Pb) is well documented (Guilizzoni 1991; Rai et al. 1995; Gupta et al. 1996; Sinha et al. 1993, 1997, 2002). Plants remove these metals by surface adsorption and/or absorption and incorporate them into their own system or store them in a bound form. Metals also form complex with the ligands found in the natural environment, which also affects their bioaccumulation and toxicity potential. The bioaccumulation and effects of metals, such as Mn, Cu, Fe, Cr, Cd and Hg on the submerged plant, *Hydrilla verticillata* has been studied by several authors (Sinha et al. 1993, 1997; Gupta et al. 1996,1998; Rai et al. 1995), however, no reports are available on the effects induced by Ni accumulation in the plants under repeated metal exposure. The plant, *Hydrilla verticillata* is commonly used as fish food and the metal accumulated in the plants may biomagnify through food chain. Thus, it was considered worthwhile to undertake the present study.

MATERIALS AND METHODS

The submerged plants of *Hydrilla verticillata* (l.f.) Royle were grown and propagated in controlled environmental conditions. Healthy plants (6" long) were cut off from

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mother plants with similar biomass (3.0 g fresh weight). These plants were further acclimatized in 10% Hoagland's solution for 6 weeks under laboratory conditions. Using analytical grade reagent $NiCl_2$ (nickel chloride, minimum assay 98%, procured from CDH), different concentrations of nickel (5, 10, 25, 50 and 100 µM) were prepared in 10% Hoagland's solution. The measured test concentrations were 4.97, 9.94, 25.08, 50.07 and 99.7 µM. Three replicates of each concentration were kept in 250 ml beakers (200 ml solution). The plants with approximately the same biomass (3.0 g fresh weight) were kept in all the treatment and control sets under submerged conditions. Plants in 10% Hoagland's solution served as the control. The experiment was performed under standard physiological conditions providing 14 h per day fluorescent light of 114 µmoles m^{-2} s^{-1} intensity at 26 ± 2^0 C temperature. The plants were harvested after 2, 4 and 6 d of exposure and the metal solutions were changed every 2^{nd} day. The harvested plants were properly washed with distilled water to remove any nickel adhering to the plant surface and dried using blotting paper to remove excess water and used for the estimation of chlorophyll, protein, cysteine, non-protein thiol and malondialdehyde contents. The remaining parts of the plants were oven dried at 80^0C for metal estimation.

Dried plant tissues were digested in concentrated HNO_3: $HClO_4$ (3: 1, v/v). Nickel was estimated using an atomic absorption spectrophotometer (GBC Avanta Σ). Chlorophyll content from the whole plant was extracted using 80% chilled acetone and estimated by the method of Arnon (1949) using Spectrophotometer (GBC Cintra 10e). Protein content in the whole plant was estimated by the method of Lowry et al. (1951) using BSA (Bovine Serum Albumin) as standard. Cysteine content in plants was measured by the method of Gaitonde (1967). The level of lipid peroxidation was measured in terms of malondialdeyde (MDA), a product of lipid peroxidation, in the plant samples estimated by thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Non – protein thiol (-SH) content in plant samples was measured using the method of Ellman (1959).

Analytical data quality of nickel was ensured through repeated analysis (n=6) of EPA quality control samples in water and the results were found to be within ±3.05% of certified values. For plants, recoveries of nickel from the plant tissues were found to be 99% as determined by digesting four samples each from untreated plant with a known amount of metal. Blanks were run throughout each analysis. Analysis of variance (ANOVA) was performed at significance level $P<0.05$, in a completely randomized block design involving five Ni concentrations and three exposure periods. Duncan's multiple range test (DMRT) was used to determine significant differences between treatment means (Gomez and Gomez 1984).

RESULTS AND DISCUSSION

The effect of different concentrations of Ni on fresh weight of the plants of *H. verticillata* is shown in Table 1. There was no significant effect of Ni treatment on the fresh weight of the plants after 2 days. A significant increase in fresh weight was found after 4 d of exposure up to 10 µM followed by non-significant decrease at higher concentrations as compared to control, whereas, a significant decrease was found in fresh weight of the plants as compared to control after 6 days. Exposure to Ni after 8 days becomes lethal for the plants, thus the long -term study could not be done.

Accumulation of Ni in *H. verticillata* is shown in Table 2. Accumulation of Ni in the plants increased significantly ($P< 0.05$) with increase in concentration and exposure

Table 1. Effect of Ni on fresh wt. (g) of *H. verticillata* at different concentrations and exposure periods

Conc. (μM)	Exposure periods (d)		
	2	4	6
Control	3.59±0.11	3.85[a]±0.37	4.50[a]±0.30
5.0	3.59±0.17	4.02[a]±0.06	3.73[b]±0.31
10.0	3.59±0.95	4.21[a]±0.24	3.54[abc]±0.34
25.0	3.49±0.11	3.69±0.05	3.44[abd]±0.17
50.0	3.46±0.17	3.60±0.04	3.33[abc]±0.15
100.0	3.47±0.24	3.63±0.19	3.18[abcd]±0.05

The metal solutions were replaced with initial metal concentrations on 2nd and 4th d of exposure. All values are means of triplicates ± S.D. ANOVA P<0.05. Identical superscripts on values denote significant difference (P<0.05) between means of different treatments according to Duncan's multiple range test.

periods except between 10 and 25 μM after 2 d. At the lowest metal concentration (5 μM), the plants accumulated 149.46 μg g^{-1} dw Ni after 2 d of exposure, which increased over time after replacing the metal solution. Accumulation of metal further increased with increase in metal concentration. Maximum accumulation of 4683.76 μg g^{-1} dw Ni was found at 100 μM in the plants on 6th d of exposure.

Table 2. Accumulation of Ni (μg g^{-1} dw) in the plants of *H. verticillata* at different concentrations and exposure periods.

Conc. (μM)	Exposure periods (d)		
	2	4	6
Control	31.52±2.57	32.70±1.79	39.96±12.14
5.0	149.46±52.62	354.86±4.79	502.18±60.83
10.0	350.04±27.25[a]	770.93±19.90	1197.62±225.60
25.0	441.77±29.66[a]	1353.58±190.10	1474.47±77.30
50.0	631.86±139.26	1819.06±132.32	2168.04±251.38
100.0	1532.3±31.13	4047.96±185.66	4683.76±651.20

Metal solutions were replaced with initial metal concentrations on 2nd and 4th d of exposure. All values are means of triplicates ± S.D. ANOVA P<0.05. Identical superscripts on values denote non-significant difference (P<0.05) between means of different treatments according to Duncan's multiple range test.

The effect of Ni concentration on chlorophyll content in *H. verticillata* is shown in Table 3. Chlorophyll content increased with increase in concentration of the metal at 2 d of exposure as compared to control. At 2 d, maximum increases (P <0.05) of 28.76, 26.39 and 21.88% were found in total chlorophyll, chlorophyll-a and chlorophyll-b contents, respectively, at 25 μM as compared to control. However, carotenoid content increased with increase in concentration at 2 d with maximum increase of 78.18% (P<0.05) at 100 μM. Maximum decreases (P <0.05) of 48.91, 35.66, 41.94 and 66.67% were found in total chlorophyll, chlorophyll-a, chlorophyll–b and carotenoid contents, respectively, at 100 μM after 6 d of exposure as compared to control.

The effect of Ni on protein content in *H. verticillata* is shown in Table 4. Protein content increased with increased metal concentrations up to 4 d followed by a decrease at 6 d as compared to their respective controls. At 100 μM Ni, the maximum increase

Table 3. Effect of Ni on photosynthetic pigments (mg g^{-1} fw) of *H. verticillata* at different concentrations and exposure periods.

Conc. (µM)	Exposure periods (d)			Photosynthetic pigments (mg g^{-1} fw)
	2	4	6	
Control	2.26 ± 0.16^{c}	2.24 ± 0.20^{bcde}	2.29 ± 0.12^{a}	Total chlorophyll
	1.44 ± 0.06	1.45 ± 0.14	1.43 ± 0.07	Chlorophyll a
	0.64 ± 0.02^{bcde}	0.65 ± 0.05^{ab}	0.62 ± 0.02	Chlorophyll b
	0.55 ± 0.12^{e}	0.54 ± 0.11^{de}	0.54 ± 0.10^{a}	Carotenoid
5.0	2.35 ± 0.08^{b}	2.42 ± 0.12^{a}	2.51 ± 0.10	Total chlorophyll
	1.63 ± 0.03^{abc}	1.24 ± 0.15^{a}	1.20 ± 0.03^{a}	Chlorophyll a
	0.66 ± 0.04^{bcde}	0.68 ± 0.04^{a}	0.52 ± 0.01^{a}	Chlorophyll b
	0.62 ± 0.14^{de}	0.58 ± 0.10^{cde}	0.50 ± 0.17^{ab}	Carotenoid
10.0	2.69 ± 0.19^{a}	2.36 ± 0.09^{ab}	2.14 ± 0.12^{a}	Total chlorophyll
	1.70 ± 0.05^{a}	1.20 ± 0.04^{ab}	1.16 ± 0.08^{ab}	Chlorophyll a
	0.69 ± 0.09^{bc}	0.60 ± 0.08^{abe}	0.50 ± 0.08^{ab}	Chlorophyll b
	0.69 ± 0.16^{cd}	0.64 ± 0.16^{bcd}	0.48 ± 0.15^{abc}	Carotenoid
25.0	2.91 ± 0.15	2.33 ± 0.11^{abc}	1.75 ± 0.14^{b}	Total chlorophyll
	1.82 ± 0.08	1.18 ± 0.08^{abc}	1.08 ± 0.10^{bc}	Chlorophyll a
	0.72 ± 0.06^{ab}	0.55 ± 0.03^{c}	0.47 ± 0.03^{abc}	Chlorophyll b
	0.80 ± 0.13^{bc}	0.76 ± 0.14^{ab}	0.35 ± 0.11^{bcd}	Carotenoid
50.0	2.68 ± 0.13^{a}	2.30 ± 0.27^{abcd}	1.68 ± 0.10^{b}	Total chlorophyll
	1.68 ± 0.06^{ab}	1.10 ± 0.11^{cd}	1.04 ± 0.10^{c}	Chlorophyll a
	0.78 ± 0.02^{a}	0.47 ± 0.08^{d}	0.41 ± 0.04^{cd}	Chlorophyll b
	0.87 ± 0.12^{ab}	0.82 ± 0.15^{a}	0.25 ± 0.13^{de}	Carotenoid
100.0	2.32 ± 0.13^{bc}	2.20 ± 0.17^{bcde}	1.17 ± 0.11	Total chlorophyll
	1.62 ± 0.12^{abc}	1.02 ± 0.11^{d}	0.92 ± 0.08	Chlorophyll a
	0.68 ± 0.04^{bcd}	0.45 ± 0.04^{d}	0.36 ± 0.05^{d}	Chlorophyll b
	0.98 ± 0.05^{a}	0.73 ± 0.12^{abc}	0.18 ± 0.07^{e}	Carotenoid

The metal solutions were replaced with initial metal concentrations on 2nd and 4th d of exposure. All values are means of triplicates ± S.D. ANOVA $P<0.05$. Identical superscripts on values denote non-significant difference ($P<0.05$) between means of different treatments according to Duncan's multiple range test.

of 123.53% ($P<0.05$) and maximum decrease of 57.98% ($P<0.05$) in protein content were found at 2 and 6 d, respectively, as compared to their respective controls.

Analysis of the results (Figure 1) showed significant increase in cysteine content at all metal concentrations up to 4 d of exposure period as compared to their respective controls. Overall analysis of the results showed maximum increase of 40.94% in cysteine content at 50 µM after 2 d of exposure, whereas, maximum decrease of 21.03% was observed at 100 µM after 6 d of exposure as compared to their respective controls. The effect of different metal concentrations on thiol (– SH) content at different exposure periods is shown in Figure 2. Thiol content significantly increased with increase in metal concentrations at all exposure periods as compared to control except at 5 µM at 2 d. At 100 µM, maximum increases ($P<0.05$) of 42.5, 46.82 and 65.44% in – SH content were found after 2, 4 and 6 d of exposure, respectively, as compared to their respective controls.

Table 4. Effect of Ni on protein content (mg g^{-1} fw) of *H. verticillata* at different concentrations and exposure periods.

Conc. (μM)	Exposure periods (d)		
	2	4	6
Control	33.40±1.44	34.11±1.89	36.44±1.17
5.0	36.33±3.28	42.72±2.11[b]	33.27±1.46
10.0	42.44±1.82	49.41±1.63	30.65±1.03
25.0	56.68±1.36	53.97±1.60	24.33±1.61
50.0	64.72±2.10	45.05±1.06[a]	20.87±0.97
100.0	74.66±2.10	43.88±1.56[ab]	15.31±0.94

The metal solutions were replaced with initial metal concentrations on 2nd and 4th d of exposure. All values are means of triplicates ± S.D. ANOVA P <0.05. Identical superscripts on values denote non-significant difference (P <0.05) between means of different treatments according to Duncan's multiple range test.

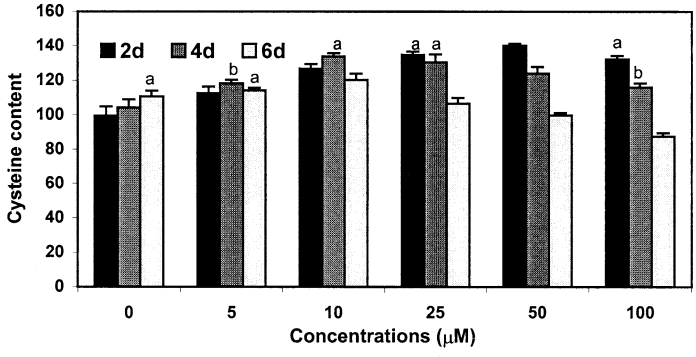

Figure 1: Effect of Ni on cysteine content (nmol g^{-1} fw) of *H. verticillata* at different concentrations and exposure periods. The metal solutions were replaced with initial metal concentrations on 2nd and 4th d of exposure. All values are means of triplicates ± S.D. ANOVA P <0.05. Identical superscripts on values denote non-significant difference (P <0.05) between means of different treatments according to Duncan's multiple range test.

Formation of malondialdehyde (MDA) was considered as a measure of lipid peroxidation in the plant samples. Decrease in MDA content of the plant was observed up to 4 d of exposure at all the metal concentrations as compared to their respective controls (Table 5). In contrast, the level of MDA content of the plant increased significantly (P<0.05) with increase in metal concentrations at 6 d of exposure after replacing the metal solution at 2 and 4 d of exposure. At 100 μM, the maximum level of MDA content of 8.66 μmol g^{-1} fw (48.03% increase over its control value) was found after 6 d of exposure.

In the present study, the plants have shown increase in fresh weight of the plants at lower concentrations and initial exposure periods followed by decrease with increase in metal concentration and exposure periods, which is in conformity with the study of Schickler and Caspi (1999). Submerged plants possess significant potential to

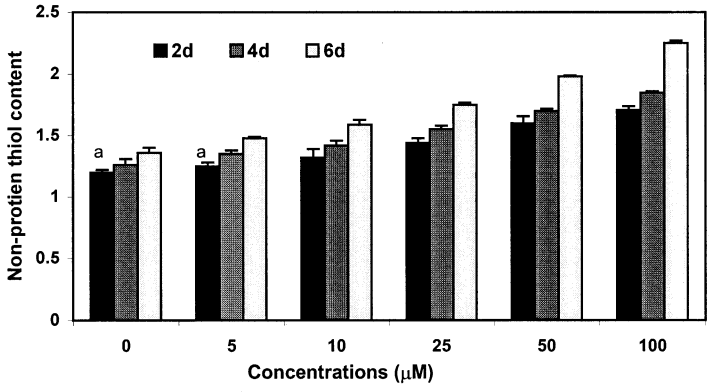

Figure 2: Effect of Ni on non-protein thiol content (μmol g^{-1} fw) of *H. verticillata* at different concentrations and exposure periods. The metal solutions were replaced with initial metal concentrations on 2^{nd} and 4^{th} d of exposure. All values are means of triplicates ± S.D. ANOVA $P<0.05$. Identical superscripts on values denote non-significant difference ($P<0.05$) between means of different treatments according to Duncan's multiple range test.

bioconcentrate metals due to their greater surface area as compared to non-submerged plants (Guilizzoni 1991; Rai et al. 1995; Sinha et al. 1997). In the present study, the metal accumulation in the plants of *H. verticillata* was found more than 3 times higher at 6 d of exposure by replacing the metal solutions at a regular interval, which is in conformity with earlier reports on metals (Sinha et al. 2002, 2003).

An increase in chlorophyll content has been reported at lower concentration of Ni, followed by decrease at higher metal concentration in different species of Lemnaceae (Xylander and Augsten 1992). Similar to the present study, Pandey et al (1999) reported stimulation in chlorophyll content at 25 μM Ni after 24 h followed by inhibition at 50 μM and above in *Spirodela polyrrhiza*. In an another study, an increase has also been reported in chlorophyll content of *Hydrilla verticillata* at lower concentration of essential metals, Cu and Fe, and initial exposure periods (Gupta et al. 1996; Sinha et al. 1997). Recently, Sinha et al. (2003) reported similar findings in submerged plant of *Najas indica* treated with Fe under repeated metal exposure. The decrease in chlorophyll content with increase in metal concentration and exposure periods might have occurred due to iron deficiency induced by the presence of nickel in the growth medium as reported by Pandey and Sharma (2002) in 42 d old cabbage plants exposed to 500 μM Ni. Carotenoid, a non-enzymatic antioxidant, is a part of photosynthetic pigment, playing an important role in protection of chlorophyll pigment under stress conditions. Recently, Sinha et al (2003) reported an increase in carotenoid content in the submerged plant of *Najas indica* at lower concentration of Fe under repeated metal exposure after 3 d. An increase in carotenoid content under metal stress may be attributed to the strategy of plants to overcome the metal induced oxidative stress (Kenneth et al. 2000).

Gupta et al. (1996) reported an increase in protein content with increase in Cu concentration (up to 80 μM) in plants of *Hydrilla verticillata* up to 96 h, however, protein content decreased at 8 μM onwards after 168 h. In a study on different species

Table 5. Effect of Ni on malondialdehyde content (μmol g^{-1} fw) of *H. verticillata* at different concentrations and exposure periods.

Conc. (μM)	Exposure periods (d)		
	2	4	6
Control	5.53±0.33[a]	5.67±0.66	5.85±0.54[abc]
5.0	5.11±0.84	5.22±0.94	6.07±0.47[abc]
10.0	4.93±0.95	5.01±0.61	7.24±0.47[ab]
25.0	4.56±0.78[a]	5.11±0.97	7.73±0.32[ac]
50.0	4.40±0.43[a]	5.23±1.04	8.24±0.45[b]
100.0	4.82±0.38	5.34±1.01	8.66±0.55[a]

The metal solutions were replaced with initial metal concentrations on 2nd and 4th d of exposure. All values are means of triplicates ± S.D. ANOVA $P<0.05$. Identical superscripts on values denote significant difference ($P<0.05$) between means of different treatments according to Duncan's multiple range test.

of Lemnaceae, Xylander and Augsten (1992) reported an increase in protein content at 30 μM Ni at first day of exposure, however, it decreased at higher Ni concentration (100 μM). Results of the present study are in agreement with findings of these authors showing increased protein content at lower metal concentrations and initial exposure periods.

It has also been shown that plants resist stress by increasing components of their intrinsic defensive system. For example, some antioxidants like Glutathione (GSH), thiols, cysteine, carotenoids and ascorbate may also play a role in inducing resistance to metals (Halliwell and Gutteridge 1993). Antioxidant system in plants render the tolerance at lower metal concentration and initial exposure period, however, when exposed to elevated concentrations of metals for prolonged period, plants eventually fail to maintain metal homeostasis and develop stress symptoms. Further, the increase in cysteine and thiol contents in Ni treated plants of *H. verticillata* is in conformity with the findings of other workers (Sinha et al. 1997; Gupta et al. 1998) on the same plant treated with Fe and Hg. Since non-protein thiol content is considered to be an indicator of phytochelatin (PC) synthesis in plants, it is quite possible that Ni treated plants of *H. verticillata* may have synthesized PCs to account for detoxification of metals as reported in Hg treated plants of *Hydrilla verticillata* (Gupta et al. 1998).

Metal-induced membrane destablization is mostly attributed to increased peroxidation of membrane via increased production of free radicals (Halliwell and Gutteridge 1993). Several metals such as, Cu and Ni are known to induce peroxidation of lipids in plants (De Vos et al 1989; Rao and Sresty 2000). Similar to other reports, higher concentrations of Ni in this study resulted in an accumulation of lipid peroxidation product (MDA) in plants of *H. verticillata* at longer (6 d) exposure period under repeated Ni exposure. Sinha et al. (1997) also reported an increase in MDA content in Fe treated plants of *Hydrilla verticillata*. The reason for inhibition of MDA by Ni at earlier exposure periods (up to 4 d) could be due to stimulation of reducing capacity of plant tissue by increasing sulphydryl content, which protects the membrane from oxidative attack by oxygen free radicals (Halliwell and Gutteridge 1993). Moreover, long-term repeated exposures of *H. verticillata* to Ni were found to be extremely toxic leading to plant death.

In conclusion, the plants of *H. verticillata* were found to be tolerant to Ni stress up to 4

Table 6: Regression analysis of various responses in the Ni treated plant of *H. verticillata* at different exposure periods.

Parameters	Regression analysis at different exposure periods		
	2d	4d	6d
Accumulation	Y= 81.63+13.93x, r=0.98	Y= 198.14+37.84x, r=0.99	Y= 323.29+42.77x, r=0.98
Fresh wt.	Y= 3.57-0.001x, r=-0.81	Y= 3.97-0.004x, r=-0.67	Y= 3.90-0.009x, r=-0.71
Total chlorophyll	Y= 2.55-0.001x, r=-0.08	Y= 2.35-0.001x, r=-0.63	Y= 2.30-0.01x, r=-0.94
Chlorophyll a	Y= 1.63+0.0005x, r=0.15	Y= 1.30-0.003x, r=-0.83	Y= 1.26-0.004x, r=-0.84
Chlorophyll b	Y= 0.68+0.0004x, r=0.33	Y= 0.64-0.002x, r=-0.90	Y= 0.55-0.002x, r=-0.89
Carotenoid	Y= 0.63-0.004x, r=0.93	Y= 0.62-0.002x, r=0.64	Y= 0.50+0.004x, r=-0.94
Protein	Y= 38.39+0.41x, r=0.94	Y= 44.09+0.02x, r=0.14	Y= 33.11-0.20x, r=-0.94
Cysteine	Y= 116.42+0.25x, r=0.62	Y= 121.32-0.003x, r=-0.01	Y= 115.58-0.28x, r=-0.93
Non-protein thiol	Y= 1.26+0.005x, r=0.95	Y= 1.34+0.006x, r=0.96	Y= 1.47+0.008x, r=0.97
MDA	Y= 5.06-0.005x, r=-0.50	Y= 5.26-0.0001x, r=-0.01	Y= 6.48+0.02x, r=0.86

d under repeated metal exposure, however, there was significant negative effect after 6 d. The high concentration of metal and increased exposure periods decreased chlorophyll and protein content, but increased antioxidant levels helped the plants to cope with the stress under repeated metal exposure.

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