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Full Length Research Paper

Nickel accumulation by *Colocassia esculentum* and its impact on plant growth and physiology

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The remediation of heavy metal-contaminated sites using plants presents a promising alternative to current methodologies. In this study, the potential of *Colocassia esculentum* for Nickel (Ni) accumulation was determined. *C. esculentum* plants exposed to Ni, demonstrated capability to accumulate on average, more in shoots as compared to roots, suggesting better translocation of Ni from root to shoot. High metal content in soil caused reduction in growth parameters and an increase in oxidative stress. Under heavy metal stress, an increase in catalase, peroxidase, ascorbic acid and proline were observed in the roots along with some anatomical changes. Lipid peroxidation showed a slight increase by Ni treatment along with some anatomical changes in the root. This work demonstrates that metal induced oxidative stress occurs by the presence of heavy metals at higher concentrations. It also suggests that superior antioxidative defenses, particularly catalase activity, may play an important role in *C. esculentum*. The outcome of this study corroborate that *C. esculentum* is a suitable candidate for the phytoremediation of Ni contaminated soil and could be considered as a potential Ni hyperaccumulator plant species.

Key words: *Colocassia esculentum*, heavy metal, nickel, oxidative stress, tolerance.

INTRODUCTION

Since the beginning of the industrial revolution, pollution of the biosphere with toxic metals has accelerated dramatically. Plants are one pathway for toxic metal mobilization into the human food chain, and paradoxically they may also provide an elegant means of reducing this spread. Elements such as Cu, Zn, Ni, Co, Fe, Mo and Mn are essential mineral nutrients, and play a significant role in gene expression, biosynthesis of proteins, nucleic acids, growth substances, chlorophyll and secondary metabolites, and carbohydrate and lipid metabolism (Rengel, 1999). At high concentrations, nickel reduce or inhibit shoot and root growth (Lyngby and Brix, 1984; Brune and Dietz, 1995) though low concentrations of nickel may also stimulate the germination and growth of various crop species (Mishra and Kar, 1974).

The main sources of aerial copper and nickel contamination are mining and smelting. Nickel is frequently found in phytotoxic concentrations in soils derived from serpentine, which is an ultra basic Ni and Cr-rich rock containing ferromagnesium minerals (Woolhouse, 1983). Increased acidity of soils has increased the mobility and solubility of metals, presenting phytotoxic problems (Hutchinson and Whitby, 1977).

Heavy metals, unlike organic pollutants, cannot be chemically degraded or biodegraded by microorganisms. An alternative biological approach to deal with this problem is Phytoremediation, which removes pollutants, including toxic metals, from the environment using plants (Salt et al., 1995; Cunningham and Ow, 1996). Hyper - accumulators of Co, Cr, Cu, Pb, or Ni have concentrations of 1000 ppm dry mass, whereas hyperaccumulators of Mn or Zn are defined as those containing 10 000 ppm dry mass (Baker and Brooks, 1989). As the scope of the study is to evaluate the response of the plant species toward heavy metal (Ni) stress we used *Colocasia esculentum* reported as hyperaccumulator in the present study.

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Abbreviations: AAS, Atomic absorption spectroscopy; **DTPA-TEA,** diethyltriamine penta acetic acid-triethanol amine; **PVP,** poly vinyl pyrrolidone.

High concentrations of heavy metals generate stress responses in plants, including oxidative stress. Plants exposed to Cd, Ni and Zn often accumulate AOS and undergo oxidative stress likely due to metal induced disturbance of AOS-generating metabolism and deactivation of the process required for AOS destruction (Dietz et al., 1999). Accumulation of hydrogen peroxide is a typical response of many plant species to toxic concentrations of metals (Schutzendubel et al., 2001). Protection against AOS and peroxidation reactions is provided by antioxidative enzymes and other nonenzyme antioxidants.

The aim of this research was to study the efficacy of *Collocassia esculetum* for Ni uptake and its response to overcome metal stress along with some adaptations by elucidating few anatomical changes in the roots.

MATERIALS AND METHODS

Experimental design

C. esculentum were washed under running tap water and the rhizomes were transferred to black plastic pots containing garden soil (1 Rhizome/2.5 kg soil/pot). Rhizomes were acclimatized for three days in the pots and were exposed to increasing concentration of Ni in form of Nickel nitrate (0, 50, 100, 150, 200 and 250 mg/kg).

Heavy metal analysis

Plant sample preparation

Different tissues of *C. esculentum* were harvested after 10 weeks and dried at 80°C to constant weight. Known weight of dry plant biomass (root, stem and leaf) was digested in an acid mixture of nitric acid: sulphuric acid: perchloric acid (2:1:1, v/v). Acid mixture was allowed to evaporate and the metal residues were dissolved in 25 ml of 0.1 N HCl. Ni concentrations in the plant digest was measured using AAS (GBC-911), on dry weight basis (Ganje and Page, 1974).

Soil sample preparation

To analyze the residual Ni, the soil samples were prepared by DTPA- TEA method (Lindsay, 1972). Mixture of 10 gm dry soil sample and 20 ml of DTPA-TEA reagent was kept on a shaker for 2 h at 250 rpm at 25°C. The mixture was filtered through filter paper and the filtrate was subjected to AAS (GBC-911) for Ni analysis.

Biochemical analysis

Total chlorophyll content

Total chlorophyll determination in *C. esculentum* leaves was performed (Arnon, 1949).

Total protein content

Protein content in the extracts was determined according to Lowry method (Lowry et al., 1951). Fresh root tissues were homogenized in an ice cooled mortar in extraction buffer containing 20 m*M* Tris (pH 8.0), 0.25*M* sucrose, 5% PVP and 3 µl of β- mercaptoethanol. The homogenate was centrifuged at 12,000 g for 20 min. and the supernatant was used for protein assays. The protein amount was determined following a standard curve obtained with bovine serum albumin as standard (1 mg/ml).

Enzyme assay

For enzyme assay, roots of Ni treated plants were homogenized in an ice cooled mortar in phosphate buffer (pH 6.8). The homogenate was centrifuged at 12,000 g for 20 min at 4°C and the supernatant was used for enzyme assays. Catalase activity was estimated by permanganate method (Povolotskaya and Sedenka, 1956; Gopalachari, 1963), whereas peroxidase activity was determined following the method of Kar and Mishra (1976). The catalase activity was calculated as mg H_2O_2 destroyed in 5 min by 1 g plant tissue and the peroxidase activity was expressed as absorbance units (0.1 difference in absorbance value was taken as one unit of enzyme activity) per mg protein.

Proline

Free proline content was estimated following the procedure (Bates et al., 1973). Fresh roots tissues were homogenized in 3% aqueous sulphosalicylic acid and the homogenate was filtered. To an aliquote of 2 ml filtrate, 2 ml of acid ninhydrin was added followed by addition of 2 ml glacial acetic acid and boiling for 1 h. The mixture was extracted with toluene and the free proline was estimation for the organic phase at 520 nm. The amount of proline in the sample was calculated using a standard curve prepared from pure proline.

Lipid peroxidation

TBARS (thiobarbituric acid reacting substance) were determined according to the methods of Heath and Packer (1968). Fresh Ni treated root tissues were homogenized in trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid and incubated at 95°C in water bath for 30 min. Then, the mixture was quickly cooled in ice bath and centrifuging at 10,000g for 5 min and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of malondialdehyde was calculated by using extinction coefficient of 155 mM^{-1} cm^{-1}.

Ascorbic acid content

Fresh Ni treated roots were homogenized in ice cold 6% TCA at 4°C and was centrifuged at 10,000 g for 20 min. The supernatant was analyzed by dinitrophenylhydrazine method (Mukherjee and Chaudhuri, 1983) using standard ascorbic acid to compare the changes in vitamin C content of control and Ni treated plants.

Anatomical studies

To study the anatomical changes, temporary slides of cross section of control and 100 mg/kg Ni treated roots of *C. esculentum* were observed and photographed using Carl-Zeiss Image Analyzer at a magnification of 320X.

Statistical analysis

All values reported in this work are mean ± SD of three replicates.

Table 1. Ni accumulation in root, stem and leaf of *C. esculentum* treated with increasing concentrations of heavy metal.

Metal (mg)/kg soil	Ni (mg/g) root	Ni (mg/g) stem	Ni (mg/g) leaves
Control	0.00±0.00	0.00±0.00	0.00±0.00
50	10.194±3.607	10.17±1.702	9.545±2.040
100	10.861±1.403	10.49±1.365	9.869±2.885
150	12.072±2.811	11.549±1.847	10.335±3.183
200	12.753±3.056	11.873±1.915	10.738±1.872
250	14.638±2.551	12.761±1.647	11.04±3.005

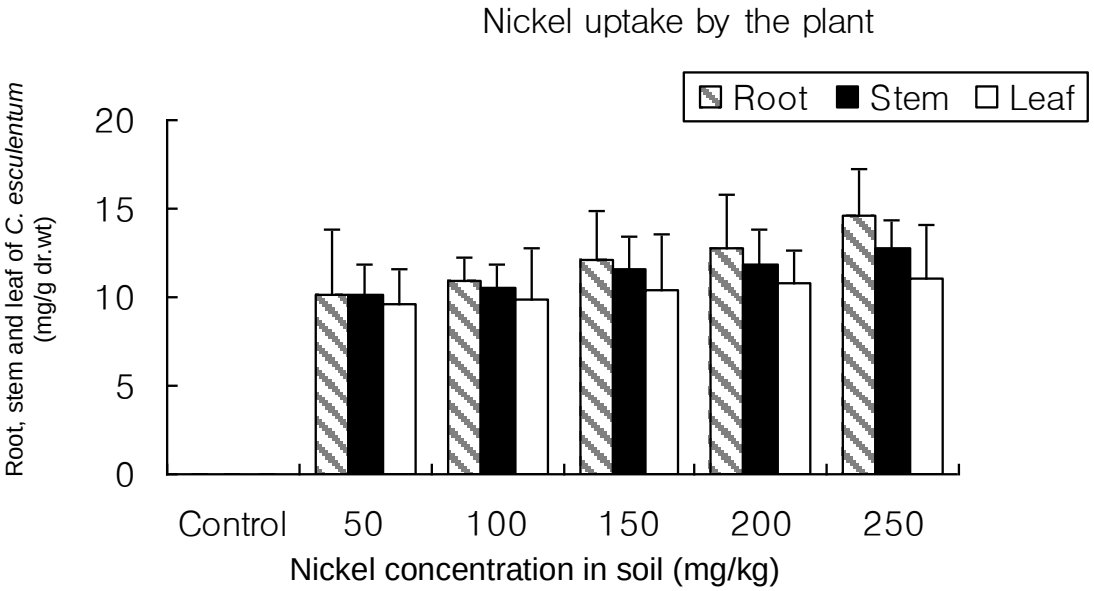

Figure 1. Ni accumulation in root, stem and leaf of *C. esculentum* treated with increasing concentrations of heavy metal.

RESULTS

The results of this study showed elevated concentrations of Ni in shoots and roots of *C. esculentum* (Table 1). The residual Ni in the soil decreased on growing *C. esculentum* (Figure 1). The average Ni concentrations were 21.67 mg/g (21670 ppm) in shoots and 12.1 mg/g (1210 ppm) in roots of *C. esculemtum*. Ni concentrations >1000 mg/kg in shoots confirm Ni hyperaccumulation. These results indicated the extent of the tolerance to Ni of this plant species. The plant showed a slight reduction in growth with respect to increase Ni concentration in soil. Biomass production of plant was reduced to 8.91% than that of the unexposed controls (Figure 2).

The chlorophyll pigment of *C. esculentum* leaves declined slightly with the increase in heavy metal concentration. The relationship was inversed with respect to Ni accumulation in the plant (Figure 3).

C. esculentum showed a tremendous rise in total protein content in root. The increase in root protein is almost 1.5 times more than the protein in untreated roots (Figure 4).

Ni assimilation induces a greater increase in the enzymatic activities. Both catalase and peroxidase activity increased under Ni stress (Figures 5 and 6). The average increase in catalase and specific peroxidase activity was 1.2 and 1.1 times, respectively, in Ni treated roots of *C. esculentum* at the higher concentration.

The data indicated that the Proline in roots was directly correlated with the heavy metal concentration in soil. With increase in Ni concentration, the proline content was also increased (Figure 7). The thiobarbituric acid reacting substance (TBARS) content of roots increased slightly with respect to control, by about 1.15 to 2.02 fold at low and high Ni concentration, respectively (Figure 8). The ascorbic acid content in the root increased significantly under Ni stress. The average increase of ascorbate under Ni stress was around 3.9 fold (Figure 9).

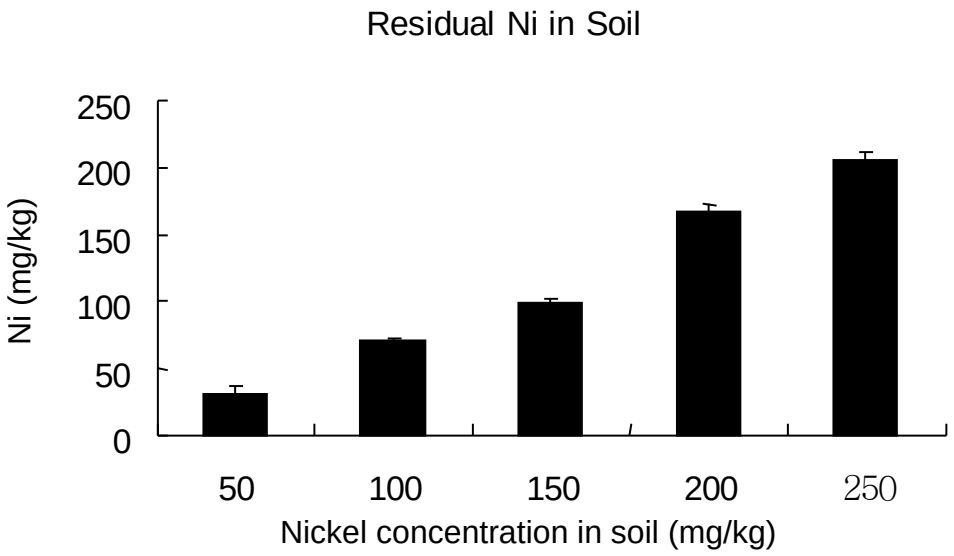

Figure 2. Residual Ni in soil after harvesting *C. esculentum.*

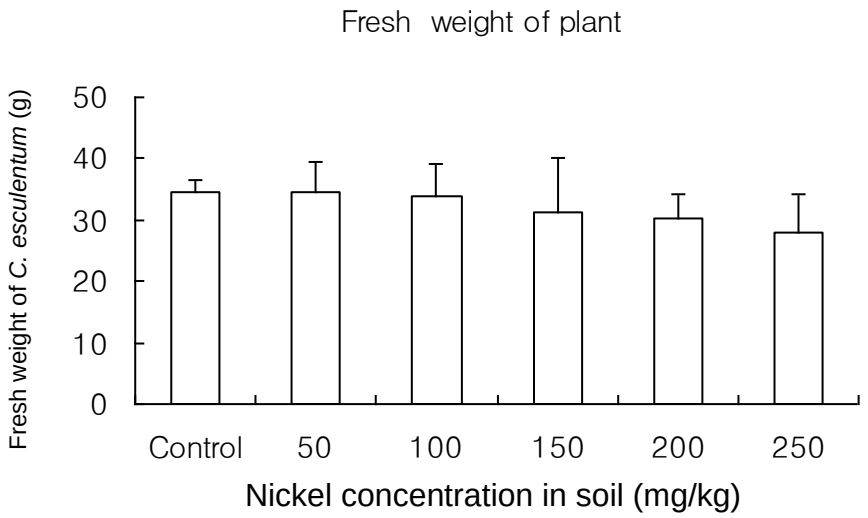

Figure 3. Effect of increasing concentrations of Ni on fresh weight of *C. esculentum.*

C. esculentum roots treated at 100 mg/kg of Ni showed significant changes, against control. The Ni roots treated showed a decrease in number of the cortical cells, with some elongated aerenchyma like cells instead of the normal parenchymatous tissue along with increase in number of xylem tissues as compared to control. The cell walls showed thickening in metal treated plants with respect to control (Figure 10).

DISCUSSION

In the present study, the Ni uptake and several biochemical and physiological responses representing the oxidative damage and protection in roots of *C. esculentum* was examined. The plant readily took up Ni from the soil. Also *C. esculentum* is a reported Cd hyperaccumulator (Patel et al., 2005). Accumulation of more than one metal is one of the characteristics of metal hyperaccumulator. *C. esculentum* showed typical characteristics of a hyperaccumulator by its ability to uptake both Cd and Ni under field conditions and in the present study, it can be classified under the species as a Ni hyperaccumulator (Reeves et al., 1983). Ni stress in plant led to decrease chlorophyll pigment in *C. esculentum*. It can cause ultra structural changes in the chloroplast leading to inhibition in photosynthesis and reduced growth in plants. Accumulation of Cr by plants

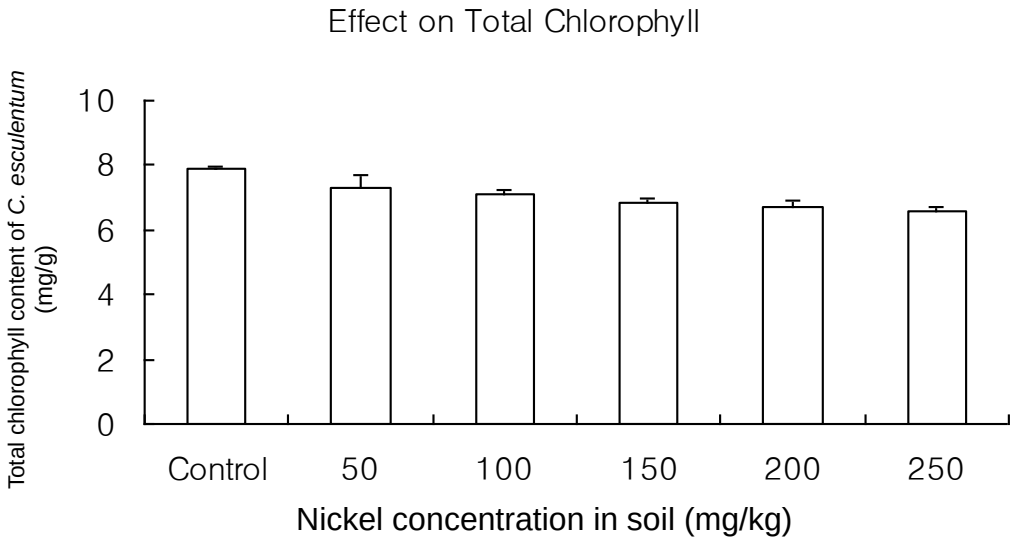

Figure 4. Effect on total chlorophyll content of *C. esculentum* treated with increasing concentrations of Ni.

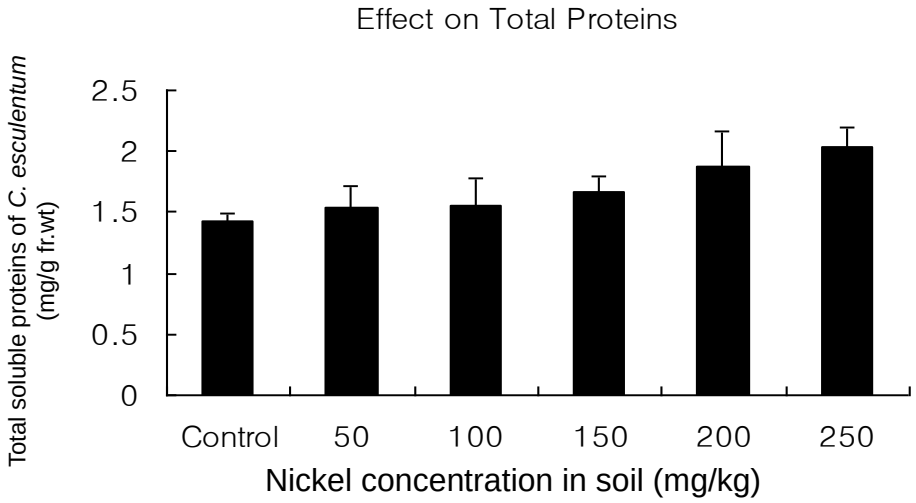

Figure 5. Effect of increasing concentrations of Ni on total soluble proteins of *C. esculentum.*

can reduce growth, induced chlorosis in young leaves, reduce pigment content and caused ultra structure modification of the chloroplast and the cell membrane (Panda et al., 2003; Hu et al., 2004).

In plants, both essential and nonessential heavy metals induce the formation of thiol rich peptides, known as metal binding peptides or Phytochelatins. It probably plays a central role in the homeostatic control of metal ions in plants (Steffens, 1990). The increase in total protein under Ni stress in our study may be due to induction of phytochelatins or the metallothionein protein, which may be involved in the physiological mechanism of metal tolerance in plant (Tomsett et al., 1389; Salt et al., 1989).

It is known that a variety of abiotic stress cause molecular damage to plants, either directly or indirectly, through the formation of AOS. To scavenge excess AOS and to avoid oxidative damage, plants have evolved various protective mechanisms, one of which is the enzymatic antioxidant system operating with the simultaneous and sequential action of a number of enzymes, such as SOD, CAT, POD and GR. An increase in ROS production was also evidenced by the enhanced catalase and peroxidase activities.

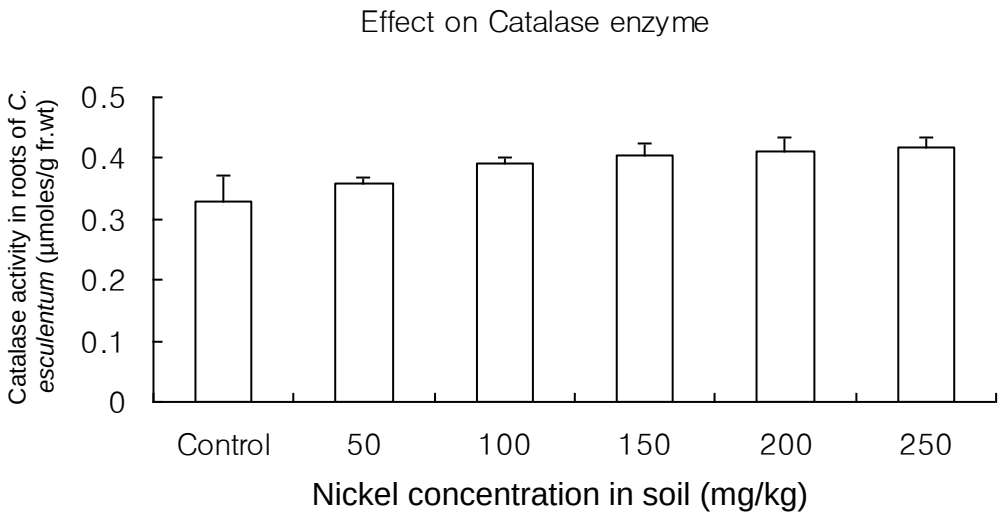

Figure 6. Catalase activity in roots of *C. esculentum* treated with Ni.

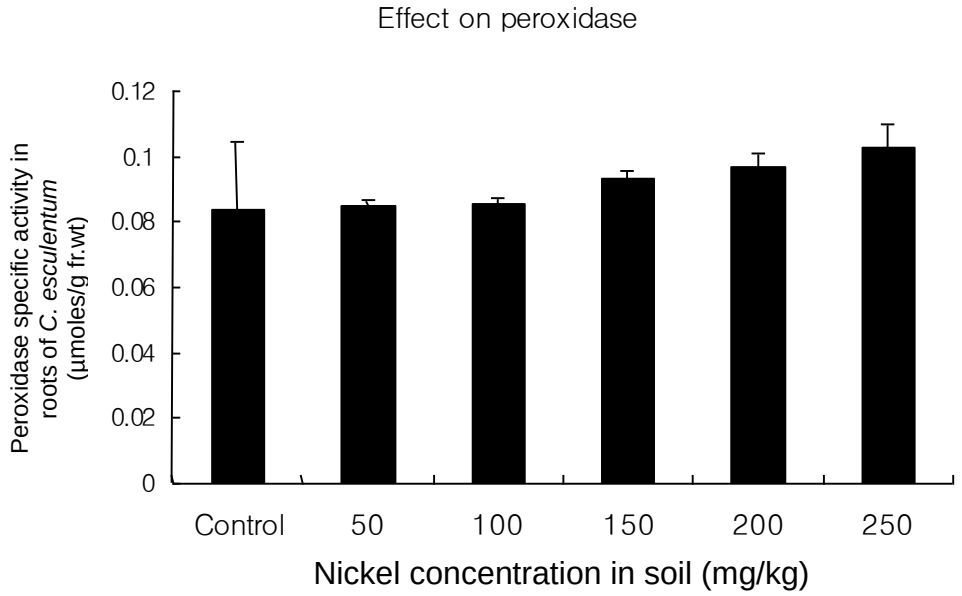

Figure 7. Peroxidase specific activity in roots of *C. esculentum* treated with Ni.

The catalase and peroxidase exhibited an inducible activity in the presence of increasing concentration of Ni. Catalase, located in peroxisomes, mitochondria, and the cytosol, can scavenge H_2O_2 without co-substrates. Peroxidases, distributed in the cytosol, vacuoles, and cell walls, as well as in extracellular spaces, can use guaiacol or ascorbate as an electron donor and can also eliminate H_2O_2, having a higher affinity for H_2O_2 than CAT. The significant increase in the activities of these enzymes at latter stages might greatly contribute to the degradation of H_2O_2. This indicated that the moderate concentration of Ni activated a sufficiently defensive mechanism against oxidative stress by inducing the potent antioxidant enzyme in the roots of *C. esculentum*. Schutzendubel et al. (2001) reported that treatment of Cd at 50 µM induced increases in POD activities in pine root tips, which was accompanied by accumulation of phenolics and lignification. While a variety of reactions were catalyzed by POD for cell wall rigidification, H_2O_2 served as a necessary substrate for these processes (Schopfer, 1996; Schutzendubel et al., 2001).

The control of reactive oxygen species levels can be also obtained by non-enzymitic antioxidants composed of metabolites such as ascorbate or tocopherol and also the induction of proline (Schutzendubel and Polle, 2002). The enhanced levels of lipid peroxides in roots indicated that

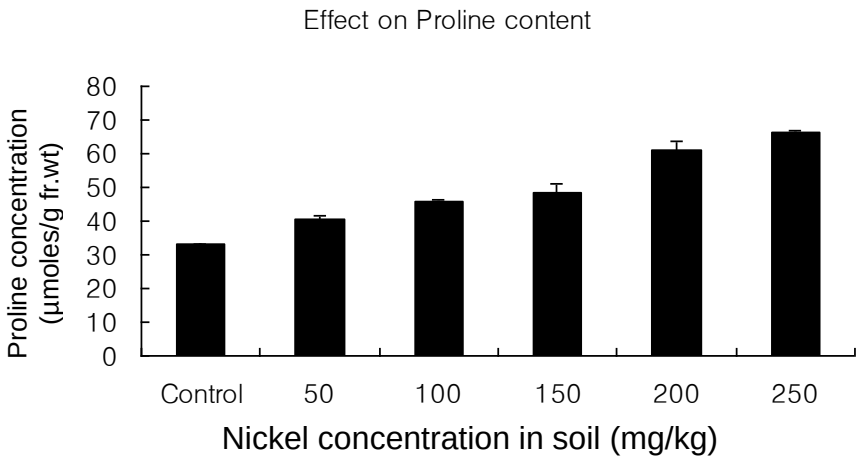

Figure 8. Effect of increasing concentration of Ni on Proline content of *C. esculentum.*

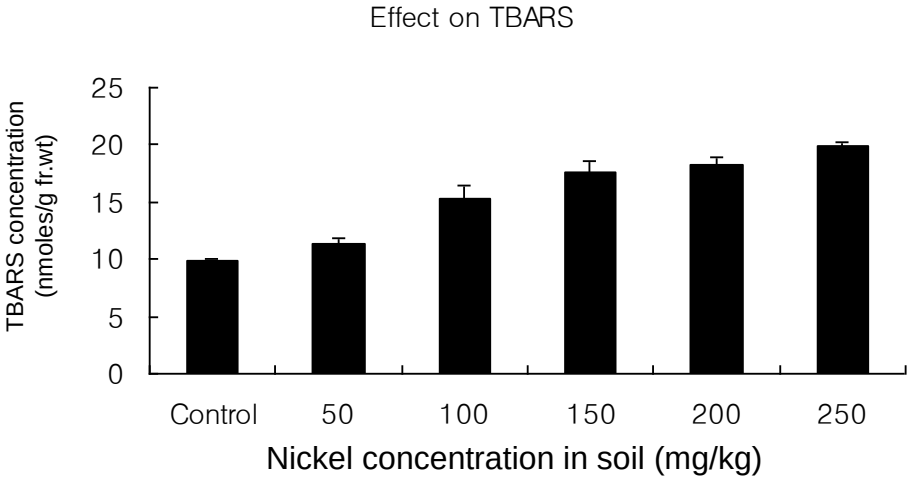

Figure 9. Effect of increasing concentrations of Ni on TBARS of *C. esculentum.*

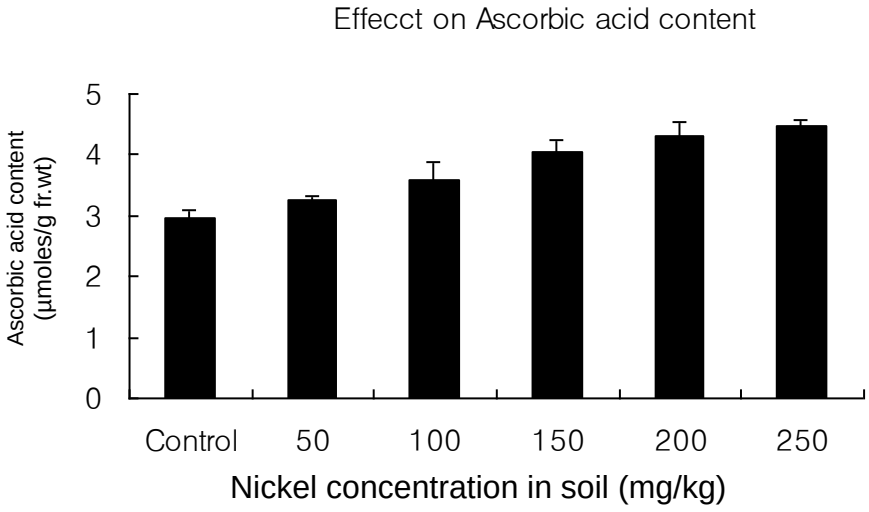

Figure 10. Effect of increasing concentrations of Ni on ascorbic acid content of *C. esculentum*.

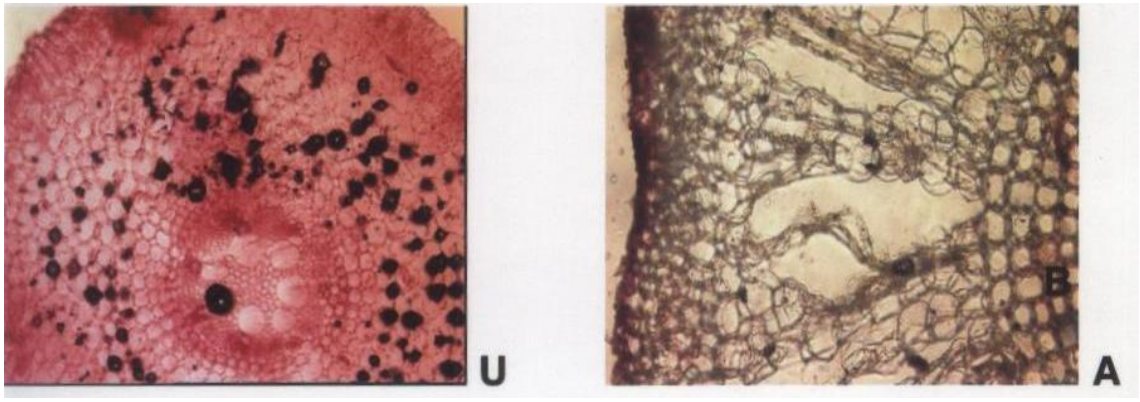

Figure 11. Anatomical changes observed in the roots of *C. esculentum* treated with 100 mg/kg of Nickel heavy metal (320×). U=untreated root, A=Nickel treated root.

excess Ni accumulation triggered the production of ROS, which caused the oxidative damage to plasma membrane. Moreover, the lipid peroxidation caused under Ni stress is very low as compared to arsenic stress in *C. esculentum*. Therefore, we measured the contents of ascorbate, the major components for plant cells to dispose of H_2O_2 in some cellular compartments (Del et al., 2002). Our results indicated that the increasing trend in the cellular non enzymatic antioxidant like ascorbic acid content in roots after Ni treatment, suggesting its role in scavenging of H_2O_2, a reaction catalyzed by APX and their ability to detoxify the reactive oxygen species directly (Gallego et al., 1996). Thus, the consumption of H_2O_2 might lead to a decrease in Ni-induced oxidative stress in plants. Proline is a substance which induces osmotic adjustment. It has been suggested that proline is a source of energy, carbon and nitrogen for the recovering tissues (Stewart and Lee, 1974). From the results obtained, it is suggested that proline can protect cells against damage induced by metal stress.

The increase in number of xylem tissues may be due to increased rate of transpiration under Ni stress. The thickening of the cell wall and the reduction in number of cells in the cortical region of the roots may be due to accumulation of Ni in the cell wall and vacuoles of the cortical cells. Transmission electron microscopy and electron energy loss spectroscopy data, showed a distribution pattern of electron dense granules of Ni in vacuoles existed in the vacuolar precipitates of meristematic or cortical parenchyma cells of the differentiating and mature roots, in between cell wall and,plasmalemma and plasmolysis of cortical cells of *Allium cepa* when treated with 1 mM Cd for 72 h (Donghua and Ingrid, 2004).

Conclusion

In conclusion, *C. esculentum* can prove to be a Ni hyper accumulator by elevating the antioxidant levels like ascorbic acid and some antioxidant enzyme activities, especially catalase and peroxidase activity, directly or indirectly inhibiting AOS generation and reducing MDA production and undergoing few anatomical changes to adapt under Ni stress. The results of this study, along with the fact that this plant species is capable of growing in harsh conditions, the data reported, corroborates that *C. esculentum* is a suitable candidate for the reclamation of Ni contaminated soil.

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