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Nickel Tolerance and the Complexing Role of Histidine in *Raphanus sativus*

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The effect of nickel (Ni) on growth and some tolerance strategies with regard to heavy metal tolerance mechanism was investigated in radish (*Raphanus sativus*) seedlings. The protective effect of histidine on nickel stress conditions was also monitored. The seedling growth decreased with an increase in metal concentrations. The inhibitory effect was more pronounced in the root elongation than in the shoot elongation. Increasing Ni supply showed a progressive increase of Ni concentrations in the roots and shoots. Ni content was higher in the shoots than in the roots. In the presence of nickel, radish exhibited an antioxidative defense mechanism, as evidenced by the elevated malondialdehyde(MDA), showing that nickel is an efficient inducer of lipid peroxidation. Exposure of radish to elevated concentrations of nickel was accompanied by an increase in the proline content. Supplemental histidine in the presence of Ni ameliorated metal-induced growth inhibition and lipid peroxidation. Combinations of Ni and histidine resulted in a significant decline in proline content compared with Ni stress alone, indicating that histidine may provide protection against the adverse effect of Ni stress. From the results it is suggested that histidine is an efficient chelator by complexing metal ion within the plant and may play a role in nickel tolerance implicated in metal detoxification.

Key Words : Nickel tolerance, Lipid peroxidation, Proline, Histidine, Radish(*Raphanus sativus*)

1. Introduction

Plants are exposed to natural climatic or edaphic stresses, for example, high irradiation, heat, chilling, frost, drought, flooding and nutrient imbalances. Since plants are sessile organisms and have only limited mechanisms for stress avoidance, they need adaptable means for acclimation to changing environmental conditions. In order to improve a plant's protection, it is important to understand the mechanisms contributing to stress tolerance.

In soils influenced by human activities a range of different problems such as overexploitation, salinity, acidification and contamination by various pollutants including heavy metals have been reported. Nickel pollution of soils and waterways occurs mainly as a result of effluent disposal from the mining, smelting and electroplating industries and from sewage sludge and compost[1]. For the recultivation of degraded soils and the reclamation of industrial sites, stress-tolerant plants are required. In order to devise new strategies for phytoremediation and improved tolerance, it is important to understand the basic principles as to how the pollutants are taken up and act at the cellular and tissue level.

Nickel has been defined as an essential micronutrient for plants. The nickel requirement of plants is generally very low, 1.7 nmol g^{-1} Ni or less in tissue dry biomass[2,3]. Nickel is strongly phytotoxic at high concentrations and has a destructive effect on growth, mineral nutrition, photosynthesis and membrane function[4,5]. Nitrogen-fixing microorganisms require nickel for the enzyme that reprocesses some of the hydrogen gas generated during fixation (hydrogen uptake hydrogenase). Nickel has been known to involve in enzymatic activity in legumes[6]. Urease is the only known nickel- containing enzyme in higher plants. Nickel-deficient plants accumulate urea in their

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leaves and, consequently, show leaf tip necrosis. The most common deficiency symptoms of nickel are chlorosis, and inhibited photosynthesis and respiration[7]. However, the mechanisms governing nickel toxicity are not well understood.

Plants display a wide range of adaptations to soil with contrasting metal contents. Instead of excluding toxic metals, so-called hyperaccumulators accumulate metals such as Ni, Zn or Co in their aboveground biomass. This trait is highly attractive for the development of technologies aimed at the decontamination of metal polluted environments using plants[8]. Methods for phytoremediation in Ni-contaminated sites are being developed using Ni hyperaccumulator[9,10]. Nickel hyperaccumulator species are able to take up and store Ni without detrimental effect on growth or cell function[11]. The mechanisms how hyperaccumulators are protected from the toxic effects of Ni in their tissues are not yet fully understood. The high amount of metals in the hyperaccumulator plant's tissues suggests the existence of defense mechanisms to avoid the harmful effects caused by the metals. Metal chelation by specific low molecular mass ligands is another major process determining metal tolerance of plants. More is known in terms of Ni tolerance in hyperaccumulator plants where two mechnisms, complexation and compartmentalization, appear to be responsible for Ni detoxification. In the hyperaccumulator plants, chelators such as free histidine[12,13] and citrate[14] have been implicated in metal detoxification. The chelators have been shown to be coordinated with Ni *in vivo* and to play a role both in Ni tolerance and transport to the shoot[14,15]. The complexation of Ni by histidine and organic acids has been reported in a number of hyperaccumulor plants such as *Alyssum lesbiacum*[12] and *Thlaspi goesingense*[16].

Heavy metal toxicity can cause oxidative stress damage by overproduction of toxic oxygen species, particularly H_2O_2[17], proposed as part of the signaling cascade leading to protection from the stress. Increasing evidence has indicated that much of the injury to plants due to various environmental stresses is associated with oxidative damage through direct or indirect formation of reactive oxygen species. These oxygen species attack lipids, proteins and nucleic acids, causing lipid peroxidation, protein and DNA mutation[18]. Plant cells have evolved efficient strategies to counteract oxidative stress.

Nickel, a non-redox-active metal, cannot generate free radicals directly in single-electron reactions. However, Ni could indirectly trigger oxidative stress by disrupting the balance of formation and destruction of active oxygen species associated with normal cellular metabolism.

The purpose of this study was to elucidate the effect of Ni on seedling growth and tolerance strategies with regard to heavy metal tolerance mechanism in *Raphanus sativus*. This study also monitored whether histidine as a metal chelator play a role in the tolerance mechanism toward Ni involved in Ni-induced oxidative stress.

2. Materials and Methods

2.1. Plant materials and growth conditions

Seeds of radish (*Raphanus sativus* L.) were germinated in a mixture of Perlite and vermiculite moistened with deionized distilled water in the dark for 7 days at 25±1℃ in a growth chamber. Seven-day-old seedlings were transferred to vessels containing nutrient solution containing 200 μM $Ca(NO_3)_2 \cdot 4H_2O$, 100 μM $MgSO_4 \cdot 7H_2O$, 100 μM $K_2HPO_4 \cdot 3H_2O$, 5 μM FeNaEDTA, 1 μM $MnSO_4 \cdot 4H_2O$, 5 μM H_3BO_3, 0.1 μM $(NH_4)6Mo_7O_{24} \cdot 4H_2O$, 0.2 μM $ZnSO_4 \cdot 7H_2O$ and 0.15 μM $CuSO_4 \cdot 5H_2O$ in deionized water. The nutrient solution was buffered to pH 5.7 with MES/NaOH, aerated, and changed every day. After a week, nickel (Ni, as $NiSO_4 \cdot 6H_2O$) was added to the nutrient solution at a concentration of 0, 0.5, 50, 100, 250 and 500 μM. 100 μM L-histidine (Sigma Chemical Co.) was supplied as a metal chelator. Plants were grown in 100 ml glass culture tube in a growth chamber (Eyelatron, FLI-301N, Japan) at a day/night cycle of 16 h /8 h, at 25±1℃, with a relative humidity between 50 and 60% and a light intensity of 80 μmol $m^{-2}s^{-1}$ under white fluorescent lamps. Growth culture solutions were changed every week. After an additional 7 days, plants were harvested, washed with distilled water and analyzed for further studies.

2.2. Growth measurements

After a quick wash with destilled water, each individual plant was gently dried on blotting paper and the shoot and root lengths were measured. The fresh weight and dry weight were also measured to

0.1 mg accuracy with an analytical balance. Plants wrapped in aluminum foil were dried at 80℃ until no weight changes could be detected, and dry weight was measured.

2.3. Ni analysis

The plants grown in the conditions of the nutrient solution containing test solutions for a week were rinsed with distilled water. The plants were then dried in a heating block at 100℃ and digested for liquid scintillation counting[12]. Plant samples (1 g) were dried at 60℃ for 3 day, and then digested at 180℃ for 105 min in 5 ml of concentrated nitric acid (HNO_3, 70%). After samples were cooled to room temperature, 1 mL of 30% (w/v) hydrogen peroxide was added. The mixture was heated at 180°C for 20 min, cooled, and added deionized water to a final volume of 12.5 mL. Ni concentrations were then measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Thermo Jarrell Ash ICP-IRIS, U.S.A)[19].

2.4. Malondialdehyde (MDA) assay

Lipid peroxidation was measured as the amount of MDA, determined by the thiobarbituric acid reaction[20]. A known quantity (0.1-0.5 g) of roots was homogenized with 0.2 g activated charcoal and 5 ml of 5% w/v trichloroacetic acid in an ice bath using a prechilled mortar and pestle. The homogenate was filtered through a 0.45 μm filter. The colorimetric reagent was a 1:1 v/v mixture of 0.6 mM 4-(2-pyridylazo) resorcinol (disodium salt) and 0.6 mM potassium titanium-oxalate. To a known volume of supernatant, 1 ml of colorimetric reagent was added and the mixture was incubated at 45℃ in a heating block for 60 min. The absorbance was measured at 508 nm against a reference solution containing 50 μl of 5 % w/v TCA and 1.95 ml of 100 mM potassium phosphate buffer (pH 8.4). The MDA contents were determined from a standard curve.

2.5. Proline assay

The proline contents in the plant material extracts were determined according to the method described by Bates *et al.*[21]. One g of the plant material was homogenized and resuspended in 10 ml of 3% (v/v) aqueous sulfosalicylic acid and the homogenate was centrifuged at 1,500 g for 10 min. Two ml aliquot of the extract supernatant was treated with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100℃ and the reaction terminated in an ice bath. The reaction mixture was extracted with 5 ml toluene and mixed vigorously for 15-20 sec. The chromphore containing toluene was separated and the absorbance was spectrophotometrically determined at 520 nm using toluene for a blank. The proline concentration was then calculated on a dry weight basis. All the experiments mentioned above were conducted at least twice.

2.6. Statistical analysis

Data were statistically evaluated by the standard deviation and T-test. The results presented are combined from at least 3 replicated experiments for survival data, or at least 2 replicated experiments for biochemical measurements.

3. Results

3.1. Effects of Ni on seedling growth

Toxic effects of Ni on *R. sativus* were evident both morphologically and physiologically. Young leaves of plants affected by Ni toxicity showed chlorosis between the veins and developed necrotic lesions at higher concentrations. Ni toxicity was evaluated by its influence on plant weight and the data are summarized in Table 1. Notable differences in Ni tolerance were observed and the degree of

Table 1. Fresh and dry weight of *R. sativus* exposed to different concentrations of Ni for 7 days

Ni in culture solution (μM)	Fresh weight (mg)		Dry weight (mg)	
	1 d	7 d	1 d	7 d
Control	13.1±0.24	28.4±1.60	1.12±0.15	1.45±0.15
0.5	12.7±0.31	28.1±1.03	1.11±0.35	1.55±0.20
50	12.4±0.12	27.3±0.71	1.08±0.24	1.85±0.15
100	9.8±0.23	16.5±0.54	0.91±0.13	1.62±0.21
250	9.7±0.32	11.0±0.27	0.70±0.31	1.40±0.17
500	8.5±0.21	9.1±0.21	0.63±0.12	1.10±0.11

reduction was dependent upon the Ni supply. Reduced fresh weight was observed at high concentrations of Ni, and was not significant at low concentrations of Ni. This result may be due to the high variability found in the low concentrations of Ni treatment, looking smaller and yellowish. The reduction in fresh weight observed at elevated Ni concentrations was not accompanied by a parallel reduction in dry weight.

The changes of weight was investigated in plants grown in the 100 μM Ni treatment with or without histidine (Fig. 1). The fresh weight decreased concurrently with the growth of the seedling. Treatment with exogenous histidine in the presence of 100 μM Ni resulted in an increase in weight, especially in fresh weight.

The elongation of the shoots and roots decreased by the external Ni treatment, and the growth rate of shoot was almost reduced during the development (Fig. 2). In Ni concentration of 100 μM, the shoot growth was limited to approximately 50% after 7 days of treatment. Notable differences in Ni tolerance were observed between 100 μM Ni and Ni+histidine. Exogenous histidine in the presence of Ni ameliorated the inhibitory effect of Ni in seedling growth, especially in root elongation. The beneficial effect of histidine was less pronounced in shoot growth.

3.2. Nickel concentration in plant

Ni accumulation was measured by digestion of plant dry material with concentrated nitric acid, and concomitant reading of metal concentrations by ICP. Shoot and root systems of radish were sampled for measurement of Ni concentration from plants provided with a hydroponic solution supplemented with 100-500 μM Ni. A progressive increase of Ni concentration in different parts of seedlings was observed with increasing Ni supply.

At the end of an exposure period of 7 day, Ni concentrations in shoots and roots differed markedly (Fig. 3). Shoot Ni concentrations ranged between 8.8 and 52.9 μmol Ni g^{-1} dry biomass and root Ni concentrations contained 5.2 to 42.7 μmol Ni g^{-1} dry biomass at the end of the 7-d-exposure period. The concentration of Ni in the shoots was higher than in

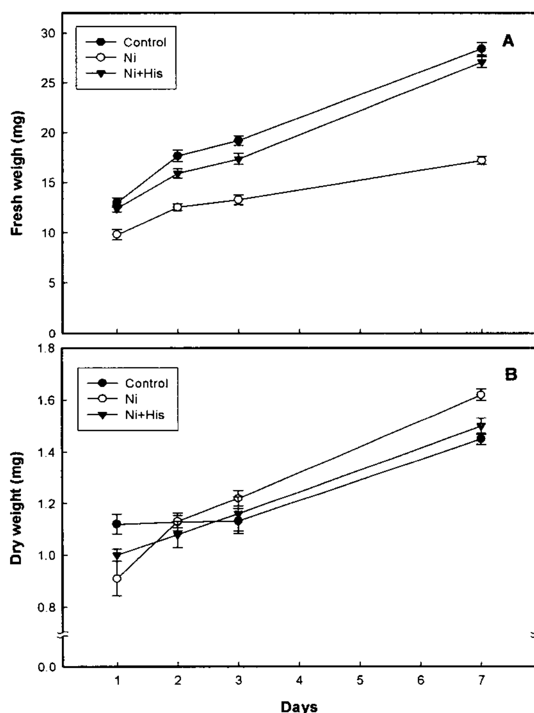

Fig. 1. Changes in fresh weight (A) and dry weight (B) of *R. sativus* exposed to 100 μM Ni with or without 100 μM histidine.

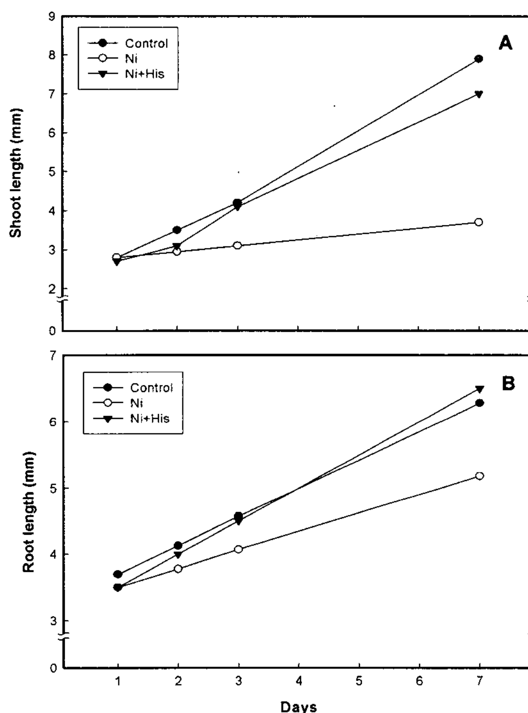

Fig. 2. Time course of shoot(A) and root(B) elongation of *R. sativus* exposed to 100 μM Ni with or without 100 μM histidine for 7 days.

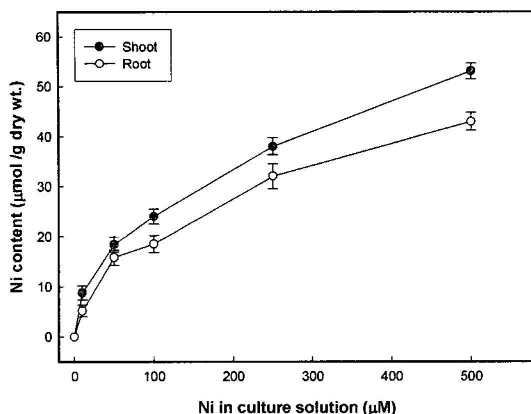

Fig. 3. Ni content in shoot and root of *R. sativus* treated with different concentrations of Ni.

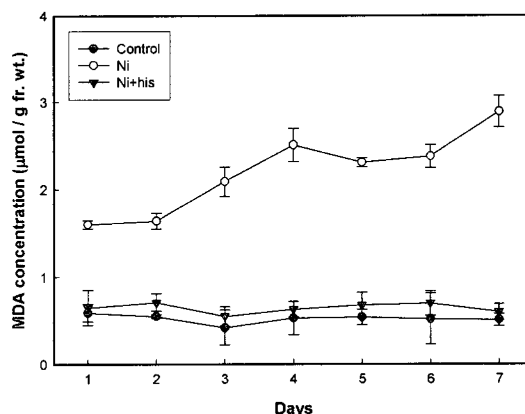

Fig. 4. MDA concentration in *R. sativus* root treated with 100 μM Ni for 7 days.

Table 2. Nickel concentration in *R. sativus* exposed to 100 μM Ni with or without 100 μM histidine

Treatment	Ni concentration (μmol/g dry wt.)	
	1 d	7 d
Control	-	-
Ni	0.12±0.03	0.66±0.15
Ni +Histidine	0.53±0.19	1.32±0.12

the roots.

The effects of Ni and histidine, administered separately and in combination, on Ni content during seedling growth were determined (Table 2). When radish was supplied with a combination of histidine and Ni, Ni concentrations more increased substantially in seedlings than those in Ni stress alone.

Ni levels in the radish roots decreased with time after 10 days, reflecting the continuous removal of Ni from the medium as subsequent growth occurred. In contrast, because Ni-treated roots did not grow, the Ni content in roots remained relatively constant.

3.3. MDA content

To investigate the effect of Ni on lipid peroxidation, which leads to disruption of membrane functions and harmful effects on plant cells, malondialdehyde (MDA) concentration in radish was measured. Lipid peroxidation measured as the amount of MDA was remarkably increased and symptoms of oxidative injury have been observed in plants exposed to Ni (Fig. 4).

MDA levels in Ni-treated radish roots were, on average, 3.6-fold higher than those without Ni treatment. The average increase in MDA concentration in shoots was substantially higher 8.6-fold, following a 5.5-fold increase in MDA levels just after the beginning of the culture period. MDA levels in the roots were significant (< 0.05) throughout the culture period except the days 1 and 7. Exogenous application of histidine to Ni-treated roots induced a significant reduction in MDA concentration and restored their ability to survive metal stress.

3.4. Proline content

The proline levels found in the separate and simultaneous effects of Ni and histidine are given in Fig. 5. Metal stress caused a large increase in the free protein levels. As the concentration of Ni in the

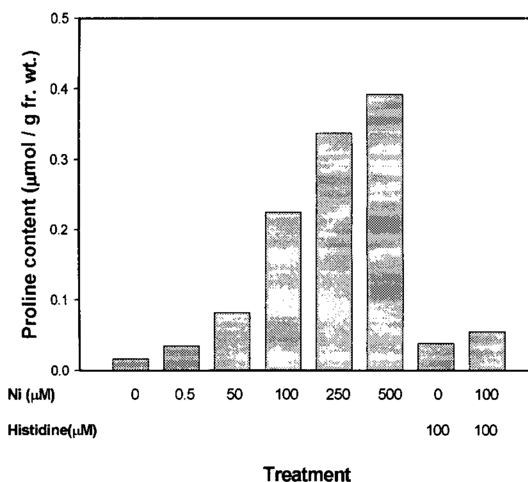

Fig. 5. Proline content in *R. sativus* exposed to different concentration of Ni with or without histidine.

external medium increased, there was a concomitant increase in the amount of proline. During the exposure to deleterious concentrations of Ni, the level of intracellular proline remarkably increased as Ni increased to 500 μM. Combinations of Ni and histidine decreased proline content, and the proline content decreased with an elevated of Ni concentration, suggesting protective role of histidine against the adverse effects of the metal.

4. Discussion

High concentrations of Ni in the growth culture solution were found to be extremely toxic, up to plant death. The toxicity was expressed by reduction in plant biomass. This decrease in fresh weight was not accompanied by a parallel decrease in dry weight, the latter remaining little change. Slight reduction in fresh weight was observed for *Alyssum argenteum*, from Ni concentration of 0.1 mM, and plants survived up to the highest concentration analyzed (1.0 mM)[22]. The dry weight of plant was not significantly changed during the development, suggesting that the water loss at high Ni concentrations may be the result of membrane damage[23]. The reduction in fresh weight was also not accompanied by a parallel reduction in dry weight. These results suggest a mechanism for *R. sativus* by which protection against damage may allow sustained transport, uptake and cellular compartment integrity enabling continued accumulation as well as growth. The scheme that Ni induces defense pathways is also consistent with the observation that root and shoot growth stops or is significantly inhibited after Ni-exposure. In the Ni-sensitive population of *Silene paradoxa*, the Ni induced a significant inhibition of the root growth even at the lowest concentrations of Ni[17]. As a result of Ni-induced defence reactions, root seems to have lost their capacity for nutrient uptake and, thus, their ability to sustain plant growth. This would lead to growth retardation at the whole-plant level.

At 100 μM concentration of Ni, the species accumulated Ni up to 0.47 % of their dry weight, defining it as a non-Ni-hyperaccumulator. The pronounced Ni accumulation was followed by severe growth inhibition, reflected in decrease in fresh and dry weight. The observed decrease in fresh weight as a result of Ni accumulation suggests a change in the plant's water status which may be the result of decreased water uptake or enhanced water loss, both of which may occur following membrane damage. Plant cell membranes are generally considered primary sites of metal injury[23].

Metal-based plant defence hypothesis received the most experimental support among the current hypotheses seeking to explain the evolution of metal hyperaccumulation. Hyperaccumulation raises interesting questions such as the mechanisms by which toxicity is avoided and the possible adaptive significance of high levels of heavy metals. Because metals are complexed with common metabolic products such as citrate, malate, amino acids, or oxalate, translocation and compartmentalization may represent the direct metabolic costs for metal-based defences. Obviously the Ni-hyperaccumulator *Psychotria* should contain more Ni than the nonhyper- accumulator *Ficus*[24]. Compared with the Ni-tolerant *Thlaspi goesingense*, *T. arvense* was sensitive to Ni and accumulated higher concentrations in the roots than in the shoots, thus behaving as a nonaccumulator[23].

The concentration of Ni in the shoots of *R. sativus* was higher than in the roots, showing the characteristic of a Ni-tolerant species. The results obtained confirm its efficient transport of Ni from root to shoot under nonphytotoxic conditions. Most Ni accumulated by *Berkheya coddi* (71%) was found in shoots, especially in the leaves[25]. It was suggested that the apparent metal accumulation in roots may be partly due to metal precipitation in the apoplast and an increased net influx into the shoot symplast of the nonaccumulator.

Exposure of plants to non-redox reactive metal such as cadmium, mercury and nickel resulted in oxidative stress as indicated by lipid peroxidation, H_2O_2 accumulation and an oxidative burst[26]. To underline some effects and responses to toxic concentrations of Ni on plants, the relation between heavy metal and oxidative stress involved in metal tolerance was evaluated. Lipid peroxidation was markedly increased when the plants were exposed to Ni, indicating a efficient inducer of lipid peroxidation. Taking these observations, it may be suggested that Ni induces a transient loss in antioxidative capacity, perhaps accompanied by a stimulation of oxidant producing enzymes, which results in intrinsic

H_2O_2 accumulation. In Ni-sensitive plant, Ni was able to induce lipid peroxidation to a greater extent, in agreement with the fact that different metals have different capacities for inducing oxidative stress owing to their different action mechanisms[27]. High levels of H_2O_2 could in part explain the Ni-induced oxidative stress. H_2O_2, then, would act as a signalling molecule triggering secondary defences.

The elevated proline level was observed in the Ni-stressed plants and proline may be partly attributable to the reduction in Ni toxicity. The present study shows that increasing amounts of intracelluar proline reduces the internalization of Ni from the culture solution. This could result from enhanced efflux of Ni from the cytoplasm, assuming that the efflux of Ni is enhanced when it is complexed by proline. The present observations showing a positive relationship between metal toxicity and proline accumulation suggest a protective role of the amino acid against heavy metal toxicity. There are well established metabolic traits leading to proline accumulation under heavy metal stress conditions, suggesting that proline provides protection by maintaining the water balance which is often disturbed by heavy metals, or chelating heavy metals in the cytoplasm[28]. However, the survival value of proline accumulation under heavy metal stress has been a matter of debate.

Supplemental histidine in the presence of Ni ameliorated metal-induced growth inhibition and lipid peroxidation by reducing the production of harmful radicals, or sequesters them. Alternatively, histidine might complex with metal ions inside the cell thereby protecting sensitive cellular sites from toxic effects. Combined treatment of Ni and histidine provided protection by reducing metal-induced proline contents. It is likely that histidine protect plants from being affected by toxic Ni, suggesting that histidine possibly lowers the toxicity of Ni via its complexing with Ni.

If free histidine is involved in the accumulation of Ni, we could predict that histidine binds Ni within the plant. From the data it was clear that free histidine or free histidine-like molecule is involved in coordinating Ni in *R. sativus*. The addition of exogenous histidine to the hydroponic solution in the presence of Ni resulted in an increase in Ni concentrations in seedlings, as observed in *Alyssum lesbiacum*[13] showing the enhancing effect of histidine on Ni flux in the xylem sap.

An important component of Ni-tolerance mechanism appears to be based on the efficient intracellular compartmentalization of the Ni into the vacuole or cytoplasm. Ni-sensitive non-accumulator has only a limited capacity for vacuolar storage of Ni and Ni accumulate in the cytoplasm, thereby poisoning sensitive cellular processes and ultimately causing cell death[29,30]. A Ni storage mechanism efficient enough to prevent metal toxicity appears to be lacking in the non-accumulator. However, the hyperaccumulator can more efficiently compartmentalize Ni in the vacuole as a Ni-organic acid complex, indicating the presence of a more efficient vacuolar sequestration mechanism for Ni[30]. It is possible that, in addition to efficient Ni transport into the vacuole, an as-yet-unidentified cytoplasmic chelator, possibly histidine, or accumulation in an organelle other than the vacuole, may also contribute to Ni tolerance.

Since histidine ameliorates the symptoms of Ni toxicity by complexing metal ions within the plants, we may conclude that histidine is an effective chelator of Ni during the transport and storage of Ni, and histidine provide a protective effect against the consequences of Ni stress. Our further research efforts will address localization of Ni accumulation within the plant body involved in antiherbivore defensive function regarding the adaptive significance of the accumulation trait.

References

1) Déportes, I., J. L. Benoit-Guyod and D. Zmirou, 1995, Hazard to man and the environment posed by the use of urban waste compost, Science of the Total Environment, 172, 197-222.
2) Brown, P. H., R. M. Welch, E. E. Cary and R. T. Checkai, 1988, Nickel : a micronutrient essential for higher plants, Plant Physiol., 85, 801-803.
3) Dalton, A. D., S. A. Russel and H. J. Evans, 1998, Nickel as a micronutrient element for plants, Biofactors, 1, 11-16.
4) Pandolfini, T., R. Gabbrielli and C. Comparini, 1992, Nickel toxicity and peroxidase activity in seedlings of *Triticum aestivum* L., Plant Cell Environ., 15, 719-725.
5) Moya, J. L., R. Ros and I. Picazo, 1993, Influence of cadmium and nickel on growth, net photo-

synthesis and carbohydrate distribution in rice plants, Photosynthesis Research, 36, 75-80.
6) Welch, R. M., 1995, Micronutrient nutrition of plants, Crit. Rev. Plant Sci., 14, 49-82.
7) Foy, C. D., R. L. Chaney and M. C. White, 1978, The physiology of metal toxicity in plants, Annu. Rev. Plant Physiol., 29, 511-566.
8) Salt, D. E., M. Blaylock, N. P. B. A. Kumar, V. Dushenkov, B. D. Ensley, I. Chet and I. Raskin, 1995, Phytoremediation : a novel strategy for the removal of toxic metals from the environment using plants, Biotechnol., 13, 468-474.
9) Robinson, B. H., A. Chiarucci, R. R. Brooks, D. Petit, J. H. Kirkman, P. E. H. Gregg and V. De Dominicis, 1997, The nickel hyper-accumulator plant *Alyssum bertolonii* as a potential agent for phytoremediation and phytomining of nickel, J. Geochemical Exploration, 59, 75-86.
10) Robinson, B. H., R. R. Brooks, A. W. Howes, J. H. Kirkman and P. E. H. Gregg, 1997, The potential of the high-biomass nickel hyper-accumulator *Berkheya coddii* for phytoremediation and phytomining, J. Geochemical Exploration, 60, 115-126.
11) Baker, A. J. M., R. D. Reeves and A. S. M. Hajar, 1994, Heavy metal accumulation in British population of the metallophyte *Thlaspi caerulescens* J. and C. Presl (*Brassicaceae*), New Phytol., 127, 61-68.
12) Krämer, U., J. D. Cotter-Howells, J. M. Charnock, A. J. M. Baker and J. A. C. Smith, 1996, Free histidine as a metal chelator in plants that accumulate nickel, Nature, 379, 635-638.
13) Kerkeb, L. and U. Krämer, 2003, The role of free histidine in xylem loding of nickel in *Alyssum lesbiacum* and *Brassica juncea*, Plant Physiol., 131, 716-724.
14) Sagner, S., R. Kneer, G. Wanner, J. P. Cosson, B. Deus-Neumann and M. H. Zenk, 1998, Hyperaccumulation, complexation and distribution of nickel in *Sebertia acuminata*, Phytochem., 47, 339-347.
15) Gabbrielli, R., C. Mattioni and O. Vergnano, 1991, Accumulation mechanisms and heavy metal tolerance of a nickel hyperaccumulator, J. Plant Nutrition, 14, 1067-1080.
16) Persans, M. W., X. Yan, J. M. M. L. Patnoe, U. Krämer and D. E. Salt, 1999, Molecular dissection of the role of histidine in nickel hyperaccumulation in *Thlaspi goesingense* (Halacsy), Plant Physiol., 121, 1117-1126.
17) Foyer, C. H., H. Lopez-Delgado, J. F. Dat and I. M. Scott, 1997, Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signalling, Physiol. Plant., 100, 241-254.
18) Bowler, C., M. Van Montague and D. Inze, 1992, Superoxide dismutase and stress tolerance, Ann. Rev. Plant Physiol., Plant Mol. Biol., 43, 83-116.
19) Zhao, F., S. P. McGrath and A. R. Crosland, 1994, Comparison of three wet digestion methods for the determination of plant sulphur by inductively coupled plasma atomic emission spectrometry (ICP-AES), Communications in Soil Science Plant Analysis, 25, 407-418.
20) Buege, J. A. and S. D. Aust, 1978, Microsomal lipid peroxidation, Methods Enzymol., 52, 302-310.
21) Bates, L. S., R. P. Waldren and I. D. Teare, 1973, Rapid determination of free proline for water-stress studies, Plant and Soil, 39, 205-207.
22) Schickler, H. and H. Caspi, 1999, Response of antioxidative enzymes to nickel and cadmium stress in hyperaccumulator plants of the genus *Alyssum*, Physiol. Plantarum, 105, 39-44.
23) Krämer, U., R. D. Smith, W. W. Wenzel, I. Raskin and D. E. Salt, 1997, The role of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Hálácsy, Plant Physiol., 115, 1641-1650.
24) Davis, M. A., S. G. Pritchard, R. S. Boyd and S. A. Prior, 2001, Developmental and induced responses of nickel-based and organic defences of the nickel-hyperaccumulating shrub, *Psychotria douarrei*, New Phytol., 150, 49-58.
25) Robinson, B. H., E. Lombi, F. J. Zhao and S. P. McGrath, 2003, Uptake and distribution of nickel and other metals in the hyperaccumulator *Berkheya coddii*, New Phytol., 158, 279-285.
26) Schützenbübel, A. and A. Polle, 2002, Plant responses to abiotic stresses : heavy metal-induced oxidative stress and protection by mycorrization, J. Exp. Bot., 53, 1351-1365.
27) Baccouch, S., A. Chaoui and E. El Ferjani, 1998, Nickel-induced oxidative damage and antioxidant responses in *Zea mays* shoots, Plant Physiol.

Biochem., 36, 689-694.

28) Schat, H., S. S. Sharma and R. Vooijs, 1997, Heavy metal-induced accumulation of free proline in metal-tolerant and a nontolerant ecotype of *Silene vulgaris*, Physiol. Plantarum, 101, 477-482.

29) Brune, A., W. Urbach and K. J. Dietz, 1995, Differential toxicity of heavy metals is partly related to a loss of preferential extraplasmatic compartmentation : a comparison of Cd- Mo- Ni- and Zn-stress, New Phytol., 129, 403-409.

30) Krämer, U., I. J. Pickering, R. C. Prince, I. Raskin and D. E. Salt, 2000, Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species, Plant Physiol., 122, 1343-1353.