Pak. J. Bot., 45(S1): 495-500, January 2013.

EFFECT OF NICKEL AND CADMIUM ON GLUCOSINOLATE PRODUCTION IN *THLASPI CAERULESCENS*

SAEED AHMAD ASAD[1, 2*], SCOTT YOUNG[2] AND HELEN WEST[2]

[1]*Department of Environmental Sciences, COMSATS University-22060 Abbottabad, Pakistan.*
[2]*Division of Agricultural and Environmental Sciences School of Biosciences, University of Nottingham LE12 5RD, United Kingdom*
[*]*Corresponding author e-mail:asadenviro@hotmail.com;saeedasad@ciit.net.pk*

Abstract

Hyperaccumulator plant species are studied because of their potential for cleaning up land contaminated with heavy metals, but another aspect of study relates to the reason for hyperaccumulation. The most accepted hypothesis over the last decades is elemental defence hypothesis stating that accumulated heavy metals defend the plants against herbivores and pathogens. Glucosinolates in hyperaccumulators are also known to defend the plants against these environmental stresses. Current study was designed to test any trade-off between these 2 types of defences in *Thlaspi caerulescens*. *Thlaspi* plants were grown in glass house at different Ni and Cd concentrations where clipping damage with scissors was applied to substitute herbivory. The maximum foliar Ni was observed as 233.20 mg kg^{-1} whilst, maximum Cd uptake was reported to be 119.63 mg kg^{-1} of dry mass of *Thlaspi* shoots. The maximum uptake of Ni was 2-4 times higher while that of Cd was 4-10 times higher as compared to that applied in substrate. The Ni uptake by *Thlaspi* was far below than the threshold value, the concentrations commonly used to define hyperaccumulation. There was a positive correlation between soil metal addition and concentration of shoot metals. Generally, lower concentrations of glucosinolates were observed in plants with higher foliar Cd concentrations, while in case of Ni treated plants, glucosinolates were induced at elevated metal application. Trade-offs between 2 types of chemical defences was only observed at highest Cd concentrations while Ni application increased the level of glucosinolates. The overall conclusion partially supported the 'trade- off hypothesis'.

Introduction

Transition elements with a specific gravity of >5 and an atomic mass of over 20 are toxic to both plants and animals even at very low concentrations and are termed "heavy metals" (Rascio & Navari, 2011). Heavy metals form only 1% of the earth's crust, the other 99% is constituted by major elements, Mg, Na, Ti, P, K, Fe, Al, Ca, O and Si (Alloway, 1995). Heavy metals that are ecotoxic and of great concern to agriculture and human health include As, Pb, U, Hg, Se and Cd. Some heavy metals are essential for normal growth of fauna and flora and without the presence of these elements it is impossible for plants and animals to complete their life cycle (Llabjani *et al*., 2012). For example crops need Mn, Zn and Cu and livestock need Mn, Co, Cu and Zn for normal growth and productivity (Alloway, 1995).

Agricultural inputs, use of fossil fuels and sewage sludge, metallurgical and electronics industries, waste disposal and warfare and military operations have resulted in significant additions of trace elements to soil particularly in regions where intensive farming occurs. Heavy metals in soil may ultimately pass into the food chain in addition to causing yield losses, reduced microbial activity and decreased fertility (Kafeel *et al*., 2011; McGrath *et al*., 1995). These heavy metals may result in either direct hazardous effect to the human health or indirectly by leaching which contaminates both surface and underground water (Murtaza *et al*., 2008).

No doubt, the occurrence of heavy metals can be both beneficial and harmful for plants. For example, Zn acts as a cofactor for several enzymes like oxidases, peroxidases, anhydrases and dehydrogenases (Hewitt, 1983) and regulates nitrogen metabolism, cell multiplication, photosynthesis and hormone (auxin) synthesis in plants (Shier, 1994). On the other hand radicle elongation is more adversely affected than plumule extension with enhanced Zn concentrations (Sresty & Madhava, 1999).Unlike Zn, Cd is not involved in any known physiological function within the plant body, so regarded as a non-essential element and has been reported to cause deleterious effects on plants and seed germination (Ahmad *et al*., 2012). For example, Cd resulted in stunted growth and chlorosis in plants, symptoms that resemble those induced by iron (Fe) deficiency. In some other studies, cadmium toxicity appeared to induce phosphorus deficiency or reduce manganese transport and interfere with uptake and transport of several essential nutrients (Ca, Mg, P and K) and water by plants (Godbold & Hutterrmann, 1985).

Although Ni is required by some plant species it is not really essential, unlike Zn, Cu, and Mg, its elevated concentrations have been reported to be toxic for plants (Seregin *et al*., 2006). For example, excess Ni in the environment lowers the uptake of Mg and Fe, because of the chlorosis caused by it (Piccini & Malavolta, 1992) & Seregin *et al*., (2006) reported that excess Ni concentrations specifically affect the ionic balance in different plant organs.

A group of plants called "hyperaccumulators" with extra high ability to accumulate metals has attracted the attention of a number of research groups in the last two decades to investigate the reasons of hyperaccumulation. Three basic questions; (1) Why some plants species hyperaccumulate heavy metals, (2) what physiological functions are attributed to these accumulated heavy metals and (3) what benefits do these hyperaccumulators reap from the metals or metalloids made the basis of this investigation (Rascio & Navari, 2011; Rafia & Sana, 2012). Another hypothesis called the "defence hypothesis" (Boyd & Martens, 1992) describes the role of hyperaccumulated elements as defence compounds

against a wide range of insect herbivores and pathogens and so far, this hypothesis has received the most supporting evidence despite some contradictory results (Boyd, 2007). Almost all the hyperaccumulated heavy metals have proved to be effective against herbivores in different hyperaccumulator species. For example, Zn (Behmer *et al*., 2005), Se (Galeas *et al*., 2008), Cd (Jiang *et al*., 2005), (Rathinasabapathi *et al*., 2007) and Ni (Jhee *et al*., 2006) all have been reported to be involved in plant defence.

Brassica plants have also been known to contain organic defense compounds called glucosinolates (GS) present naturally as well as induced as a result of tissue damage. Intact glucosinolates exhibit very little defense role, but it is their hydrolysis products (isothiocyanates, nitriles etc.) which provide effective defense against herbivores and pathogens. These hydrolysis products are produced when glucosinolates are hydrolyzed by the enzyme myrosinase. Glucosinolates are metabolically expensive to synthesize. For example, it has been estimated that 90 ATP and 12 $NADH_2$ are required in addition to 9 enzymatic steps, but the real costs involve myrosinase production, compartmentalization, transport and maintaining production (Wallace & Eigenbrode, 2002).

Heavy metals are present naturally in the environment and taken up by plants where they are known to defend the plants whilst; glucosinolates are produced at the expense of plant energy. The question arises if heavy metals are providing defense to plant then why should plants invest energy on the production of organic defense compounds (glucosinolates in this case).

Current study was designed to explore any possible relationship between heavy metals and glucosinolate production i.e. do heavy metals have any impact on the production of glucosinolates? Second objective of this study was to investigate any trade-offs between inorganic (heavy metals) and organic (glucosinolates) defences in the Ni and Cd hyperaccumulator *Thlaspi caerulescens*.

Materials and Methods

Two independent experiments were conducted under glass house conditions in compost filled pots. Plants of *Thlaspi caerulescens* (Ganges ecotype) were grown for 6 weeks in a glasshouse fitted with supplementary lights to support the natural radiation at 16h/8h (day/night) photoperiod and temperature at 20/16ºC (day/night).

Experiment 1: Soil was amended with one of 5 Ni treatments applied as $NiCl_2$ prior to planting. Treatments were: 0, 10, 25, 40 and 60 mg kg^{-1} dry weight of compost. Controls received no additions. After 6 weeks of growth, leaves of 5 replicates per treatment were damaged by clipping with scissors (without removing any leaf material) whilst the remaining 5 replicates remained undamaged. Shoots were harvested 24 h after clipping, immediately frozen in liquid nitrogen and stored at -80ºC.

Experiment 2: Soil was amended with one of 5 Cd treatments applied as $CdSO_4$ prior to planting. Treatments were: 0, 1, 3, 4, and 10 mg kg^{-1} dry weight of compost. Controls received no additions. Irrigation was applied with tap water when required. Clipping and harvesting procedure were same as applied in experiment 1. There were 5 replicates for each treatment. Harvested plant material (both damaged and un damaged plants) were dipped in liquid nitrogen to stop the metabolic process and stored at -80ºC until further analysis.

Plant metal analysis: Prior to analysis these shoots were lyophilized for 17 hours at -40ºC using a Hem Lab SB4 freeze drier. After lyophilisation, shoot dry weights were recorded after which they were stored in desiccators containing silica gel. Prior to analyses, dried leaves were ground manually with a pestle and mortal and passed through sieve of 2 mm mesh size.

Approximately 50mg of ground plant material was weighed (exact weights recorded) and digested in concentrated (70% v/v) HNO_3, H_2O_2 and H_2O in the ratio of 3:2:3 respectively using a microwave digestion system (Model Multiwave 3000, Anton Paar Ltd., Hertford, UK). After digestion, the volume of digested plant material was made up to 20ml by adding Milli- Q H_2O. For analysis, digestates were further diluted to 1:10 with Milli-Q water and analyzed using Inductively Coupled Plasma Mass Spectrometry (Thermo Scientific- x series II ICP-MS) against appropriate standards.

Glucosinolate analysis: For the extraction of total glucosinolates, 200 mg of freeze-dried ground plant material was weighed in a disposable centrifuge tube and 3ml of aqueous methanol (70% v/v) heated to boiling temperature was added to it. The test tube was put in a boiling water bath for 5 minutes and then stirred continuously for 4-5 minutes using a whirl mixer. Following mixing, samples were then centrifuged at 3500 rpm for 15 minutes using a bench top centrifuge (MSE, Centaur 2, Sanyo, UK). After centrifugation the supernatant was collected and the pellet was re-extracted using the same method as before. The extraction procedure was done 3 times in total for each sample. The three liquid phases were amalgamated and concentrated using a rotary evaporator (Rotvapor-R, Buchi, Switzerland) at 45ºC to evaporate the methanol. After evaporation, the remaining sample (2.5-3.0 ml) was collected and the total volume made up to 7.5ml by adding deionized water. This solution was marked as "Solution A".

Myrosinase treatment: Four ml of "Solution A" were loaded onto a DEAE-Sephadex A-25 column and allowed to drain slowly so that glucosinolates were absorbed onto the Sephadex ion exchange filter. After draining, the column was washed through with deionized water and subsequently with 0.02M sodium acetate buffer. These 'drainings' were discarded. Myrosinase (250µl) was added to the column and left at room temperature for 15 hours. After 15 hours, liberated glucose was eluted by adding two aliquots of 0.5 ml deionized water to the

column; this solution was collected for analysis. Final volume of 1.25 ml (0.25 + 0.5 + 0.5 ml) was collected in screw capped plastic vials and stored at -20ºC until analyzed. This solution was marked as "Solution B".

Quantification of total glucosinolates: A measured volume of "Solution B" was incubated with the assay reagents provided in the GAGO 20 kit obtained from Sigma, St. Louis, MO, USA. Released glucose was determined according to the manufacturer's instructions. Solutions were mixed thoroughly and absorbance was recorded against the reagent blank at 540 nm using a spectrophotometer (CECIL instruments, CE 1011, 1000 series). Total glucosinolates in the plant and reference materials were quantified by using equation 1.

$$\text{Glucosinolates} = \frac{\text{Glucose in solution B x 1.25x7.5x1000}}{\text{M xVx180}} \quad \text{(Eqn. 1)}$$

where M is the weight (mg) of plant sample, V is the volume of "solution A" applied to the Sephadex column and 7.5 is the total volume (ml) of solution after methanol evaporation, 1.25 is the volume (ml) collected from the Sephadex column after incubation with myrosinase enzyme and 180 denotes the relative molecular mass of glucose.

Data analysis: Each treatment had 5 replicates and average was obtained for data analysis. All analyses were performed using GenStat version 13.1 (Lawes Agricultural Trust, UK).

Results and Discussion

Relationship between metal application to soil and uptake by *Thlaspi caerulescens:* There was a positive correlation between soil Ni addition and concentration of shoot Ni (Fig.1). Plants grown in high-Ni soil had greater shoot Ni concentrations than those grown in low Ni soil. There was no effect of clipping on shoot Ni concentration which would be expected because plants were harvested 24 h after treatment. Plants grown in Cd amended soil showed a similar trend where increasing Cd concentration in substrate resulted in elevated level of metal uptake by *Thlaspi* (Fig. 2). Both Ni and Cd taken up by plants were significantly higher ($p<0.001$) than that applied in the growing medium (Figs. 1-2). Although Ni and Cd uptake did not reach their maximum potential in this trial, foliar accumulation still occurred. Enhanced metal uptake relative to application is because *Thlaspi caerulescens* is a Ni and Cd hyperaccumulator. According to Brooks, (1987) plants accumulating 10,000 mg kg^{-1} of Zn, 100 mg kg^{-1} of Cd and 1000 mg kg^{-1} of Ni are considered hyperaccumulators. More recently, Papoyan & Kochian, (2004) observed concentrations of 30000 mg kg^{-1} Zn and 1000 mg kg^{-1} Cd in shoots of *Thlaspi* without any toxicity symptoms. Brown *et al*., (1994) recorded concentrations of up to 51000 mg kg^{-1} for Zn and 1740 mg kg^{-1} for Cd in plants grown on Zn and Cd amended soils. Due to this hyperaccumulation characteristic, *Thlaspi* has been highly studied to determine its phytoremediation potential. Nevertheless, relatively little work has been done on the physiology of metal hyperaccumulation in *Thlaspi*, but its homolog *Arabidopsis thaliana*, has been extensively studied and genes involved in metal hyperaccumulation identified (Papoyan & Kochian, 2004).

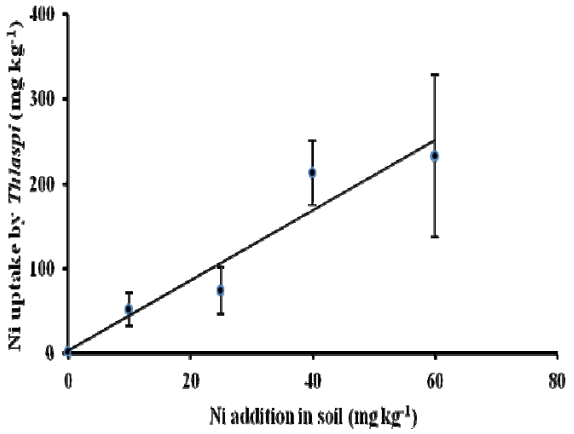

Fig. 1. Relationship between Ni added to the soil and uptake by *Thlaspi caerulescens* exposed to different Ni concentrations. Data are means of five replicates ± SE. ANOVA: $p<0.001$.

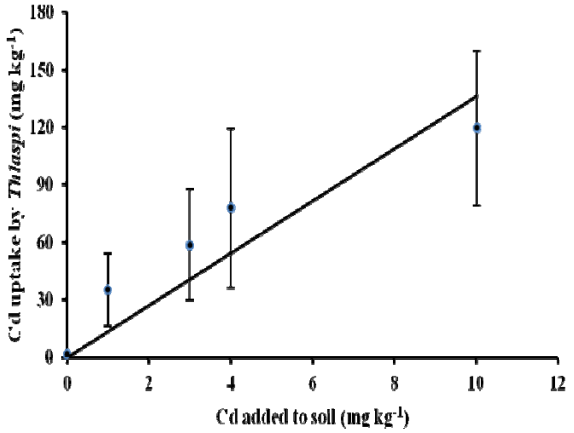

Fig. 2. Relationship between Cd application to the soil and uptake by *Thlaspi caerulescens*. The data are means of five replicates ± SE. ANOVA: $p<0.001$.

Effect of clipping damage on glucosinolates: Effect of clipping damage (simulated herbivory) was observed on the production of glucosinolates in *Thlaspi caerulescens* exposed to different Ni and Cd concentrations. As evidenced in Table 1 there was no significant difference in total glucosinolates in damaged and undamaged plant leaves at low Ni application (including control). However, at higher concentrations of Ni i.e., 40 and 60 mg kg^{-1}, damaged leaves showed significantly higher concentrations of these secondary metabolites as compared to undamaged plant leaves exposed to same Ni concentrations. This could be an artifact or it could be the clipping which induced glucosinolates because

glucosinolates despite being present naturally in brassica are also induced as a result of biotic or abiotic stresses. According to trade-off hypothesis hyperaccumulating plants will not induce glucosinolates as a result of any environmental stress. In the current study glucosinolates were induced as a result of clipping damage in plants growing at elevated Ni concentrations as compared to the lower concentration. The results presented here disagree with the previous work (Davis *et al.,* 2001: Jhee *et al.,* 2005) who concluded the defence role of hyperaccumulated Ni against biotic stress in *Psychotria douarrei* and S*treptanthus polygaloides* respectively. There could be two possible explanations for induction of glucosinolates in the presence of Ni. Firstly, Ni concentration in leaves was far below 1000 mg, the concentration commonly used to define Ni hyperaccumulation (Reeves & Baker, 2000; Boyd, 2004). Secondly, it could be the Ni itself which enhanced the level of glucosinolates due to its stressing effect. In fact, plant was exposed to two types of stresses in this case, one was clipping damage and the other was Ni itself. No doubt, *Thlaspi caerulescens* is a Ni hyperaccumulator, but this element has stressing effect. According to Boyd & Marten, (1994), one benefit of metal based defense is that they divert less cellular resources towards the production of organic defense compounds, but in our findings this was not true as glucosinolates (organic defense compounds) were induced as a result of clipping damage despite elevated levels of Ni. Both Ni and glucosinolates might be involved in different functions including defence. For example, as observed by Boyd & Martens, (1992) that Ni might be involved in drought resistance or metal tolerance and therefore glucosinolate concentrations were induced to enhance defence.

Table 1. Effect of clipping damage on glucosinolates exposed to different Ni and Cd concentrations.

Heavy metal	Concentration of heavy metal in soil (mg kg^{-1})	Total glucosinolates (µmolg^{-1}) in *Thlaspi*	
		Damaged leaves	Undamaged leaves
Ni	0	2.04 ± 0.1	2.75 ± 0.24
	10	51.64 ± 19.5	19.47 ± 11.24
	25	74.29 ± 27.9	76.64 ± 38.21
	40	212.96 ± 37.8	60.56 ± 25.99
	60	233.2 ± 95.4	155.19 ± 23.67
Cd	0	1.73 ± 0.59	0.81 ± 0.04
	1	35.39 ± 18.91	36.06 ± 13.23
	3	58.73 ± 28.99	43.08 ± 8.55
	4	77.88 ± 41.61	47.58 ± 9.51
	10	119.63 ± 40.45	186.19 ± 29.55

Data are means of 5 replicates ±SE

Cd had no effect on the concentration of glucosinolates while growing on lower concentration of metal (0, 1, 3 and 4 mg kg^{-1}). However, at maximum availability of Cd to plants, glucosinolate concentrations were significantly higher in undamaged plants as compared to the damaged ones. These findings are in line with the trade-off hypothesis stating that "when plant is hyperaccumulating heavy metals it would induce less glucosinolates if any stress is applied". As at lower concentration of Cd, there was not enough Cd accumulated by *Thlaspi* to restrict the induction of glucosinolates whilst at higher concentration of metals, elevated level of element resulted in less induction of this defence compound. This seems realistic, as the maximum concentration of Cd (119.63 mg kg^{-1}, Fig. 2), was higher than 100mg kg^{-1}, the concentration commonly used to define Cd hyperaccumulation (Boyd, 2004). This argument also supports our previous observation where Ni concentration (233.20mgkg^{-1}) less than threshold value (1000 mg kg^{-1}) could not reduce the induction of glucosinolates. Jiang *et al*., (2005) observed that Cd defended the *Thlaspi caerulescens* from herbivory damage by generalist herbivore *Frankliniella occidentalis*, but these scientists studied only the defense role of Cd due to its toxicity and did not consider or evaluate the involvement of glucosinolates. Moreover, Jiang *et al.,* (2005) did not work out any possible trade-off between Cd and glucosinolates.

Interestingly, in other experiment by Asad *et al*., (not reported here) trade-off hypothesis was tested in *Thlaspi caerulescens* by studying the heavy metal Zn and glucosinolates interactions where Zn protected the plant from generalist herbivore *Frankliniella occidentalis* and not the glucosinolates. Moreover, the concentration of glucosinolate was significantly less at the highest Zn concentration and *vice versa* which is in line with the findings in current study. This is not surprising because uptake mechanisms of Cd is not selective and it is taken up along with Zn i.e., follow the same route for accumulation. Keeping in view, similar uptake mechanism of these two metals it could be assumed that their effects on the glucosinolates would be same.

Cd has been reported to decrease the concentration of individual glucosinolates (Sun *et al*., 2009) in *Arabidopsis thaliana*, a homologue of *Thlaspi caerulescens*. In the current study, although plant samples were not analyzed for glucosinolate profiles (for logistic reasons), but certainly individual composition of these secondary metabolites would formulate the total concentration of

glucosinolates and ultimately reduction in individual amounts would lead to reduced concentration of total glucosinolates. Mechanisms involved in glucosinolate regulation are controlled by jasmonic acid (Mewis *et al.,* 2006) and Jasmonic acid have been reported to be altered when brassica are exposed to heavy metal stress (Maksymiec *et al*., 2005). These researchers also found that jasmonic acid was involved in the toxic action of heavy metal. Results so far indicate that Cd may provide the adaptive advantage to hyperaccumulators. Moreover, it is also clear from the above findings that when heavy metals are present at the optimum levels they may reduce the production of glucosinolates and ultimately support the trade-off hypothesis.

Conclusion

It was established from results presented here that *Thlaspi caerulescens* is a model hyperaccumulator of Ni and Cd. It accumulated these elements, 3-10 times more as compared to that applied in the growth medium. Trade-off between glucosinolate and elemental defence compounds was only possible at maximum Cd uptake while Ni had stressing effect on *Thlaspi* and induced glucosinolates rather than suppressing their induction. Over all conclusions was that hyperaccumulation threshold values of heavy metals (Ni, Cd) may result to reduce the production of glucosinolates as a result of environmental stress.

Acknowledgment

The authors are thankful to the School of Biosciences and graduate school of the University of Nottingham, United Kingdom, for providing financial support to conduct this research.

References

Ahmad, I., M.J. Akhtar, Z.A. Zahir and J. Amer. 2012. Effect of Cadmium on seed germination and seedling growth of four wheat (*Triticum aestivum* L.) cultivars. *Pak. J. Bot.*, 44: 1569-1574.

Alloway, B.J. (Ed.). 1995. *Heavy metals in soils*. Blackie Academic and Professional, London.

Behmer, S.T., C.M. Lloyd, D. Raubenheimer, J. Stewart-Clark, J. Knight, R.S. Leighton, F.A. Harper and J.A.C. Smith. 2005. Metal hyperaccumulation in plants: mechanisms of defence against insect herbivores. *Functional Ecology*, 19: 55-66.

Boyd, R. S. and S. N. Martens. 1992. The raison dêtre for metal hyperaccumulation by plants. In: *The vegetation of ultramafic (Serpentine) soils*, *Proceedings of the First International Conference on Serpentine Ecology*. (Eds.): A.J. Baker, M.J. Procter and R.D. Reeves. Andover, Intercept, UK, pp. 279-289.

Boyd, R.S. 2004. Ecology of metal hyperaccumulation. *New Phytol.*, 162: 563-567.

Boyd, R.S. 2007. Defense hypothesis of elemental hyperaccumulation: status, challenges and new directions. *Plant and Soil*, 293: 153- 176.

Boyd, R.S. and S.N. Martens. 1994. Nickel hyperaccumulated by *Thlaspi montanum* Var. montanum is acutely toxic to an insect herbivore. *Oikos*, 70: 21-25.

Brooks, R. R. 1987 (Ed.). *Serpentine and its vegetation*. A Multidisciplinary Approach. Dioscorides press. Portland, Oregon, USA.

Brown, S.L., R.L. Chaney, J.S. Angle and A.J.M. Baker. 1994. Phytoremediation potential of *Thlaspi caerulescens* and *Bladder campion* for Zinc and Cadmium-contaminated Soil. *J. Environ. Quality*, 23: 1151-1157.

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.

Galeas, M.L., E.M. Klamper, L.E. Bennett, J.L. Freeman, B.C. Kondratieff, C.F. Quinn and E.A. Pilon-Smits. 2008. Selenium hyperaccumulation reduces plant arthropod loads in the field. *New Phytol.*, 177: 715-724.

Godbold, D.L. and A. Hutterrmann. 1985. Effect of zinc, cadmium and mercury on root elongation of *Picea abies* (Karst.) seedlings, and the significance of these metals to forest die-back. *Environment and Pollution*, 38: 375-381.

Hewitt, E.J. 1983. *Metals and micronutrients: uptake and utilization by plants*, In: (Ed.): D.A. Robb and W.S. Pierpoint. Academic Press London, pp. 277-300.

Jhee, E., R. Boyd and M. Eubanks. 2005. Nickel hyperaccumulation as an elemental defense of *Streptanthus polygaloides* (Brassicaceae): influence of herbivore feeding mode. *New Phytol.*, 168: 331-344.

Jhee, E., R. Boyd and M. Eubanks. 2006. Effectiveness of metal–metal and metal-organic compound combinations against *Plutella xylostella*: implications for plant elemental defense. *J. Chem. Ecol.*, 32: 239-259.

Jiang, R. F., D.Y. Ma, F.J. Zhao and S.P. McGrath. 2005. Cadmium hyperaccumulation protects *Thlaspi caerulescens* from leaf feeding damage by thrips (*Frankliniella occidentalis*). *New Phytol.*, 167: 805-914.

Kafeel, A., E. Abid, A. Mehwish, K.I. Zafar, A. Muhammad, F. Al-Qurainy, F. Asia, G. Sumera, B. R. Ali and V.E. Ehsan. 2011. Lead, Cadmium and Chromium contents of canola irrigated with sewage water. *Pak. J. Bot.*, 43: 1403-1410.

Llabjani, V., R.N. Malik, J. Trevisan, V. Hoti, J. Ukpebor, Z.K. Shinwari, C. Moeckel, K.C. Jones, R.F. Shore and F.L. Martin. 2012. Alterations in the infrared spectral signature of avian feathers reflect potential chemical exposure: A pilot study comparing two sites in Pakistan. *Environment International*, 48(2012): 39-46.

Maksymiec, W., D. Wianowska, A.L. Dawidowicz, S. Radkiewicz, M. Mardarowicz and Z. Krupa. 2005. The level of jasmonic acid in *Arabidopsis thaliana* and *Phaseolus coccineus* plants under heavy metal stress. *J. Plant. Physiol*., 162: 1338-1346.

McGrath, S.P., S.J. Dunham and R.L. Correll. 1995. Potential for phytoextraction of zinc and cadmium from soils using hyperaccumulator plants. In. (Eds.): N. Terry and Q. Banuelos. Lewis Publishers, Boca Raton, Florida, pp. 109-128.

Mewis, I., J.G. Tokuhisa, J.C. Schultz, H.M. Appel, C. Ulrichs and J. Gershenzon. 2006. Gene expression and glucosinolate accumulation in Arabidopsis thaliana in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochem*., 67: 2450-2462.

Murtaza, G., A. Ghafoor and M. Qadir. 2008. Accumulation and implications of cadmium, cobalt and manganese in soils and vegetables irrigated with city effluent. *J. Science of Food and Agriculture*, 88: 100-107.

Papoyan, A. and L.V. Kochian. 2004. Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiol.*, 136: 3814-3823.

Piccini, D. F. and E. Malavolta. 1992. Effect of nickel on two common bean cultivars. *J. Plant Nutr.,* 15: 2343-2350.

Rafia, A. and R. Sana. 2012. The inhibition of polymerization of glucose in carbohydrate under Cu stress in *Vigna radiata*. *Pak. J. Bot.,* 44: 95-98.

Rascio, N. and F. Navari-Izzo. 2011. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Sci,* 180: 169-181.

Rathinasabapathi, B., M. Rangasamy, J. Froeba, R.H. Cherry, H.J. Mcauslane, J.L. Capinera, M. Srivastava and L.Q. MA. 2007. Arsenic hyperaccumulation in the Chinese brake fern (*Pteris vittata*) deters grasshopper (*Schistocerca americana*) herbivory. *New Phytol.,* 175: 363-369.

Reeves, R. D. and A. J. M. Baker. 2000. Metal-accumulating plants. In: *Phytoremediation of toxic metals*: *using plants to clean up the environment*, (Ed.): I. Raskin and B.D. Ensley. John Wiley, New York, pp. 193-229.

Seregin, I. V., A. D. Kozhevnikova, E. M. Kazyumina and V. B. Ivanov. 2006. Nickel toxicity and distribution in maize roots. *Fiziol Rast,* 50: 793-800.

Shier, W. T. 1994. Metals as toxins in plants. *Toxin Reviews,* 13: 205-216.

Sresty, T.V.S. and R.K.V. Madhava. 1999. Ultra structural alterations in response to zinc and nickel stress in the root cells of pigeon pea. *Environ. Exp. Bot.,* 41: 3-13.

Sun, X., J. Zhang, H. Zhang, Q. Zhang, Y. Ni, J. Chen and Y. Guan. 2009. Glucosinolate profiles of *Arabidopsis thaliana* in response to cadmium exposure. *Water Air Soil Pollut.,* 200: 109-117.

Wallace, S.K. and S.D. Eigenbrode. 2002. Changes in the glucosinolate- myrosinase defense system in *Brassica juncea* cotyledons during seedling development. *J. Chem. Ecol.,* 28: 243-256.

(Received for publication 1 September 2012)