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Root Growth Inhibition, Photosynthetic Pigments Production, and Metal Accumulation in *Sinapis alba* as the Parameters for Trace Metals Effect Determination

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Metals in the environment may present a more insidious problem than organic chemicals because they cannot be degraded to innocuous products, such as carbon dioxide and water. Because metals are transported very well by the atmosphere, many urban areas have been loaded with considerable amounts of toxic metals from point and non-point sources due to human activity. Many plants in the natural environment have great variation in the concentration of heavy metals depending on their growth place (Tölgyessy et al. 1993). In recent years, considerable progress has been made in the assay of trace elements phytotoxicity assessment in environmental plant samples. Plant tissues may serve as indicators of environmental concentrations of contaminants. The uptake of trace metals through the root systems of plants and subsequent release of metals during the decomposition of plant material and transmission of these metals to organisms of higher trophic levels represents a pathway of cycling of trace metals in ecosystems (Samecka-Cymerman and Kempers 1996). According to Bowen (1979), the number of elements such as As(III) as well as Al, B, Be, Cd, Co, Cr(VI), Cu, Mo, Ni, Se(IV), and Tl can be harmful to plants even at quite low concentrations. Nevertheless, many of these elements are also essential for regular growth. Mechanisms of toxicity may operate by altering the permeability of cell membranes, by reacting with essential metabolites, or by replacing one another in enzymatic pathways and receptor proteins (Letardi et al. 1995). The majority of metals, when present at abnormally high available levels in solution or soil, can cause visible injury to plants, inhibit plant growth by damaging the roots, or can cause crop failure. Phytotoxicity and clastogenicity test in plants also confirmed a high genotoxicological effect of some metals (Miadoková et al. 1998). Long-term exposure of whole plants to metals, may affect chlorophyll synthesis and thus have an important role in both the chloroplast development in young leaves and the inhibition of photosynthesis (Boddi et al. 1995). Root growth inhibition as well as production of photosynthetic pigments is an early symptom of metal toxicity (Singh et al. 1996; Ebbs and Kochian 1997). Seedlings grown in aquacultures are able to accumulate various metals from artificially contaminated water over a range of environmentally relevant concentrations (Harangozó and Královic 1996). Plants can restrict the uptake of metals, but don't prevent it. The degree of enrichment depends both on the kind of metal and on the species of plant

absorbing the metal. In the same plant species the concentrations of various metals can be different in the shoots and leaves than in the roots (Harangozó and Královic 1996). Concentration of metals has been studied in some plant species (Ouzounidou 1995a; 1995b), but it is less known about the concentration of metals in different parts of plants (Tölgyessy et al. 1993).

The purpose of this study was to examine the copper, manganese, vanadium, molybdenum and nickel phytotoxicity on the terrestrial plant Sinapis alba and accumulation of these metals in the roots and hypocotyls of seedlings. Phytotoxicity was determined as root growth inhibition and photosynthetic pigments production. The metals ions under investigation were chosen on the results of the ecological monitoring over some major industrial centers in the Slovak Republic. All of them, except vanadium, are micronutrients for biological systems and become toxic to most lifeforms at only slightly higher concentrations than the minimum requirement.

MATERIAL AND METHODS

Plant material and cultivation: The seeds of mustard (Sinapis alba) were germinated in Petri dishes with a 14-cm diameter on plastic net for 3 days. The temperature in the laboratory was kept at 23 ± 1 °C and the dishes were shielded from direct sunlight and covered by a glass cap to prevent loss due to evaporation. Tap water in the amount of 50 mL was added to each dish, and 50 healthy looking and of similar size seeds were evenly spread into the surface of the plastic net. After 3 days, the plastic nets with germinated seeds produced a root length of about 1 mm, were transferred on the surface of 500 mL modified Knopp solution containing in distilled water the following chemical ingredients (mg/L): $Ca(NO_3)_2$ 0.8; KH_2PO_4 0.2; KNO_3 0.2; $MgSO_4.7H_2O$ 0.2; KCl 0.2; $FeSO_4$ 0.01; pH=5.2. Plants were grown hydroponically in 500 mL containers under a natural sunlight routine for the next 8 days. After an 8-day growth period the root length (cm) was measured.

Photosynthetic pigments determination: In the plants after the 8-day growth in Knopp solution supplemented with metals photosynthetic pigments - chlorophyll *a*, chlorophyll *b* and carotenoids were determined in hypocotyls. The extraction of pigments was done in ethanol (95 % v/v). For extractions 1.0 g of fresh above-ground parts of plants and 25 mL of ethanol were used. The extraction lasted until all mashed leaves and canes were completely bleached. Then the extraction solution was filtered and absorbance at the wave length 665, 649 and 470 nm was measured by using a spectrophotometer. For the calculation of pigment amounts, the following equations were used:

$$\text{chlorophyll } a\ (C_a) = 13.95\ A_{665} - 6.88\ A_{649}$$

$$\text{chlorophyll } b\ (C_b) = 24.96\ A_{649} - 7.32\ A_{665}$$

$$\text{carotenoids } (C_{x+c}) = \frac{1000\ A_{470} - 2.05\ C_a - 104\ C_b}{245}$$

The amounts of pigments were calculated as µg/mL of plant extract (Hartmut et al. 1983).

Element analysis: Eight days after exposure to metals, the plants were divided into hypocotyls and roots. Roots were washed in distilled water and all samples were dried for 24 h at 80 °C. Metals were determined by atomic absorption spectrophotometry with flame and electrothermic atomization (AAS 3, Carl Zeis 1) after mineralization. For mineralization, the dry samples (0.05 g of roots and 0.2 g of hypocotyls) were dissolved in 2 mL of HNO_3 (65 % p.a. MERCK, Darmstadt, FRG) with two drops of H_2SO_4 (95-97 % p.a. MERCK, Darmstadt, FRG). After 24 h the samples were autoclaved at the temperature 120 °C for 45 min. Cooled samples were diluted with distilled water at the volume 25 mL. By the same way were also treated the control samples. The results are introduced as means values of 3 replicates.

Statistics: All experiments were set up in a completely randomized design with 3 replications. The significant difference between the treated and control samples was analyzed by Student's t-test. The values of growth analysis presented here are means of 30 measurements, while the values of photosynthetic pigments and elemental content are means of 3 measurements. Concentrations for a 50 % effect, EC_{50} values, were derived from the dose-response curves. Nonlinear regression analysis was used to determine relationships between metal concentration and root growth (Lentner and Bishop 1993).

Metals: The tested metals were used in the following compounds: Cu^{2+} - $CuSO_4.5H_2O$; Ni^{2+} - $NiSO_4.7H_2O$; Mn^{2+} - $MnSO_4.H_2O$; MoO_4^{2-} - $(NH_4)_6Mo_7O_{24}.4H_2O$; VO_4^{3-} - V_2O_5 (MERCK, Darmstadt, FRG). Each metal was tested for EC_{50} value calculation in 10 various concentrations in the next ranges (mg/L): Cu^{2+} 0.1 - 10.0; Ni^{2+} 5.0 - 50.0; Mn^{2+} 5.0 -30.0; MoO_4^{2-} 1.0 - 10.0; VO_4^{3-} 10.0 - 30.0. For photosynthetic pigments determination as well as for accumulation tests metals were used only in concentrations corresponding with calculated EC_{50} values for root growth inhibition.

RESULTS AND DISCUSSION

Effect of metals on seedlings root growth: The inhibitory effects of tested metals on root prolongation of S. alba were evaluated by using probit analysis as EC_{50} values and their 95 % confidence intervals (CI) (Table 1.). On the base of these values and their statistical evaluation, metals can be arranged in a rank order of inhibition as follows: $\mathbf{Cu^{2+} > MoO_4^{2-} > Ni^{2+} \geq Mn^{2+} > VO_4^{3-}}$. Judging from EC_{50} values for root growth inhibition of S. alba it is evident that Cu^{2+} ion was the most, and VO_4^{3-} the least toxic one. It can be concluded that a low toxic effect was also determined for Mn^{2+} and Ni^{2+} ions. The toxicity of Ni^{2+}, Mn^{2+} and VO_4^{3-} ions was approximately 10-times lower than that of Cu^{2+} and MoO_4^{2-} metal ions. For plants, copper is one of several heavy metals that is essential to their life.

Table 1. Inhibitory effects of metals on root growth and photosynthetic pigment production of Sinapis alba and their statistical evaluation

Metal	EC_{50} (mg/L) (95 % CI)	Root length ± SD (cm)	Chl*a* ± SD (μg/mL)	Chl*b* ± SD (μg/mL)	Carotenoids ± SD (μg/mL)
Contr.		2.2 ± 0.077	140.5±4.57	72.52±2.68	56.9±2.08
Mn^{2+}	13.0 (11.22 – 14.60)	1.1 ± 0.032 ***	87.08±3.39 [38 %] ***	43.49±1.65 [40.2 %]***	34.04±1.35 [40.2 %]***
VO_4^{3-}	15.8 (13.95 – 16.32)	1.3 ± 0.031 ***	104.35±4.10 [25.7] %**	55.49±2.54 [23.5 %]**	42.13±2.15 [26.0 %]**
Cu^{2+}	4.3 (3.72 – 4.86)	1.4 ± 0.042 ***	103.47±3.05 [26.7 %]**	57.22±1.70 [21.3 %]**	42.15±1.44 [25.9 %]**
Ni^{2+}	11.5. (10.75 – 12.17)	2.0 ± 0.028 ***	133.93±6.69 [4.7 %]*	30.10±1.41 [58.5 %]***	55.96±2.68 [1.7 %]*
MoO_4^{2-}	6.1 (5.82 – 7.08)	1.2 ±0.036 ***	131.11±6.00 [6.7 %]*	83.56±2.47 [15.2 %]*	60.41±2.89 [+6.2 %]*

SD – standard deviation; [] percentage of inhibition in comparison with the control
* no significant difference in comparison with the control (P>0.05); ** significant difference in comparison with the control (P<0.05); *** highly significant difference in comparison with the control (P<0.01);

Exceptionally copper may also be toxic to plants, affecting thereby mainly the growth of the roots (Ouzounidou 1995a; 1995b). As Ouzounidou (1995b) and Ebbs and Kochian (1997) described, the root growth is influenced not only by the concentration of the present metal but also by the plant species. Mustard Sinapis alba used in our tests belongs to Capparidales, Brassicaceae and seems to be more sensitive to copper than the species of Caryophyllaceae and Cruciferae which were tested by Ouzounidou (1995b). The concentration of copper that inhibited the root length of mustard by 50 % was about half the times lower than those introduced by Ouzounidou (1995a; 1995b). For nickel, Berrow and Burridge (1991) introduced that it is as essential as toxic to plants. Its EC_{50} value in our tests was 11.5 mg/L and did not overreach the values of nickel in the hydrosphere (Tölgyessy 1993) and lithosphere (Wedepohl 1991). Manganese in its inorganic species is a ubiquitous, essential element in nature, and in its occurring concentrations as well with molybdenum is hardly toxic, and this statement agrees with our observations. The obtained EC_{50} value for manganese in our tests exceeds the level of this metal in the environment 1,000-times (Tölgyessy 1993).

Effect of metals on photosynthetic pigments production: All tested metals in used concentrations inhibited the photosynthetic pigment production no more than 58.5 % (chlorophyll *b* content inhibition by Ni^{2+}). Very strong inhibitory effect on

production of all three determined pigments had in used concentration Mn^{2+} ion which inhibited the production of chlorophyll *a* by 38.0 %, chlorophyll *b* and carotenoids by 40.2 % (Table 1.) in comparison with control. The strongest inhibitory effect on chlorophyll *b* had Ni^{2+} (58.5 %) (Table 1.). No significant differences in comparison with control were determined for Ni^{2+} and chlorophyll *a* and carotenoids content, and for MoO_4^{2-} and all photosynthetic pigments (Table 1.). From these results it can be concluded that MoO_4^{2-} did not influence the production of the determined photosynthetic pigments. In scientific literature, attention is concentrated infrequently on pigment production in various plant species under metal effect (Ouzounidou 1995a). It is usually introduced as the effect of metals upon the photosynthetic apparatus (Šeršen al. 1997) and on chlorophyllase activity measurement (Abdelbasset et al. 1995). Chlorophyllase is an enzyme which decompose chlorophyll. Some metals (Cd^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}) and their mixture enhanced activity of this enzyme on chlorophyll *a* decomposition higher than on chlorophyll *b* (Abdelbasset et al. 1995). No differences between effects of Mn^{2+}, VO_4^{3-} and Cu^{2+} on individual pigments were confirmed during our observations. Nickel inhibited stronger chlorophyll *b* than chlorophyll *a* and carotenoids production and it is completely in opposition of the results introduced by Abdelbasset et al. (1995). Molybdenum also inhibited more chlorophyll *b* production but this metal enhanced the production of carotenoids in S. alba. As described Singh et al. (1996), metals affect generally chlorophylls more than carotenoids, and this statement agrees with our results obtained for Ni^{2+} and MoO_4^{2-} ions. When the effect of Cu^{2+} on pigments production was compared with effects of other metals tested, no significant differences were confirmed between copper and vanadium. In comparison with manganese, the Cu^{2+} effect was significantly lower for all determined pigments, and in comparison with Ni^{2+} and MoO_4^{2-} significantly higher, except chlorophyll *b* content in presence of Ni^{2+}.

Accumulation of metals: The results from the bioaccumulation of tested metals in the concentrations responding to estimated EC_{50} values are given in Table 2. The accumulation of all tested metals was higher in the roots than in the hypocotyls. Usually in literature, it is also introduced that the accumulation of metals is most intensive in roots, than in cane and leaves, and the lowest is in the seeds (Sawert et al. 1987). Distribution of metals in plants depends on the vegetation stage and part of plant as on the species and metal concentration in the surrounding environment (Harangozó and Královic 1996). Extremely high was especially the accumulation of the MoO_4^{2-} ion. The accumulated amount of MoO_4^{2-} in the roots was 70.5 % and in the hypocotyls 24.6 % from the growth solution. The real conceptions connected with the amount of Mo as well as Mn, Cr, Cu, Zn and other metals is always joined with their positive versatility as microelements. Small amounts of molybdenum appear to be essential for maximum growth (Rajagopalan 1987).

Plant tissue metal concentrations are generally a function of the metal concentration in the growth solution or in the soil, but the relationship differs according to plant species and tissue (Kabatta-Pendias and Pendias 1984).

Table 2. Bioaccumulation of tested metals from the hydroponic medium into the roots and hypocotyls of Sinapis alba seedlings

Metal (mg/L)	Conc. in root ± SD (mg/g DW)	Conc. in hypocotyl ± SD (mg/g DW)
Mn^{2+} [13.0]	1.1 ± 0.026 8.5 %	0.4 ± 0.009 3.1 % (***)
VO_4^{3-} [15.8]	1.2 ± 0.035 7.6 %	0.5 ± 0.013 3.2 % (***)
Cu^{2+} [4.3]	1.8 ± 0.056 41.9 %	0.3 ± 0.007 7.0 % (***)
Ni^{2+} [11.5]	1.1 ± 0.030 9.6 %	0.6 ± 0.019 5.2 % (***)
MoO_4^{3-} [6.1]	4.3 ± 0.127 70.5 %	1.5 ± 0.056 24.6 % (***)

SD – standard deviation; DW – dry weight; [ ] used EC_{50} values;
* - statistical evaluation was done in comparison between metal amount in root and in hypocotyl; (***) highly significant difference ($P<0.01$); % - percentage from the metal amount added into the hydroponic medium

Absorbed trace elements are not uniformly distributed throughout the plant, so that different organ may vary in their ability to concentrate heavy metals. Particularly in roots of some plants, the cell wall is important in acting as a heavy metals accumulator. In leaves and stems however, the cell vacuole system is the main place for heavy metals deposition. The metal that was also accumulated in high amount in roots was copper. It accumulates in the roots and in the cell walls and is transported into plants in various ways and may also be excreted. As Havránek et al. (1983) described, the accumulated amount of Cu in roots of corn was significantly lower than in above-ground parts whereas in clover and rape approximately the same content in both above- and under-ground parts was found. For samples of Taraxacum officinale that were collected at various distances from the margin of the highway the higher accumulated amount of Cu was for all distances also introduced in the roots. In some cases the content of copper was more than 6-times higher in the roots than in the above-ground parts of plants (Tölgyessy et al. 1993). Ouzounidou (1995a) determined 15 times higher Cu^{2+} content in the roots of Koeleria splendens C. Presl. (Gramineae) than in the above-ground parts when 10.8 mg/L of Cu^{2+} was in the growth solution. In this case, it was also confirmed that the application of increasing Cu^{2+} concentrations in the growth solution induced changes on elemental distribution in the roots and shoots of the plants. In our case, the accumulated amount of Cu in S. alba roots was significantly higher than in above-ground parts. Other tested metals V, Mn and Ni were accumulated in the root's part in the amount of about 7-10 %. They

completely indicated higher accumulation in roots. There are no uniform data about the preference accumulation of these metals (Havránek et al. 1983).

It can be concluded that from the metals, the most toxic were for root growth Cu^{2+} and MoO_4^{2-} and the least toxic was VO_4^{3-}. All tested metals in concentrations corresponding with calculated EC_{50} values inhibited the photosynthetic pigments production no more than 58.5 % (chlorophyll *b* content inhibition by Ni^{2+}). Very strong inhibitory effect on all three pigments production had especially Mn^{2+}, while MoO_4^{2-} did not significantly influence the pigments amount. In general, tested metals were accumulated in higher amounts in the roots. Very high accumulation was determined in both roots and hypocotyls for MoO_4^{2-} while copper was accumulated in high amount especially in the roots.

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