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REGULAR ARTICLE

Growth, root and leaf structure, and biomass allocation in *Leucanthemum vulgare* Lam. (Asteraceae) as influenced by heavy-metal-containing slag

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Abstract Effects of heavy metal contamination on growth, leaf turnover, biomass allocation and leaf and root structure of *Leucanthemum vulgare* Lam. were investigated. Plants were grown in two outdoor experiments, for 5 weeks or for 3 months, respectively, on sand with different additions of slag containing elevated levels of heavy metals, especially Cu and Ni. In the 3-month experiment nutrients were provided as composted manure, in the 5-week experiment as a solution. Slag contamination reduced plant growth, biomass allocation to roots, specific root length and specific leaf area, while root tissue density and leaf dry matter content increased. Fine root diameter increased, whereas coarse root diameters showed a non-significant decreasing trend. Toxicity of slag was lower in the 3-month experiment, probably due to organic matter in the substrate. We conclude that heavy metals in the soil around Cu–Ni smelters may, besides directly reducing growth of the plants, increase their susceptibility to other stresses such as drought, by reducing the root length to leaf area ratio. Fine and coarse roots show distinct responses, indicating that different root diameter classes should be regarded separately to fully understand stress responses of root systems.

Keywords Biomass allocation · Heavy metals · Leaf area · Root diameter distribution · Root length · Slag

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Introduction

Elevated levels of heavy metals in soil reduce plant growth and may even kill the plants (Woolhouse 1983). Different metals vary greatly in their detailed biochemical effects, but they usually cause some kind of oxidative damage, resulting in phenotypic effects which often are similar for the different elements (Shaw et al. 2004). For example, chlorophyll content and photosynthesis are reduced by exposure to elevated levels of Cu, Ni and other heavy metals (Burzynski and Kłobus 2004; Martins and Mourato 2006; Ouzounidou et al. 2006; Gajewska et al. 2006; Alam et al. 2007). Heavy metals reduce the number of chloroplasts and the area of thylacoid membranes, and cause damage to root tips (Barceló and Poschenrieder 2004). In contrast to effects on physiology or cell ultrastructure, the structural effects of heavy metals at the whole plant level are less investigated. For example, the well-known effect of reduced root elongation and branching (Bradshaw 1952; Barceló

and Poschenrieder 2004), frequently used as a test to assess heavy metal toxicity (Wilkins 1957), is rarely regarded in the context of the whole plant, i.e., it is usually not established whether the root growth reduction exceeds the reduction which can be expected based on the smaller plant size.

Evidence about heavy metal effects on plant biomass turnover rate is controversial: high levels of heavy metals cause early mortality (Kjær and Elmegaard 1996) and there is some evidence for accelerated senescence in detached leaves (Jana and Choudhuri 1982; Fuhrer 1983), but also for a slower leaf turnover due to delay and reduction of flowering (Brun et al. 2003; Ryser and Sauder 2006). Shortened root life span in the vicinity of a Cu–Ni smelter has been found for *Pinus silvestris* (Helmisaari et al. 1999).

The purpose of the present study was to investigate above- and below-ground aspects of response to heavy metals in plant structure, growth and leaf turnover. Our intention was to study these effects at levels of contamination which do not cause obvious damage, but nevertheless reduce growth. As well as growth and leaf turnover, we investigated the responses of biomass allocation patterns and structural traits such as specific leaf area, specific root length, leaf and root tissue density and root diameter distribution. These traits are associated with acquisition capacities for above- and below-ground resources and respond to stresses such as nutrient limitation or drought (Ryser and Lambers 1995; Kalapos et al. 1996). A metal-effect on these traits may influence the susceptibility of a plant to other stresses.

Effects of heavy metals are often tested individually using single metals. However, the effects of a metal are influenced by the presence of other metals, as shown for example by Wallace and Berry (1989) for effects of Ni, Zn and Cd on lettuce root growth, by Taylor et al. (1998) for effects of Al and Mn on foliage of *Vigna unguiculata*, or by Kukkola et al. (2000) for effects of Cu and Ni on biomass allocation pattern in *Pinus silvestris*. In contaminated soils several metals usually co-occur in high concentrations. Our approach was to investigate the effects of a mixture of metals, found in soils contaminated by the Cu–Ni mining and smelting industry around Sudbury, Ontario, Canada, by creating a gradient of similar contamination by mixing different amounts of slag produced by that industry with sand. Slag is a by-product of smelting and contains residual metals, the proportional contamination of which can be expected to be similar to the soil contamination around the smelter, as particulate matter escaping from the smelting process is the main contributor to this soil contamination. Slag is also spread in the environment as construction material for roads and railways, and as filling material (Jansons and Rousell 2002; Nieminen 2005). In Sudbury, the elevated concentrations of Cu, Ni and Co, for example, in slag from the Cu–Ni smelting industry (Dresler et al. 1997) correspond to the soil contamination in the region (SARA-Group 2004).

Achieving predetermined concentrations of bioavailable heavy metals by adding salts is difficult in the long term, even in a solution culture (Sheldon and Menzies 2005; Kopittke and Menzies 2006). Root characteristics may also be quite different in nutrient solution compared to solid substrates. In solid substrates it is even more difficult to predetermine metal availability, as it strongly depends on substrate characteristics (Evans et al. 2003; Chen et al. 2004). We assume that adding slag to sand produces over time more stable metal levels in the substrate than an addition of metal salts. Leaching of metals from slag is usually not considered to be very high, but it occurs, and metals in leachate may be taken up by plants, as shown by Lind et al. (2001) in the case of Cr. An analysis of extractable metals confirmed the similarity of relative metal contamination by different metals in our substrates with the Sudbury area soils.

Material and methods

As test species we used a naturalized perennial herb of European origin, *Leucanthemum vulgare* Lam. (Asteraceae), common in the Sudbury area along roadsides and in grasslands. The species is not found on the most heavily contaminated sites, such as former roast yards, indicating that it has not developed a tolerance to heavy metals like the grasses which grow on such sites, such as *Agrostis scabra* or *Deschampsia caespitosa* (Rauser and Winterhalder 1985; Archambault and Winterhalder 1995).

Two outdoor experiments were conducted to investigate the response of *L. vulgare* to gradients of heavy metal contamination: a 3-month experiment lasting from May to August, and a 5-week experiment in August–September. The gradients were achieved by mixing slag from a Cu–Ni smelter in Copper Cliff,

Sudbury, Ontario, Canada, with sand. Slag was collected on April 24, 2006, from a slag crushing plant. The collected crushed slag had particle size up to 4 mm, but was mostly below 2 mm in size. The slag was mixed with glaciofluvial sand from Sudbury area with a pH of 7.8 (Ethier Sand and Gravel, Sudbury, Ontario, Canada). In the 3-month experiment composted sheep manure was added as source of nutrients, in the 5-week experiment the nutrients were given as a solution twice a week. Extractable heavy metals in the substrates were analysed at the end of the experiment.

Average temperatures during the 3 months of the first experiment were 17.3, 19.7 and 18.9°C, the precipitation 49, 66 and 101 mm, respectively (Sudbury Airport, 24 km ENE of the experimental garden; Environment Canada 2007). Day length varied between 15.8 and 13.7 h, with values of PAR well over 2,000 μmol m^{-2} s^{-1} on sunny days (Light intensity data for 2007, Watchdog Ministation, Spectrum Technologies, Plainfield, Illinois, USA). Average temperature during the second experiment was 13.7°C, the precipitation 84 mm, and daylength between 13.8 and 12.0 h.

Seeds of *L. vulgare* were collected in summer of 2005 in a residential backyard in Sudbury. Seedlings were germinated on sand in a Petri dish and planted in PVC pipes of 10 cm diameter and 36 cm height containing the substrate mixtures outdoors in an experimental garden (46°35.62′N, 81°49.00′W). The pipes were standing in pools filled with approximately 10 m groundwater, filled with a solar pump. Capillary force kept the substrate moist throughout the experiment. In each of the experiments, all pots were standing in one pool. The solar pump was running during daylight hours, continuously exchanging the water in the pools.

3-month experiment

The aim of this experiment was to investigate the response of plants to various slag concentrations over an entire growing season, with the focus on growth, turnover and root system structure. The growth substrate contained slag to 0% (control), 10% or 50% (volumetric). All substrates contained 10% composted sheep manure (Home Hardware Stores Limited, St. Jacob, Ontario, Canada) to provide the plants with nutrients throughout the season. The remaining volume of the substrate, i.e., 90%, 80% and 40% in the three treatments, respectively, was sand.

Seedlings were planted on May 24, 2006, in the PVC pipes containing the three substrate mixtures, 17 replicate plants in each treatment. Ten randomly selected replicate plants of each treatment were harvested on August 24 and 25. The plants were washed out of the sand and placed between moist paper towels to be transported to the laboratory.

In the laboratory all living and dead leaves were counted, and the chlorophyll content of one non-senescent leaf per plant was measured using a Konica–Minolta SPAD 502 chlorophyll meter (Konica–Minolta Sensing, Osaka, Japan). Then the plants were dissected into leaves, roots and the remaining short stem. The area of living leaves was measured using a LI-3100 area meter (LICOR, Lincoln, Nebraska, USA), placing the leaves between two glass sheets of 2.3 mm in thickness.

Root length was measured using the grid-intersection method (Newman 1966; Tennant 1975). Root diameter distribution was assessed by measuring the diameter of approximately 100 randomly selected root sections with a microscope using a 40-fold magnification (Boot and Mensink 1990; Ryser and Lambers 1995).

Dry mass of all plant parts was determined after drying at 75°C for at least 24 h.

5-week experiment

The aim of this short-term experiment was to investigate the effects of slag in the substrate on plant growth and plant biomass allocation, as well as on leaf and root structure. In the 3-month experiment biomass allocation, and root length to leaf area ratio could not be properly assessed due to leaf (and possibly root) mortality before the final harvest.

L. vulgare seedlings were planted in PVC pipes on August 22, 2006. Five treatments were applied: Control on pure sand, and four different mixtures of that sand and slag, containing 5%, 10%, 20%, and 40% slag (volumetric). No organic matter was added in the substrate. There were ten replicate pots per treatment.

Nutrients were added twice a week as a solution, 120 ml per occasion and pot. The solution was made dissolving 20 g of All Purpose water soluble 20–20–20 fertilizer with micronutrients (Home Hardware Stores, St. Jacobs, Ontario) in 18 l of water resulting, for example, in an addition of 27 mg of N, 12 mg P and 22 mg K per pot at a time.

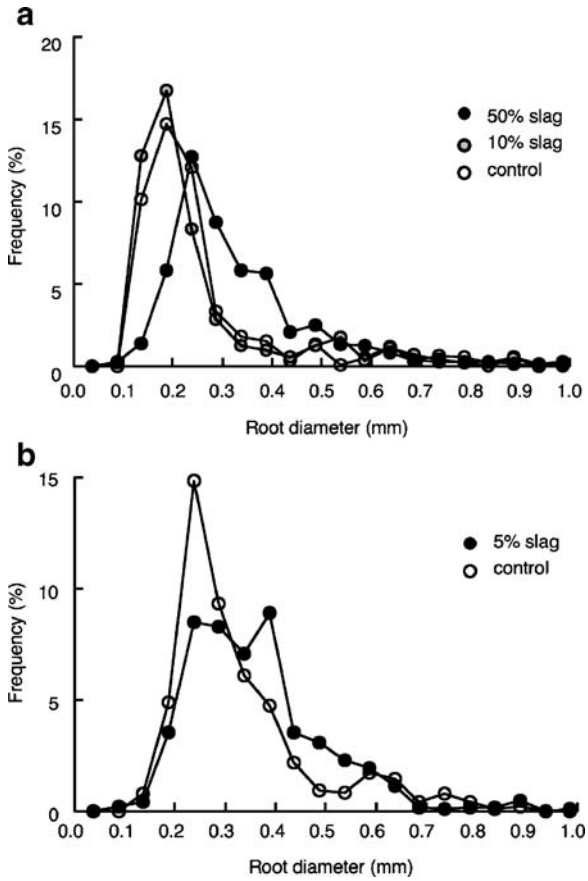

Fig. 1 Root diameter distribution for *L. vulgare* grown at three levels of slag concentration in the 3-month experiment, and two levels at the 5-week experiment. The *symbols* indicate the mean values in each of the treatment

The plants were harvested on September 26, 36 days after planting. The roots were carefully washed out of the substrate and the plants were stored between moist paper towels for transportation to the laboratory. The number of dead and living leaves was counted. Leaf chlorophyll content and leaf area were measured as described for the 3-month experiment. The plants were dissected into leaf blades, roots, and the remaining part (stem). Leaf fresh mass, dry mass of all parts, root length and root diameter distribution were measured as described for the 3-month experiment.

Calculated traits

Based on the measured traits, leaf mass ratio (LMR; leaf dry mass/total dry mass), root mass ratio (RMR; root dry mass/total dry mass), stem mass ratio (SMR; stem dry mass/total dry mass), specific leaf area (SLA; leaf area/leaf dry mass), leaf dry matter content (DMC; leaf dry mass/leaf fresh mass) and specific root length (SRL; root length/root dry mass) were calculated.

Root diameter distribution is usually expressed as the mean diameter. However, as fine and coarse roots may respond differently to metal contamination (Bushamuka and Zobel 1998) we investigated their responses separately. For the mean fine root diameter the average diameter of the finest 25% of root length was calculated, and for the mean coarse root diameter the average diameter of the 10% of roots with the largest diameter was calculated. These ranges were chosen, based on the actual distribution pattern, to express diameter of the finest and the coarsest roots, which we assumed would maximize the sensitivity of the analysis.

Based on root diameter distribution, root volume per root length was calculated, assuming a cylindrical form of all roots. This, together with the SRL, enabled the calculation of root tissue density (dry mass per volume).

Analysis of the growth substrate

In both experiments, substrate samples from three pots per treatment were collected. pH was analysed in a 1:1 mixture with water. The elemental content of extracts of these samples was analysed using inductively coupled plasma atomic emission spectroscopy (ICP-AES; Thermal Jarrell Ash ICAP 61). The samples were oven dried at 600°C and a 0.5 g sample was treated with 3 ml HNO_3 and 4 ml HCl and left overnight for a room temperature digestion and then placed on a hot plate at 100°C for 1 h. Thereafter the samples were allowed to cool and the volume was adjusted to 50 ml using deionized water. The samples were allowed to settle over night and decanted for ICP analysis.

Statistical analyses

Treatment effects on the amounts of extractable metals in the substrate mixtures were tested with an ANOVA, the experiment (3-month and 5-week) and treatment levels (three in each experiment) being the factorial variables, logarithmically transformed element concentrations in the extract the dependent variables. Due to larger variance in plant traits in the slag-containing treatments compared to the control treatment (hetero-

Table 1 Extractable amounts (ICP-AS, acid extraction) of elements in the growth substrate in the two experiments at harvest, expressed in mg g^{-1} of the dry mass

	B	Ca	Co	Cr	Cu	Fe	Ni	S	Sr	Ti	V	Zn	Zr
5-week experiment													
C	0.012±0.007	4.0±0.6	0.09±0.05	0.020±0.004	0.15±0.13	28±13	0.11±0.09	0.21±0.15	0.0080±0.0006	0.33±0.02	0.015±0.002	0.038±0.020	0.011±0.007
5%	0.033±0.017	3.7±0.6	0.27±0.16	0.034±0.011	0.36±0.18	95±61	1.57±1.43	2.20±1.88	0.0073±0.0019	0.30±0.01	0.015±0.002	0.101±0.056	0.001±0.009
10%	0.067±0.021	2.5±0.0	0.49±0.15	0.052±0.012	0.77±0.22	206±78	1.81±1.15	2.23±1.18	0.0053±0.0003	0.28±0.01	0.008±0.002	0.202±0.061	0.000±0.009
3-month experiment													
C	0.002±0.001	2.1±0.3	0.03±0.01	0.012±0.004	0.01±0.00	11±2	0.02±0.01	0.00±0.01	0.0050±0.0006	0.31±0.04	0.014±0.002	0.012±0.002	0.021±0.005
10%	0.013±0.000	2.2±0.1	0.09±0.01	0.019±0.001	0.14±0.02	27±1	0.12±0.02	0.18±0.02	0.0063±0.0009	0.31±0.02	0.014±0.000	0.039±0.002	0.019±0.003
50%	0.026±0.004	2.0±0.1	0.21±0.04	0.027±0.004	0.71±0.25	60±14	0.51±0.21	0.71±0.26	0.0042±0.0002	0.23±0.01	0.010±0.001	0.086±0.015	0.007±0.003

Data given for elements with significant treatment effects. Amounts of Ba, Be, K, Mg, Mn, Na and P did not respond to treatments. As, Cd, and Pb were generally below the detection limit.

scedasticity: Levine's test), we conducted Kruskal–Wallis tests for treatment effects on plant traits.

Results

Substrate

The extractable amount of several elements increased with increasing slag content (Tables 1 and 2). Notably Cu and Ni concentrations were high at the highest levels of slag addition. Other elements which increased with slag addition were B, Co, Cr, Fe, S and Zn.

The substrate was slightly acidified by slag addition during the experiment. At the end of the experiments the pH values in treatments containing slag were below the values in the control treatments. In the 5-week experiment the pH values for control and 5% slag mixture slag substrates were 6.7±0.1 and 6.4±0.1 (ANOVA, N=18, F=7.7, P=0.013). In the 3-month experiment the pH values for control, 10% slag and 50% slag were 7.1±0.1, 6.8±0.1 and 6.8±0.1 (ANOVA, N=12, F=6.8, P=0.016).

3-month experiment

The numbers of both living and dead leaves at the time of harvest decreased with increasing slag content in the substrate (Table 3). Leaf turnover, i.e., the ratio of dead leaves to the total number of leaves produced increased with slag concentration in the substrate, but the differences were not significant. Total plant biomass at harvest significantly decreased with increasing slag content of the substrate. Specific leaf area decreased, and leaf dry matter content increased with increasing slag content. Leaf area-based chlorophyll content was lower with 50% slag, compared to other treatments, and the leaves in that treatment were red indicating high levels of anthocyanins. Specific root length showed a weakly significant response, being higher at 10% slag compared to 50% slag or the control treatment. Average root tissue density was higher in the 50% slag treatment compared to other treatments, but due to large variation in that treatment the difference was not significant. The average diameter of the fine roots was the same for the control treatment and 10% slag treatment, but in the 50% slag treatment it was 43%, higher. The average diameter of the coarse roots was not significantly influenced (Fig. 1; Table 4).

Table 2 R^2 and P values of ANOVAs with log-transformed element concentrations in growth substrate as independent variables, and the two experiments (3-month, 5-week) and three slag concentrations in each of the experiments as factorial variables (three replicate measurements, except for the 50% slag level in the 3-month experiment with two replicates)

	B	Ca	Co	Cr	Cu	Fe	Ni	S	Sr	Ti	V	Zn	Zr
R^2	0.60	0.70	0.61	0.60	0.66	0.62	0.50	0.587	0.44	0.45	0.41	0.61	0.60
Treatment	0.005	0.017	0.004	0.009	0.002	0.003	0.013	0.022	0.040	0.036	0.014	0.003	0.015
Experiment	0.447	<0.001	0.438	0.231	0.600	0.438	0.688	0.110	0.039	0.584	0.406	0.751	0.162
$T\times E$	0.036	0.021	0.032	0.049	0.024	0.021	0.072	0.185	0.092	0.236	0.045	0.009	0.053

5-week experiment

All plants growing on slag contents of 10%, 20% and 40% died within about a week after transplanting. At harvest, plants grown on 5% slag had a total dry mass of 18% of that of plants grown on sand only (Table 4). Plants in the 5% slag treatment allocated proportionally less biomass to the roots and more to leaves and stems. RMR, for example, was 30% below the value of the control plants (Table 4). Leaf area was reduced by 86%, total root length by 94%, and the root length to leaf area ratio by 69% in the 5% slag treatment, compared to control. Specific leaf area was 22% lower in the slag-treated plants, leaf dry matter content 38% higher. The 5% slag treatment reduced specific root length by 55%, and root tissue density was triple of that in control. There was no significant effect on leaf chlorophyll content.

Fine roots (the thinnest 25% of the total root length) of the slag-treated plants were 15% thicker than fine roots of control plants. The average diameter of the coarsest 10% of the roots was slightly reduced by the slag-treatment, but the effect was statistically not significant (Fig. 1; Table 4).

Discussion

The range of extractable amounts of metals in our substrates corresponds well to levels found in Sudbury soils. For example, the ranges for Cu and Ni in our experiments were 10–770 mg g^{-1} and 20–1,810 mg g^{-1}, in samples of Sudbury soils measured by Adamo et al. (2002) 11–1,890 mg g^{-1} and 23–2,150 mg g^{-1}, respectively.

Addition of slag in the substrate reduced plant growth in both experiments, with generally similar responses in plant structure. However, the toxicity levels strongly differed between the two experiments. In the 3-month experiment 10% slag in the substrate resulted in a growth reduction of about 50%, whereas

Table 3 Characteristics of *L. vulgare* grown in substrates with 0% slag, harvested three months after planting

	0% slag	10% slag	50% slag	P value
Number of leaves alive	23.3±3.1	11.2±1.4	6.9±1.4	<0.001
Number of leaves dead	6.±1.2	3.9±0.6	2.3±0.5	<0.001
Proportion of dead leaves	0.23±0.05	0.26±0.03	0.36±0.13	0.284
Total dry mass (g)	0.92±0.06	0.44±0.06	0.11±0.03	<0.001
SLA (m^2 kg^{-1})	17.7±1.0	15.6±0.6	13.9±1.2	0.021
Leaf DMC (g g^{-1})	0.16±0.01	0.18±0.01	0.23±0.01	<0.001
Chlorophyll (SPAD units)	41±2	46±2	23±3	0.006
SRL (m g^{-1})	188±33	26±24	173±127	0.033
Root tissue density (mg mm^{-3})	0.039±0.005	0.033±0.006	0.070±0.031	0.719
Fine root diameter (mm)	0.15±0.01	0.16±0.01	0.22±0.03	0.009
Coarse root diameter (mm)	0.67±0.04	0.60±0.04	0.63±0.03	0.447

Chlorophyll concentrations are expressed as area-based SPAD units. Mean fine root diameter is the average of diameters of the finest 25% roots (length). Mean coarse root diameter is the average of diameters of the coarsest 10% roots (length). The P value is based on a Kruskal–Wallis test (Ten, nine and seven replicates in the three treatments).

Table 4 Characteristics of *L. vulgare* grown in substrates with 0% slag (control) and 5% slag harvested 5 weeks after planting

	0% slag	5% slag	*P* value
Total dry mass (g)	0.168±0.016	0.030±0.005	<0.001
LMR (g g^{-1})	0.45±0.01	0.50±0.02	0.082
SMR (g g^{-1})	0.19±0.01	0.26±0.01	0.002
RMR (g g^{-1})	0.36±0.01	0.25±0.01	<0.001
Plant leaf area (cm^2)	25.9±2.4	3.7±0.5	<0.001
Plant root length (m)	13.9±2.0	0.81±0.19	<0.001
Root length/leaf area (m cm^{-2})	0.53±0.04	0.22±0.04	0.003
SLA (m^2 kg^{-1})	34.1±1.3	26.7±2.4	0.010
Leaf DMC (g g^{-1})	0.090±0.004	0.125±0.006	0.001
Chlorophyll (SPAD units)	41.5±1.1	43.3±2.0	0.472
SRL (m g^{-1})	235±29	106±14	<0.001
Root tissue density (mg mm^{-3})	0.052±0.006	0.155±0.041	0.013
Fine root diameter (mm)	0.213±0.005	0.244±0.009	0.028
Coarse root diameter (mm)	0.665±0.025	0.595±0.036	0.151

All plants grown in substrates with 10%, 20% and 40% slag died early. Chlorophyll concentrations are expressed as area-based SPAD units. Mean fine root diameter is the average of diameters of the finest 25% roots (length). Mean coarse root diameter is the average of diameters of the coarsest 10% roots (length). The *P* value is based on a Kruskal–Wallis test (ten replicates in each treatment).

in the 5-week experiment addition of 10% slag quickly killed the plants, and a 5% addition resulted in a growth reduction of about 80%. The difference in toxicity between the treatments may have been caused by the difference in organic matter content of the substrate, as the 3-month experiment contained 10% composted sheep manure. Metals such as Ni and Cu are known to complex with organic matter (McNear et al. 2007), which strongly influences the bioavailability of these heavy metals. Furthermore, the EDTA in the nutrient solution, added to dissolve iron, may have influenced the heavy metal solubility.

Despite the differences in duration, and in metal availability for a given amount of slag in the substrate, plant responses in both experiments showed similar patterns: the reduced growth was associated with thicker and denser fine roots, somewhat thinner coarse roots, and aboveground, lower SLA and higher leaf dry matter content. The short-term experiment indicated increased allocation of biomass into the roots. In the long-term experiment, no indication of an effect on leaf turnover rate was found, but slight effects may have been confounded by the large difference in leaf production.

The increased diameter of fine roots in response to slag may be a similar ethylene-mediated response as that caused by some other stresses which increase root diameter, such as soil compaction (Clark et al. 2003), as in the short term, blocking ethylene receptors effectively abolishes the inhibitory effect of Cu on root elongation (Maksymiec and Krupa 2007).

In contrast to fine root diameters, coarse root diameters were reduced. The effect was not significant, but we assume that this trend was real, as coarse root diameter is generally positively associated with plant size (Ryser 1998). However, random sampling of the few coarse roots inaccurately reflects their diameter distribution, causing a large error variation. These results of different responses of the fine and the coarse roots to heavy metal toxicity support the opinion that different root diameter classes should be regarded as functionally distinct, as suggested by Bushamuka and Zobel (1998), who found that different root types of maize and soybean differ in their tolerance to Al-toxicity.

Root thickening in response to heavy metals has often been mentioned in the literature (e.g. Potters et al. 2007), but there is a lack of quantitative data. Zobel et al. (2007) found an increased fine root diameter in 3–9 mM Al solution, and Arduini et al. (1995) describe an increase in cortex, stele and whole root diameter in response to 1 μM Cu in two pine species. In *Miscanthus sinensis*, low Cr applications lead to a decreased root diameter, whereas high concentrations increased the diameter (Arduini et al. 2006). The contrasting effects may be a result of the different response of fine and coarse roots. Most of the mentioned studies do not make a distinction between

fine and coarse roots, and an average root diameter does not necessarily characterize a response of root system structure adequately.

Slag also increased the root tissue density, and SRL in the 5-week experiment. In the 3-month experiment SRL was the highest in the intermediate treatment, probably as a consequence of a stronger response in coarse root diameter in that treatment, compared to responses in fine root diameter or root tissue density.

Increased slag content in substrate reduced biomass allocation to roots. A reduction in root elongation as a response to heavy metals is well known, but data about the effects on relative biomass allocation to roots is rare, and inconsistent. In hydroponics, Cd and Pb have been found to increase root to shoot ratio in *Beta vulgaris* (Larbi et al. 2002). In soil, (Ahonen-Jonnarth and Finlay 2001) found that Ni and Cd increased the root to shoot ratio in *Pinus silvestris*. On the other hand, Kukkola et al. (2000) found no effect of Ni or Cu addition on the root to shoot ratio of *P. silvestris*, and a marked reduction in biomass allocation to roots when the metals were added together. Ni added to a sandy substrate reduced the root to shoot ratio in *Brassica juncea* (Alam et al. 2007). Such contrasting responses may be associated with different combinations of metals, and also with general growth conditions. Xiong et al. (2006) found for *Brassica pekinensis* that Cu increased the root to shoot ratio at low N availability, whereas at high nitrogen supply Cu reduced the biomass allocation to roots. In our short-term experiment with reduced biomass allocation to roots in the 5% slag treatment, the nutrient supply was fairly high, with 270 mg N per pot over the 5-week period.

Above-ground structure showed a response to slag which is comparable to the below-ground one. Specific leaf area decreased and leaf dry matter content increased. Such responses can be caused by other stresses, such as low nutrients or drought (e.g., Ryser and Lambers 1995; Kalapos et al. 1996), but are not often described for heavy-metal influenced plants. Martins and Mourato (2006) found an increase in leaf dry matter content of tomato leaves in response to Cu.

The consequence of all the responses was at the whole plant level a strongly reduced root length to leaf area ratio, which for plants grown on 5% slag was less than half of that of the control plants. Such a reduction of root length per unit transpiring leaf area may increase a plant's vulnerability to drought.

Plants growing on slag-containing substrate produced fewer leaves, but there was no significant effect on leaf turnover. Leaf chlorophyll content was reduced in the 3-month experiment at the highest levels of slag addition, and the observed red colour of the leaves indicates high levels of anthocyanins. Elevated levels of copper have been shown to raise the levels of anthocyanins and antioxidant enzyme activities in maize (Tanyolaç et al. 2007). The effect of slag on chlorophyll and anthocyanin levels in the 3-month experiment only, even though substrate metal levels in the 5-week experiment were comparable or even higher, may have been a result of a combined effect of the metals with the older age of the leaves, and/or the lower levels of nutrients in the 3-month experiment. Despite the strong growth reduction by the 10% slag addition in the 3-month experiment, and the 5% addition in the 5-week experiment, no reduction in chlorophyll content was observed in these treatments. Several authors have found a reduced chlorophyll content as a response to Cu and Ni in growth medium, but such a loss seems to be a less sensitive indicator for heavy metal toxicity than a growth reduction, as also indicated by the data of Zheng et al. (2005) for *Capsicum annuum*. The effective level of metals which lead to reduced amounts of chlorophyll may also depend on growth conditions, such as availability of other ions, or illumination. Studies in which chlorophyll reduction in response to Cu or Ni has been observed are often conducted in a growth chamber or a greenhouse, with reported light intensities of 300 μmol m^{-1} s^{-1} or less (Gajewska et al. 2006; Kopittke and Menzies 2006; Martins and Mourato 2006; Alam et al. 2007; Bernal et al. 2007; Tanyolaç et al. 2007). Our experiment was conducted outdoors with maxima over 2,000 μmol m^{-1} s^{-1}.

Conclusions

A contamination of the growth substrate with a mixture of heavy metals, as present in slag from Cu–Ni smelting, had strong effects on plant structure. Plants allocated less biomass to roots, and the increased root tissue density and fine root diameter further reduced root length, and the root length to leaf area ratio. Such a response may lead to increased susceptibility to drought. Fine and coarse roots differed in their responses to metal toxicity, indicating

that mean diameter is an inadequate variable to describe root system responses. Comparison of our data with published literature indicates that the effects of metals on plants may strongly depend on the mixture of metals present, and on other environmental factors, such as nutrients or illumination. In order to fully understand plant responses to contaminated soils care should be taken that these responses are investigated under as natural conditions as possible.

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