

## Foliage response to heavy metal contamination in Sycamore Maple (*Acer pseudoplatanus* L.)

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### Abstract

Sycamore Maple (*Acer pseudoplatanus* L.) belongs to the typical pioneer trees found in contaminated ecosystems where it can contribute to the phytostabilisation of heavy metals (HM) in soils. In this study, foliage reactions caused by the experimental exposure to copper / zinc / cadmium / lead = 640 / 3000 / 10 / 90 mg/kg were investigated in trees following development of distinct visible symptoms of stress in crown foliage during the third year of exposure. Low amounts of HM were translocated to the foliage and no reduction of foliage biomass was recorded. Only zinc reached concentrations near the toxicity level, especially in older foliage, and its involvement in stress reactions was confirmed by the morphology of visible symptoms, cytochemical micro-localisation and structural changes in the leaves. Low quantities of zinc reached sensitive sites in the leaf blade and triggered oxidative stress. Phytoremediation potential of Sycamore Maple includes both a phytostabilisation and bioindication component. The latter can be useful for monitoring and surveillance purposes.

Keywords: phytoremediation, phytostabilisation, bioindication, zinc, cytochemistry, condensed tannins, symptom validation

## 1 Introduction

Different tree species growing spontaneously or after planting in sites with heavy metal (HM) pollution seem to have a considerable potential for the phytoremediation of contaminated soils (PULFORD and WATSON 2003). Pioneer species are not demanding for substrate structure, texture and nutrient availability and have other advantages like a deep root system and fast growth rate (PUNSHON *et al.* 1996). Species with either low or high HM uptake are best suited for phytostabilisation or phytoextraction respectively (PULFORD and WATSON 2003). As a pioneer and anemochore species, Sycamore Maple (*Acer pseudoplatanus* L.) often grows in contaminated ecosystems from central Europe and has until now mainly shown a phytostabilisation potential (PULFORD and WATSON 2003; MERTENS *et al.* 2004). A good tolerance for HM toxicity based on species-specific growth, stress avoidance and detoxification strategies can increase the phytoremediation capacity of trees (PULFORD and WATSON 2003; VOLLENWEIDER *et al.* 2006). Partitioning and rate of HM accumulation in the different organs widely varies between HM, tree species and even individuals within a single clone (LEPP 1996; PULFORD and WATSON 2003). In trees however, the micro-localisation of HM to date has rarely been characterised down to the tissue and cell level, because sizable amounts of HM are required to detect a microscopical signal with most methods. Translocation of HM to leaf threatens physiological activities in the tree crown as several

sites in the leaf blade and vein are sensitive to oxidative stress (DIETZ *et al.* 1999; PRASAD and STRZALKA 1999; VOLLENWEIDER *et al.* 2006). According to where HM have accumulated at tissue and cell level, they can trigger either little (COSIO *et al.* 2006; VOLLENWEIDER *et al.* 2006) or cascades of oxidative stress reactions leading to hypersensitive-like responses (HR-like, VOLLENWEIDER *et al.* 2003; MARTIN *et al.* 2006). Intolerant cell and tissue reactions result in visible leaf symptoms which constitute useful bioindications to (1) diagnose the stress factor, (2) rapidly assess contaminated spots in heterogeneously contaminated environments and (3) analyse the response variability between different populations and species of trees (VOLLENWEIDER and GUNTARDT-GOERG 2005). Visible symptoms can be validated with microscopical analyses: by micro-localising the deposits of HM at tissue and cell level and identifying the physiological reactions on the basis of their structural signature (VOLLENWEIDER *et al.* 2003, 2006; MARTIN *et al.* 2006), the link between the accumulation of a given HM and the occurrence of visible symptoms in foliage can be demonstrated. This combination of methods also provides insights about direct and indirect HM-effects useful to estimate the specificity of the different visible symptom features.

This study was realised within the framework project “From Cell to Tree” (GUNTARDT-GOERG 2005) after the observation of distinct visible symptoms in the foliage during the third year of exposure to a mixed HM contamination in the soil. It is focused on responses at the leaf, tissue and cell level in the foliage of Sycamore Maple. Investigations were performed to (1) elucidate the underlying structural changes and corresponding physiological reactions (VOLLENWEIDER *et al.* 2006), (2) distinguish between direct and indirect HM effects, (3) determine which HM caused the symptoms at cell, tissue and leaf level, (4) validate the symptoms and evaluate their bioindication potential and (5) analyse possible contributions of Sycamore Maple to phytoremediation. HM effects on foliage were characterised by measuring changes in foliage biomass and foliar HM concentration, micro-localising zinc (Zn) at tissue and cell level, observing microscopical symptoms of stress and analysing the leaf content for amounts of total phenols and condensed tannins. These latter compounds can complex different heavy metals (MCDONALD *et al.* 1996; MORAN *et al.* 1997), accumulate in the same apoplastic and symplastic cell compartment where HM have been translocated (VOLLENWEIDER *et al.* 2006) and contribute with antioxidant functions to detoxify oxidative stress (RICE-EVANS *et al.* 1997; SAINT-CRICQ DE GAULEJAC *et al.* 1999) caused by HM (DIETZ *et al.* 1999). They therefore constitute important markers of the defence and detoxification reactions induced by HM stress.

## 2 Materials and methods

### 2.1 Experimental design and plant material

The experiment was carried out at WSL in Birmensdorf (450 m above sea level), Switzerland, using twenty circular field plots with four treatments and five repetitions each. Each field plot (diameter: 2 m) consisted of a two-layered soil substrate isolated by concrete walls from the surrounding ground: 45 cm of an acidic forest subsoil (loamy sand [Alisol], initial pH in 0.01 M CaCl<sub>2</sub> = 4.2, from Eiken in the Rhine valley, Aargau, Switzerland) was packed inside each field plot and topped by 15 cm of an agricultural topsoil (loamy soil, initial pH in 0.01 M CaCl<sub>2</sub> = 6.4, C<sub>org</sub> = 15.1 g kg<sup>-1</sup>, from Birr, Aargau, Switzerland). This topsoil was selected to ensure sufficient nutrient availability in experiments within the framework “From Cell to Tree” and no fertiliser was added prior to or during the experimentation (see HERMLE *et al.* 2006a for physical and chemical soil properties). Ten plots received

topsoil with and 10 without artificial HM contamination. HM dust from a non ferrous smelter filter was applied manually resulting in copper (Cu) / Zn / cadmium (Cd) / lead (Pb) = 640 / 3000 / 10 / 90 mgkg<sup>-1</sup> of which 40 % (Cu), 70 % (Zn), 85 % (Cd), and 10 % (Pb) was either mobile or easily mobilizable (NOWACK *et al.* 2006). This level of soil contamination and HM bio-availability clearly exceeded Swiss VBBo limits<sup>1</sup> but remained partly below levels found at different polluted sites throughout Europe (DICKINSON 2000; HORVARTH and GRUIZ 1996; ERNST 1972). It thus reproduced, in a realistic way, soil pollution levels existing at “brown field” sites. The control treatment (CO) without HM contamination contained: Cu / Zn / Cd / Pb = 28 / 97 / 0.1 / 37 mgkg<sup>-1</sup>. The plots were irrigated by means of six sprinklers during periods with insufficient natural precipitation. The irrigation water ionic composition was taken as the 30 years mean of the ambient rain at the location. Its pH was adjusted by HCl to either 5.5 or 3.5. During winter (November to April) no irrigation was given. Four treatments were replicated 5 times each and included (1) control: uncontaminated topsoil irrigated with rainwater pH 5.5 (CO), (2) acid rain: uncontaminated topsoil plus rainwater pH 3.5 (AR), (3) HM: HM-contaminated topsoil plus rainwater pH 5.5 (HM) and (4) combination: HM-contaminated topsoil plus rainwater pH 3.5 (HMAR). Each plot was planted with different native tree and understorey plants to simulate the ecosystem conditions in a young forest plantation. The tree layer included seven different tree species at 14 equidistant places, including one Sycamore Maple (*Acer pseudoplatanus* L.) per plot (planted as six-months-old rooted cuttings before bud break in spring 2000).

## 2.2 Leaf biomass and HM content in foliage

Foliage of each tree was harvested in September 2002 and 2003, prior to the onset of autumnal coloration. The mean single leaf area and mass, specific leaf area (leaf area per leaf mass) and specific leaf mass (leaf mass per area) was determined in 10 leaves, taken randomly over each tree, after drying at 65 °C. In order to determine the leaf content of HM, an aliquot of the entire foliage dry mass per tree, was milled (ultra centrifuge mill wolfram carbide coated), digested with a high pressure microwave system (UltraClav by Milestone: 240 °C, 120 bar) and analysed in duplicates (spread <10 %) by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, Optima 3000 by Perkin Elmer) in the central lab of WSL. The influence of the treatments on differences in leaf morphology parameters, biomass and HM content was tested using analysis of variance (ANOVA) with SAS statistical program package (Trademark SAS Institute, Inc).

## 2.3 Monitoring of visible symptoms

Visible symptoms in leaves of Sycamore Maple were recorded every two weeks from their first occurrence (summer 2002) and during the 2003 vegetation season until late summer prior to the onset of autumnal coloration. They were quantified, determining the proportion of symptomatic leaves on the top shoot (out of a total of 27.4 leaves on average per tree by the end of monitoring in 2003). Symptoms induced by the treatment were differentiated according to VOLLENWEIDER and GUNTARDT-GOERG (2005) on the basis of their morphology, seasonal development and distribution along the shoot from other symptoms induced by biotic (fungi, insects) or abiotic stress factors (hail).

<sup>1</sup> Verordnung über Belastungen des Bodens (VBBo, 1998): Cu / Zn / Cd / Pb = 150 / 300 / 2 / 200 mg/kg.

## 2.4 Cytochemical and structural analyses

In late summer (20.09.2002), disks 1 cm in diameter were excised at the leaf basis from symptomatic and asymptomatic leaves at three different leaf position along the top shoot of two CO and two HM-treated trees. They were fixed either in methanol or by infiltration with buffered (pH 7.0) 2.5 % glutaraldehyde. Zinc was cytochemically revealed (VOLLENWEIDER *et al.* 2005) for the purpose of micro-localisation. Changes in the cell and tissue structure and the physiological responses to HM stress were analysed in light and fluorescence microscopy according to VOLLENWEIDER *et al.* (2003 and 2006) and GUNTARDT-GOERG *et al.* (1997).

## 2.5 Biochemical analysis of total phenols and condensed tannins

Amounts of total phenols and condensed tannins (CT) were analysed in five to seven leaves across each tree crown in 2002 harvested at the same time as disks for microscopy. Leaf samples were immediately frozen in liquid nitrogen, freeze-dried and stored at  $-20^{\circ}\text{C}$  until analysis. An aliquot of 0.5 g of dry foliar material was frozen in liquid nitrogen prior to homogenization (1 min) with steel balls in a Mikro-Dismembrator II of B. Braun, Melsungen, Germany ( $60 \times 15$  mm oscillations per second). The still frozen powder was transferred to a separating funnel and extracted 4 times during 3 min under magnet stirring at room temperature with 4.5 ml acetone 70 % containing 0.1 % ascorbic acid. The clear filtrates were then combined and partially purified (BROADHURST and JONES 1978). Total phenols (TP) were analysed using the Folin-Ciocalteu method (ESTERBAUER *et al.* 1975; WATERMAN and MOLE 1994). CT were quantified with the proanthocyanidin (mainly the CT polymers; PORTER *et al.* 1986; WATERMAN and MOLE 1994) and acid-vanillin assay (mainly the CT oligomers; BROADHURST and JONES 1978; WATERMAN and MOLE 1994). Absorbances were read on an UV-160 spectrophotometer from Shimadzu, Kyoto, Japan. The treatment effect was tested with a non parametric test (Wilcoxon).

# 3 Results

## 3.1 Leaf biomass and HM content in foliage

Treatments had only minor effects on foliage biomass: HM or AR caused no significant change in mean single leaf area or mass and total foliage mass per tree in 2002 (mean single leaf mass: CO / AR / HM / HMAR  $\pm$  SE =  $630.3 \pm 88.87$  /  $584.6 \pm 86.04$  /  $639 \pm 38.15$  /  $532.6 \pm 67.71$  mg) or 2003 (CO / AR / HM / HMAR  $\pm$  SE =  $446.6 \pm 55.41$  /  $614.6 \pm 48.21$  /  $546.8 \pm 14.50$  /  $434.6 \pm 62.52$  mg). The only significant effect was provided by the HM x AR interaction in 2003 for the leaf area ( $P = 0.022$ ) and leaf mass ( $P = 0.028$ ). On a single leaf basis, leaves in 2003 were smaller ( $P = 0.012$ ) and tended to have a reduced mass ( $P = 0.076$ ), but mean total foliage mass per tree was almost doubled ( $P = 0.002$ ) as crown volume was larger in 2003 compared to 2002. Specific leaf area and specific leaf mass remained unchanged.

HM concentration in foliage of Sycamore Maple remained very low whatever the applied treatment and year (Table 1); only Zn and Cu reached levels above the detection limit. Zinc concentration showed a slight but significant increase due to exposure to soil contamination with HM whereas that of Cu was enhanced only by the AR treatment in 2003. The interaction between the HM and AR treatment was in both years and for both elements not

significant. The above treatments had similar effects in 2002 and 2003 with a tendency ( $P = 0.052$ ) to more Cu in 2003 particularly in the AR treatment. According to KABATA-PENDIAS and PENDIAS (2001), Cu concentrations were close to the deficiency limit (about  $5 \text{ mgkg}^{-1}$  dry mass) indicated for leaves of crop species whereas those of Zn were rather normal and hardly reached toxicity level ( $100\text{--}150 \text{ mgkg}^{-1}$  dry mass) in HM-treated trees.

Table 1. Heavy metal concentration  $\pm$  SE ( $\text{mgkg}^{-1}$  dry mass) in the foliage of Sycamore maple at the end of the 2002 and 2003 vegetation season and significance of the AR and HM treatment with regard to Co, Pb and Cd concentration remained below the detection limit ( $3$  and  $0.6 \text{ mgkg}^{-1}$  dry mass, respectively).  $N = 5$  trees.

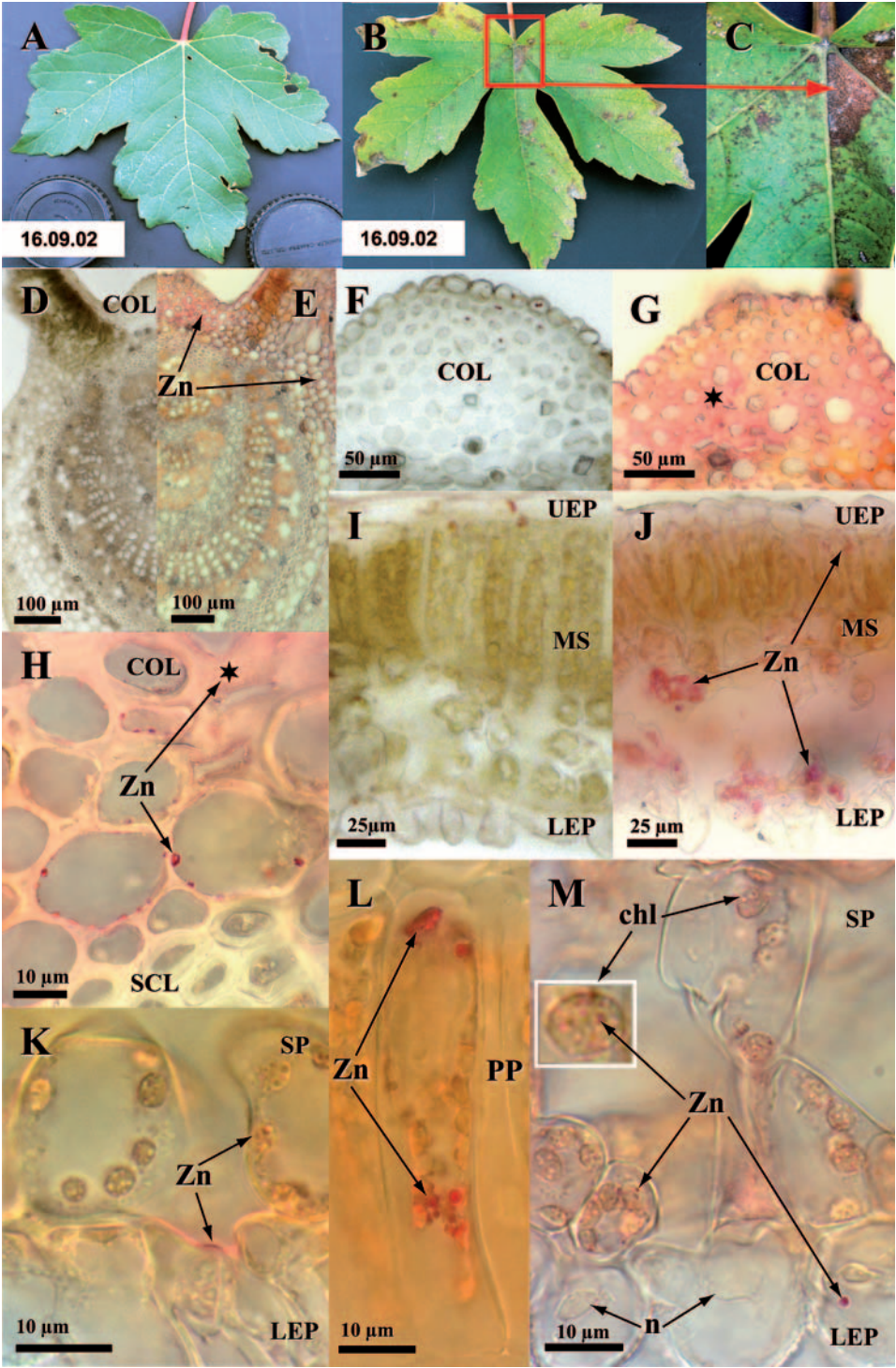
Year		Co	AR	HM	HMAR
2002	Zn	$67.0 \pm 7.00$	$61.0 \pm 5.42$	$119 \pm 10.2$	$120 \pm 9.5$
Significance (ANOVA)			n.s.	0.0001	
	Cu	$4.05 \pm 0.415$	$4.60 \pm 0.283$	$4.05 \pm 0.275$	$4.42 \pm 0.202$
Significance (ANOVA)			n.s.	n.s.	
2003	Zn	$75.2 \pm 8.05$	$76.2 \pm 8.63$	$124 \pm 7.3$	$123 \pm 8.7$
Significance (ANOVA)			n.s.	0.0001	
	Cu	$4.73 \pm 0.852$	$6.0 \pm 1.006$	$3.82 \pm 0.136$	$5.64 \pm 0.460$
Significance (ANOVA)			0.0419	n.s.	

### 3.2 Development of visible symptoms

For the first time in July 2002, stippling and chlorosis gradients starting from the annual shoot basis and decreasing towards the shoot tip were recorded in leaves of HM-exposed trees. Necrotic dots and flecks appeared later in August next to veins on the oldest leaves and also formed gradients increasing towards the leaf basis (Fig. 1B and C vs. A). These symptoms occurred again in 2003 (and 2004, not shown) starting 50 days after bud break (early June, Fig. 2). The proportion of symptomatic leaves increased until late July and was significantly different ( $P < 0.0001$ ) from that in trees without HM exposure (over all dates).

### 3.3 Cytochemical detection of zinc

In HM-treated trees, traces of Zn were observed in leaves sampled at the basis of top shoot only. No HM was detected in leaves at higher shoot positions or control trees. Zinc was micro-localised in different vein tissues (Fig. 1E, G, H vs. D, F; Table 2) noteworthy the upper collenchyma (Fig. 1G, H vs. F) which showed the largest apparent concentrations among all leaf tissues. In leaf blades (Fig. 1 J–M vs. I; Table 2) a few Zn-enriched spots scattered throughout the leaf blade showed apparently unimpaired Zn accumulation throughout most tissues (Fig. 1J vs. I). At cell level, Zn was micro-localised in different compartments (Table 2). Accumulation in cell walls was generally homogeneous except in sclerenchyma (more signal in middle lamella, not shown), leaf blade upper (more signal in outer cell wall) and lower epidermis (more signal in middle lamella of inside-facing cell wall, Fig. 1K). In symplast, Zn was never observed in vacuoles or nucleus (Fig. 1M) but in the cytosol (Fig. 1H, L and M) and mesophyll chloroplasts (Fig. 1K, M). Inside this latter organelle, several Zn granules could generally be micro-localised (detail in Fig. 1M).



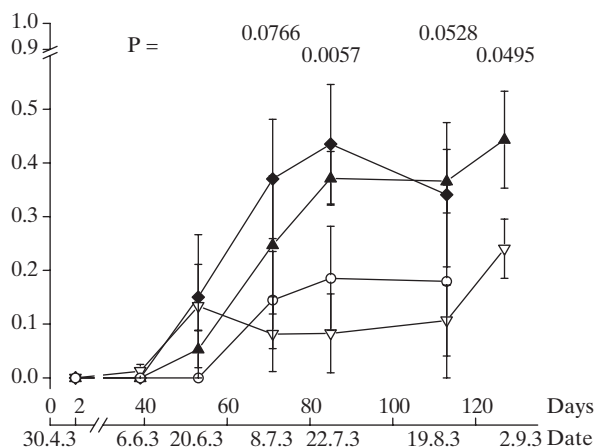


Fig. 2. Symptom development in Sycamore Maple (means  $\pm$  SE,  $N = 5$  trees) as indicated by the proportion of leaves showing visible symptoms of HM stress on the top shoot at each sampling date during the fourth year of exposure (2003). Over all data, the difference between trees in treatments with (closed symbols) or without (open symbols) HM was significant ( $P < 0.0001$ ). Level of statistical significance during the vegetation season is indicated above each sampling date. Treatments: CO ( $\circ$ ), AR ( $\nabla$ ), HM ( $\blacktriangle$ ) and HMAR ( $\blacklozenge$ ).

Table 2. Microlocalization of the Zn signal (+) at tissue, cell and organelle level in HM-exposed leaves from the top-shoot basis.

	Cell wall	periplasm	Cellular compartment		nucleus	vacuole
			cytosol	chloroplast		
<b>Leaf blade</b>						
upper epidermis	+					
palisade parenchyma			+	+		
spongy parenchyma			+	+		
lower epidermis	+		+			
<b>Leaf vein</b>						
upper epidermis	+		+			
upper collenchyma	+		+			
sclerenchyma	+					
xylem	+					
pith	+					
lower collenchyma	+		+			
lower epidermis	+		+			

Fig. 1. Visible symptoms (B–C vs. asymptomatic control in A) and micro-localization of Zn (D–M) in leaves of Sycamore Maple after experimental exposure to HM in the topsoil. Material was sampled during the third year of exposure (2002) at the end of the vegetation season. Typical symptoms included basipetally-increasing gradients of necroses along veins (B, detail in C) and leaf chlorosis. Increased amounts of Zn were observed as traces in the leaf blade (J–M) and vein (E, G, H) of the leaf samples from the HM treatment, whereas the control (D, F, I) remained unstained. Zinc was micro-localized in the apoplast (pink stain and  $\star$  in cell wall of E, G, H; K) as well as symplast (H, J–M). Zinc was stored homogeneously in cell wall of collenchyma (COL; G, H) whereas it especially accumulated in the pectin-rich outer layer of lower epidermis cells (LEP; K). Cytosolic (H, L, M) and chloroplastic (K, M) micro-localization of Zn was observed in the symplast; nuclei (n) did not contain Zn (M). Several Zn granules accumulated in the stroma of chloroplasts (chl; detail in M). UEP: upper epidermis; MS: mesophyll; SCL: sclerenchyma; PP: palisade parenchyma; SP: spongy parenchyma.

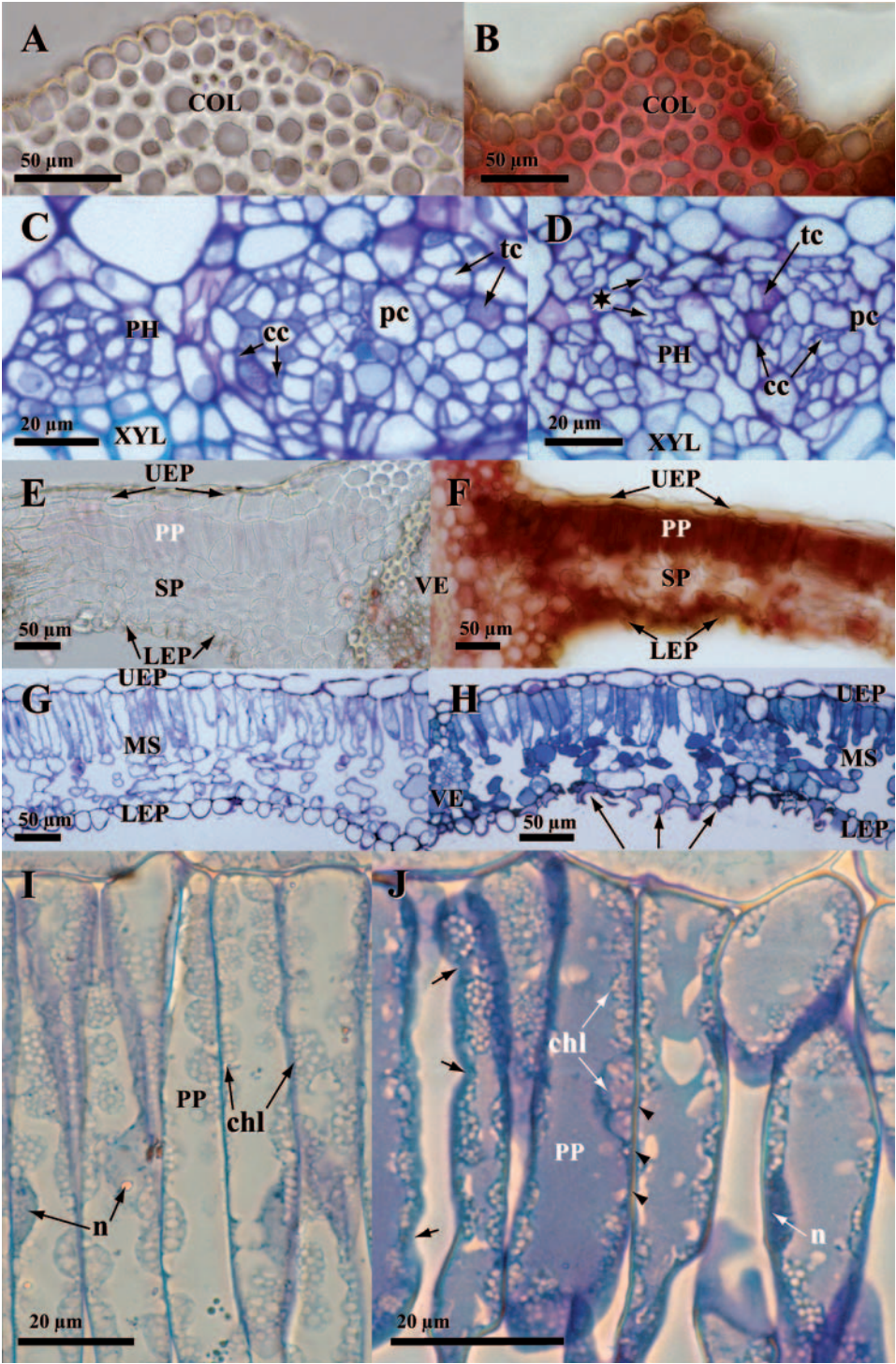
### 3.4 Structural changes and defence reactions

The HM treatment caused different stress and defence reactions in the vein and leaf blade resulting in several structural changes. In veins of leaves at lower shoot position, phloem degenerated as indicated by cell wall folding (Fig. 3D vs. C) and condensation of cell content in non conducting cells. Cell walls in adaxial collenchyma were thickened noteworthy with CT (Fig. 3B vs. A). Other apoplastic CT were found in the epidermis and xylem whereas those in the symplast occurred in the adaxial collenchyma, epidermis, phloem and medullar rays (not shown). Structural changes in the leaf blade were often linked to those in veins as shown by gradients of symptom intensity decreasing with distance from veins (Fig. 3F, H vs. E, G). The most characteristic changes included gradients of necrosis in lower epidermis (Fig. 3H vs. G) as shown by cell collapse, cell wall thickening and accumulation of secondary compounds (Fig. 3F, H). Palisadic and spongy cells in mesophyll showed different degeneration symptoms including cell wall thickening, folding and partial collapse (Fig. 3J vs. I), accumulation of secondary compounds (Fig. 3F, J vs. E, I), condensation of the cell nucleus, reduction of the chloroplast size and condensation of its stroma (Fig. 3J vs. I). In reference to VOLLENWEIDER *et al.* (2006), these changes were indicative of accelerated cell senescence processes (ACS). CT formed one class of the secondary compounds which were accumulated inside mesophyll of HM-exposed samples. They were strongly increased in comparison to control samples (Fig. 3F vs. 3E) and preferentially accumulated in the lower spongy parenchyma layers. The micro-localisation of CT thus closely matched that of Zn in the vein (Fig. 3B vs. 1G) as well as leaf blade (Fig. 3F vs. 1J). Structural injuries induced by HM decreased at higher shoot positions: necroses were no more detected and symptoms most frequently recurring included phloem degeneration, increase of secondary compounds in the vacuole and ACS gradients apart from veins (not shown).

### 3.5 Quantification of total phenols and condensed tannins in foliage

Spectrophotometrical measurements of TP and CT showed to which extent the phenolic compounds cytochemically detected in HM-treated leaves from the basis of top shoot were also quantitatively increased throughout the whole crown foliage. The acid vanillin reagent with which the oligomeric fraction of CT was assessed (Fig. 4) was the same as that used to reveal CT cytochemically (Fig. 3B, F). TP reached 6.2 % of the foliage dry mass on average in control samples of which CT represented a small fraction (less than 5 %) only (Fig. 4). The

Fig. 3. Stress and defence reactions to HM in leaves of Sycamore Maple sampled from the basis of the top shoot by the end of the third year of treatment (2002). A to D changes in vein. B vs. A collenchyma (COL) cell walls and to a lesser extent lumen contained increased amounts of CT (coloured in red) matching Zn micro-localization. D vs. C phloem (PH) showed stress reactions in the form of folded cell walls (★) and slightly condensed cell content especially in non conducting cells. E to J changes in leaf blade. F vs. E gradient of vacuolar CT close to main vein (VE). CT especially accumulated in palisade parenchyma (PP) and in the lower layer of spongy parenchyma (SP) forming a gradient culminating at the junction with the main vein. A few lower epidermis (LEP) cells contained CT, however phenolics were otherwise too oxidized to still react with vanillin, as indicated by brown tones. H vs. G stress reactions in the leaf blade next to a secondary vein. Mesophyll (MS) cells accumulated secondary compounds in their vacuole (coloured in blue). Lower epidermis became largely necrotic as shown by cell collapse (arrows). J vs. I changes in palisade cells. Cells underwent ACS as shown by thickening (arrow-heads) and folding (arrows) of cell walls, vacuoles full of secondary compounds, smaller and denser nuclei (n) and condensed chloroplasts (chl) with more lipids (dark grey shades). UEP: upper epidermis.



HM treatment did not change the TP content in the foliage whereas the polymeric and oligomeric CT fractions tended to increase (Fig. 4): amounts of the oligomeric fraction were raised from 1.2 % (CO) to 2.1 % (HM) and those of the polymeric form from 2.7 % (CO) to 5 % (HM) of the TP on average. These moderate increases thus confirmed that the exposure to HM tended to specifically enhance the leaf content of CT and that this did not only occur in older leaf material but also in the rest of the crown.

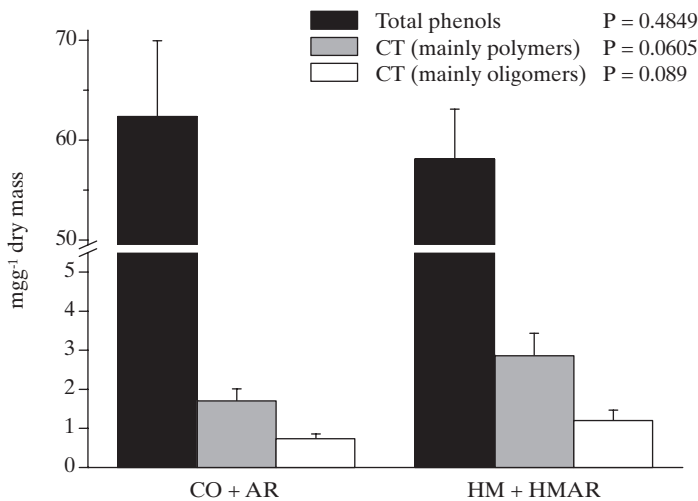


Fig. 4. Phenolic content (means  $\pm$  SE) in foliage of Sycamore Maple at the end of the third vegetation season (2002). Samples from treatments without (CO, AR) and with heavy metals (HM, HMAR) were pooled. Level of statistical significance (Wilcoxon test) between both groups is indicated in the legend. N = 10 trees.

## 4 Discussion

Since the foliage biomass and crown development was not affected by the HM and AR treatment, the phytoremediation capacity of the investigated Sycamore Maples remained unchanged, at least during the whole period of observation despite the stress reactions observed in older foliage. It could be a consequence of the low amounts of HM which were translocated to the tree crown. Among the HM enriched in foliage, the uptake level of Zn was superior to that of Cu as shown by bio-accumulation factors (BAF, calculated as the ratio between the leaf and soil concentration) around 0.04 vs. 0.006 respectively. Lower BAF for Cu than Zn is generally observed in trees (PULFORD and WATSON 2003) as well as in other vascular plants (SIEDLECKA 1995). Our BAF were at best similar to and generally smaller than those derived from data in other studies using Sycamore Maples growing on dredged sediments (0.21 for Zn and 0.11 for Cu, MERTENS *et al.* 2004), or in two woodland sites with different level of HM contamination (0.04 and 0.78 for Zn, 1.45 and 0.04 for Cu, WATMOUGH and DICKINSON 1996). This could relate to the experimental soil contamination with HM as mineral oxides in HM dust within the framework “From Cell to Tree” (NOWACK

*et al.* 2006) whereas HM might be in a more readily available form for plants with other sources of contamination. Since, in our case, unfavourable physical and chemical soil characteristics, as frequently occurring in contaminated sites (PULFORD and WATSON 2003), did not further impair tree establishment and HM uptake, the little HM uptake in treated Sycamore Maple may be attributed to HM avoidance and/or blocking in the rhizosphere. In reference to the higher HM uptake observed, noteworthy, in *Populus tremula* and *Salix viminalis* (HERMLE *et al.* 2006b) within the same “From Cell to Tree” framework, the results obtained here confirm the high phytostabilisation and low phytoextraction potential of Sycamore maple regarding the investigated HM and suggest it to be a species-related characteristic.

Stress reactions provided useful bioindications in the form of visible symptoms in older foliage without changing tree growth. Gradients of stress reactions at foliage and leaf level showing a connection to the leaf vein were typical for an abiotic soil-borne stress factor (MARSCNER 1995; VOLLENWEIDER and GUNTARDT-GOERG 2005). Symptom development resulted from accumulative effects suggesting the involvement of Zn and Cd since both elements accumulate in foliage during the vegetation season (LAUREYSENS *et al.* 2004). Zinc was the only HM however, whose concentration reached near toxic levels in the foliage of the HM-exposed Sycamore Maples. Gradients of visible leaf symptoms in foliage and Zn cytochemical signal as a function of leaf position on the shoot suggest that Zn reached higher concentrations in older foliage compared to the rest of the crown. At the leaf level, symptom distribution along gradients and its connection to veins were closely matching the accumulation and distribution of Zn (as observed after cytochemical detection) as well as the distribution of microscopical stress reactions. These distribution characteristics were also found in other species e.g. *Phaseolus vulgaris* (MARTIN *et al.* 2006) and *Populus tremula* (VOLLENWEIDER and GUNTARDT-GOERG 2005) under the same mixed HM-exposure suggesting that the visible symptoms, detected here, formed typical and specific bioindications of Zn intoxication in Sycamore maple.

The micro-localisation results for Zn helped to understand how and why so moderate Zn concentrations in older foliage nevertheless resulted in necroses visible with the naked eye. Only a part of the Zn contaminants were deposited in structures like cell walls in adaxial collenchyma where it did not threaten sensitive and vitally important processes of the leaf physiology (VOLLENWEIDER *et al.* 2006). Another part accumulated apparently unimpaired in different leaf blade sites, generally close to veins, where it was in the position to significantly increase oxidative stress as Zn is an abiotic elicitor of reactive oxygen species (ROS; DIETZ *et al.* 1999). Zinc accumulation in cytosol and chloroplasts of mesophyll cells seems particularly critical as the photosynthetic electron transport system is the major source of ROS in plants (YAMASAKI *et al.* 1997). These cell compartments contain continuously running detoxification systems (ELSTNER 1996) but supplementary sources of oxidative stress can however definitely unbalance the buffering capacities (VOLLENWEIDER *et al.* 2003). Zinc was thus in the position to directly interfere with the photosynthetic and gas exchange activities in mesophyll. No detoxification processes like Zn excretion through secretory hairs (CHOI *et al.* 2001; MARTIN *et al.* 2006), storage in vacuoles (VAZQUEZ *et al.* 1994; CLEMENS 2001), or massive inlays in cell walls (LICHTENBERGER and NEUMANN 1997; VOLLENWEIDER *et al.* 2006) was detected, suggesting that lesions to tissues resulted from insufficiently controlled Zn storage.

Structural changes caused by stress reactions were found in sites where Zn was micro-localised and through which Zn was at least translocated (phloem), but where Zn concentration remained below the detection limit. Indeed, cycling of mineral elements in plants, probably through the medullar rays, contributes to HM allocation to phloem (MARSCNER 1995; VOLLENWEIDER *et al.* 2006). In reference to the latter publication, the microscopical

changes observed here in phloem probably resulted from ACS processes. In the leaf blade, advanced ACS processes were observed in connection to Zn-induced injury gradients starting from veins. In contrast to *Raphanus sativus* (MARTIN *et al.* 2006) or *Populus tremula* (GUNTARDT-GOERG and VOLLENWEIDER 2003), no HR-like reactions were observed in mesophyll in relation to Zn deposition here, most likely because of the lower Zn concentration inside the leaves of Sycamore Maple. Changes at tissue and cell levels and the microscopical gradients were otherwise similar to those observed in *Populus tremula*. The micro-localisation of CT matched remarkably well with that of Zn as observed in the case of *Salix viminalis* under Cd stress (VOLLENWEIDER *et al.* 2006). CT were found in both apoplastic and symplastic sites and in places with or without structural injuries. They were specifically induced by the HM treatment among other phenolic compounds and formed one of the few cell defence reactions induced by Zn and recorded in HM-contaminated leaves. Their eventual Zn-chelating or oxidative stress-quenching effects cannot be estimated on the basis of our investigations. The results obtained here on Sycamore Maple show however that they form interesting stress markers for several HM and tree species.

## 5 Conclusion

In Sycamore Maples growing on soils with mixed HM contamination, low amounts of Zn triggered oxidative stress in older leaf foliage because Zn could reach, unimpaired, sensitive sites inside leaf blade. They caused ACS and necrosis resulting in unspecific leaf chlorosis and necrotic spots and flecks next to veins. The latter were arranged along gradients at leaf and shoot level and were characteristic for Zn. Visible symptoms provided a reliable bio-indication of Zn contamination without changing the phytostabilisation potential of the treated Sycamore Maple, since Zn average concentration inside crown foliage hardly reached the toxicity limit. Most stress reactions occurred in older foliage where they could little influence the growth of this pioneer tree species with unlimited apical growth (at least as a young tree). The phytoremediation potential of Sycamore Maple was found to include a bioindication component which can be useful for surveillance purposes in sites where phytostabilisation is achieved.

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