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Leaf responsiveness of *Populus tremula* and *Salix viminalis* to soil contaminated with heavy metals and acidic rainwater

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Summary Fast-growing trees such as *Salix viminalis* L. and *Populus tremula* L. are well suited to phytoremediate heavy metal contaminated soils. However, information on tree performance, particularly leaf function, under conditions of heavy metal contamination is scarce. We used yearly coppiced saplings of *S. viminalis* and *P. tremula* growing in model ecosytems to test four hypotheses: (1) heavy metal contamination impairs photosynthesis by injuring leaf structure; (2) the effects of heavy metal contamination are enhanced by acidified rainwater and low soil pH; (3) heavy metal contamination increases dark respiration and, thus, repair processes; and (4) heavy metal contamination is tolerated and remediated better by *S. viminalis* than by *P. tremula*.

We investigated heavy metal accumulation, tissue injury and gas exchange in leaves of plants subjected to controlled soil contamination with heavy metal dust. Additional treatments included acidic and calcareous natural forest subsoils in combination with irrigation with rainwater at pH 5.5 or 3.5. In both provenances of *P. tremula* that were studied, but not in *S. viminalis*, heavy metal treatment reduced photosynthesis and transpiration by varying amounts, except in the hot and dry summer of 2003, but had no effect on dark respiration. At light saturation, net CO_2 uptake and water-use efficiency were reduced by heavy metal contamination, whereas the CO_2 concentration in the leaf intercellular air space was increased. Rainwater pH and subsoil pH only slightly modified the effects of the heavy metal treatment on *P. tremula*. Gas exchange responses of *P. tremula* to heavy metals were attributed to leaf structural and ultrastructural changes resulting from hypersensitive-response-like processes and accelerated mesophyll cell senescence and necroses in the lower epidermis, especially along the transport pathways of heavy metals in the leaf lamina. Overall, the effects of heavy metals on *P. tremula* corroborated Hypothesis 1, but refuted Hypotheses 2 and 3, and were inconclusive for Hypothesis 4. Both *P. tremula* and *S. viminalis* showed appreciable potential for storing heavy metals in aging foliage.

Keywords: accelerated cell senescence, cadmium, chloroplasts, CO_2 assimilation rate, dark respiration rate, histological changes, hypersensitive response-like reaction, stomatal conductance, visible injury, water-use efficiency, zinc.

Introduction

Heavy metals in soils can cause visible leaf injury and changes in cell structure (Baryla et al. 2001). Several heavy metals from contaminated soils accumulate in plants (Siedlecka 1995) and are translocated to aboveground plant parts (Marschner 1995). As a consequence, leaf function can be affected by heavy metals directly (e.g., local heavy metal accumulation) or indirectly (e.g., injury to roots; Baszynski and Tukendorf 1984, Clijsters and Van Asche 1985, Baszynski 1986, Greger and Ögren 1991, Prasad 1995, 1997).

Heavy metals can affect carbon dioxide assimilation through damage to the photosynthetic and stomatal apparatus or to the water conducting system (vascular tissues; Barcelò and Poschenrieder 1990, Prasad and Strzalka 1999). Heavy metal treatment has been reported to cause a small decrease in biomass of *Populus tremula* L., but not in *Salix viminalis* L. (Hermle et al. 2006).

Research on the effects of heavy metals on gas exchange has mostly been limited to agricultural crop species (reviewed by Barcelò and Poschenrieder 1990). Heavy metals enhance dark respiration in barley and pea (Vassilev et al. 1998, Romanowska et al. 2002). Photosynthesis of poplar leaves is impaired by excess zinc (Zn; Di Baccio et al. 2003), and Zn, copper (Cu) and cadmium (Cd) cause oxidative stress in various species (Dietz et al. 1999). However, most studies on gas exchange of heavy metal treated trees have been conducted with young potted plants or in hydroponic systems (Hagemeyer et al. 1986, Godbold and Kettner 1991, Punshon et al. 1995, Österas and Greger 2003, Vyslouzilova et al. 2003), and the relevance of such findings to what occurs under field conditions is unknown.

In this study, yearly coppiced saplings of *S. viminalis* and *P. tremula* were grown over three years in large model forest ecosystems, comprising a full factorial split-plot design, to investigate tree responses under near-natural environmental conditions to mixed heavy metal contamination (Zn, Cd, Cu and lead (Pb)) in the topsoil in combination with acidic and calacareous forest subsoils and irrigation with rainwater at pH 5.5 or 3.5. The mixed heavy metal treatment was designed to mimic polluted sites throughout Europe. We measured the light response of photosynthesis and dark respiration in the different treatment combinations to assess the carbon source strength and stress tolerance of the trees. Structural changes underlying functional alterations in gas exchange at the tissue, cell and sub-cellular level were analyzed by light and electron microscopy. Because *P. tremula* leaves showed a greater accumulation of heavy metals than did those of *S. viminalis* and a significant decrease in photosynthesis in response to the heavy metal treatment, we focused our investigation on *P. tremula*. Four hypotheses were evaluated: (1) heavy metal accumulation leads to reduced gas exchange and alters leaf structure; (2) the effects of heavy metals are enhanced by acidified rainwater and low soil pH; (3) dark respiration is enhanced as a heavy metal induced defence response; and (4) *S. viminalis* is less sensitive to heavy metals than *P. tremula*.

Materials and methods

Experimental design

The experiment was carried out in the open-top chambers (OTCs) of the Swiss Federal Research Institute WSL at Birmensdorf near Zurich, Switzerland (47°21′54″ N, 8°27′12″ E, 450 m a.s.l.). Sixteen OTCs were arranged in a Latin square. Each OTC was 3 m in height, 6.7 m^2 in area and 20.1 m^3 in volume above ground. Temperature was regulated by ventilation with ambient air. Retractable glass roofs closed automatically at the onset of rain to exclude natural precipitation. Below ground, each OTC had two concrete-walled lysimeter compartments (1.5 m deep, with a surface area of 3 m^2 and a volume of 4.5 m^3).

In 1999, a three-tiered drainage layer of pure fire-dried quartz gravel covered the floor of each lysimeter compartment (grain size of 5–8 mm at 150–120-cm depth, 1.5–2.2 mm at 120–110-cm depth and 0.7–1.2 mm at 110–100-cm depth). On top of the drainage layer, one lysimeter compartment of each OTC contained an 85-cm layer of Calcaric Fluvisol (sandy loam, pH 7.4) subsoil and the other soil compartment contained an acidic Haplic Alison (loamy sand, pH 4.2) subsoil (for soil properties, see Menon et al. 2005 and Hermle et al. 2006). Both subsoil types were covered with a slightly acidic silty loam topsoil (15 cm, pH 6.5), precluding the need to supply nutrients (Hermle et al. 2006). For the treatments starting in 2000, water at a pH of 5.5 or 3.5 (adjusted with HCl, eight OTCs each) with an ionic composition equivalent to the mean of the last 30 years of precipitation at the experimental site was supplied from May to October by means of six sprinklers in each compartment of each OTC. Despite irrigation, soil water potentials fell during the hot and dry summer of 2003 (Menon et al. 2005). During winter (November to April), the OTC roofs were left open to expose trees to the natural precipitation regime.

In eight OTCs, filter dust from a non-ferrous metal smelter was mixed into the topsoil before planting to ensure total metal concentrations similar to those occurring around such smelters. The resulting concentrations of Cu, Zn, Cd and Pb were 640, 3000, 10 and 90 mg kg^{-1}, respectively, in the contaminated topsoil and 28, 97, 0.1 and 37 mg kg^{-1}, respectively, in the uncontaminated topsoil (Menon et al. 2005). Heavy metal speciation was assessed 18 months after heavy metal application and confirmed that the distribution of metal forms remained stable (Nowack et al. 2006). The mobile and easily mobilizable fractions amounted to 40 (Cu), 70 (Zn), 85 (Cd) and 10% (Pb) of the total heavy metal soil concentration. Amounts of soil contamination and heavy metal bioavailability exceeded Swiss VBBo limits (VBBo 1998) but remained below values encountered at some polluted sites across Europe (Ernst 1972, Horvath and Gruiz 1996, Dickinson 2000). Hence, the experimental setup reproduced soil pollution comparable to that of “brown field” sites. For each subsoil type, four combinations of heavy metal and rainwater treatments were replicated four times each within the split-plot factorial design: (1) uncontaminated topsoil plus rainwater of pH 5.5; (2) uncontaminated topsoil plus rainwater of pH 3.5 (denoted as “acid rain” in the following); (3) heavy metal contaminated topsoil plus rainwater of pH 5.5; and (4) heavy metal contaminated topsoil plus rainwater of pH 3.5. Because plant responses to rainwater pH were negligible (see Tables), data were pooled in the Figures as: control = Treatments 1 + 2; HM = Treatments 3 + 4.

In spring 2000 before bud break, each OTC compartment was planted with 14 autochthonous trees together with understory plants to simulate ecosystem conditions in a young afforestation and to ensure homogeneous ground cover. The main species were European aspen (*Populus tremula*), two unrooted cuttings each from the Swiss plateau (provenance Birmensdorf at 500 m a.s.l.) and the Jura region (provenance Orvin at 960 m a.s.l.), and common oisier (*Salix viminalis*), two rooted cuttings from the Jura region (provenance Le Locle at 1000 m a.s.l.). They were accompanied by six 3-year-old individuals of Norway spruce (*Picea abies* (L.) Kast.), two individuals each from the Swiss plateau (provenance Bremgarten at 500 m a.s.l.), the Swiss prealps (provenance Murg at 1000 m a.s.l.) and the Swiss alps (provenance Conters, 1800 m a.s.l.), and two rooted cuttings of silver birch (*Betula pendula* L.) from the Jura region (provenance Romont at 900 m a.s.l.). All rooted cuttings were six months old and had roots pruned to 10 cm in length before planting. Plants were randomly arranged in three groups: deciduous species, Norway spruce and understory plants. Inter-plant competition was not observed. Leaf samples for destructive analyses were harvested from *P. tremula* and *S. viminalis* trees once each year toward the end of the growing season, but before the onset of autumnal leaf senescence.

Foliar heavy metal concentrations

Heavy metal concentrations were determined in 1-g samples from the total dried foliage of each *P. tremula* and *S. viminalis* tree harvested in mid-September of 2001 and 2002. The samples were pooled according to the soil compartment and treatment. The milled aliquots (ultra centrifuge mill, coated with wolfram carbide) were digested in a high-pressure microwave system (UltraClav by Milstone: 240 °C, 12 MPa) and analyzed in duplicate (range < 10%) by ICP-AES (Optima 3000 by Perkin Elmer) at the central laboratory, WSL, according to ISO 17025.

Visible leaf injury

Macroscopic changes in *P. tremula* leaves were monitored during the three growing seasons based on a semi-quantitative five-point scale (0 = asymptomatic, 1 = adaxial leaf stippling, 2 = necrotic leaf spots mainly at leaf margins, 3 = expanding necrotic spots and 4 = necroses expanding to more than 30% of leaf area) (Günthardt-Goerg and Vollenweider 2003, Vollenweider and Günthardt-Goerg 2006). These abiotic symptoms were differentiated on the basis of morphology, seasonal development and distribution within the canopy from those caused by biotic (fungi, mites) or other abiotic (hail, late spring frost) stress. Symptoms of leaf injury were also assessed in *S. viminalis*, but early leaf senescence and shedding prevented assessment of seasonal development.

Leaf gas exchange

Instantaneous gas exchange was measured over diurnal courses with a gas exchange system (LI-6400, Li-Cor, Lincoln, NE) in leaves of the same age. The cuvette enclosed a leaf area of 6 cm^2. During measurements, leaves were exposed to a saturating photosynthetic photon flux (PPF; porometer light source) and ambient CO_2 concentration (c_a = 370 µl l^{-1}). Leaf temperature was kept constant at 25.3 °C, and the mole fraction difference in water vapor between leaf and ambient air (Δw) was maintained at 15 mmol mol^{-1}. Measurements from four leaves per *S. viminalis* tree were averaged in July 2002 and 2003 in twelve OTCs (on one tree per subsoil compartment). Shedding of the lower leaves prevented measurements later in the season. In the same 12 OTCs, three leaves of each *P. tremula* provenance were measured and averaged, once in July 2001 and twice in July and August 2002 and 2003 (using the same leaves in July and August). Leaf symptoms ranked between Classes 0 and 2.

Photosynthetic light response curves (LRCs) were measured in July 2001 on *P. tremula* (both provenances) by lowering the PPF from high to low (at c_a) to produce steady-state responses in net CO_2 uptake rate (but not in stomatal conductance). Two trees per subsoil compartment were investigated. Measurements were made on three leaves per tree (two replicates per treatment). Dark respiration rates were measured in the Orvin provenance of *P. tremula* in August 2002 and 2003 (between 1130 and 1430 h; 2002: T_l = 20.2 °C; 2003: T_l = 21.5 °C; each at c_a). Steady-state respiration was reached, on average, after 5 minutes.

Leaf gas exchange rates (including dark respiration) were expressed on a projected leaf area basis. Photosynthetic rate (net CO_2 uptake), transpiration rate, stomatal conductance (g_s) and water-use efficiency (WUE = CO_2 assimilation rate/transpiration rate) were calculated according to von Caemmerer and Farquhar (1981). Dark respiration rate was calculated according to Jones (1992).

Microscopy

Microscopy studies of *P. tremula* focused on the Orvin provenance because it showed a wider range of heavy metal induced injuries than the Birmensdorf provenance. During September 2000 and 2002, 1-cm-diameter leaf disks of *P. tremula* provenance Orvin were sampled for microscopic examination from representative leaves (located near the shoot base) of each of the visible symptom classes. The leaf disks were fixed by infiltration with buffered 2.5% glutaraldehyde. Samples were then processed for transmission and fluorescent light microscopy or transmission electron microscopy (TEM; for details, see Günthardt-Goerg et al. 1997 and Vollenweider et al. 2003).

Statistics

Statistical significance of treatment effects was determined by analysis of variance (ANOVA). The main effects of heavy metals and acidic rain and their interaction were tested against chamber mean squares. The effects of soil type (soils nested within chambers) and their interactions with the treatments were tested against subplot mean squares (error term = interaction HM × acidic rain × subsoil). Bars in Figures represent standard errors.

Results

Heavy metal concentrations in leaves

Tree growth on heavy metal contaminated soil (HM treatment) led to significantly enhanced Cd and Zn concentrations in the foliage of *Salix viminalis* and both provenances of *Populus tremula* (Figure 1, Table 1). Accumulations of heavy metals in the foliage tended to be lower in 2002 than in 2001, especially in *S. viminalis*, where the reduction was significant. Uptake of heavy metals was higher on acidic subsoil compared with calcareous subsoil, but did not differ significantly between the Orvin and Birmensdorf provenances of *P. tremula*. Acid rain significantly increased heavy metal uptake in leaves of *S. viminalis* in 2002 (Table 1; control pH 5.5 Cd: 2.79 ± 0.31 mg kg^{-1}, control pH 3.5 acid rain Cd: 3.26 ± 0.37 mg kg^{-1}; HM pH 5.5 Zn: 813 ± 80 mg kg^{-1}, HM pH 3.5 acid rain Zn: 913 ± 81 mg kg^{-1}), although the effect was of minor quantitative importance. Foliar Cd concentrations were higher in *P. tremula* than in *S. viminalis* in the heavy metal treatment, but lower in the control trees. Similarly, Zn concentrations were higher in heavy metal treated *P. tremula* leaves than in heavy metal treated *S. viminalis* leaves, but only on the calcareous subsoil, whereas *P. tremula* leaves from the corresponding control trees had the lowest Zn concentrations among treatments. Both species had low foliar Cu concentrations irrespec-

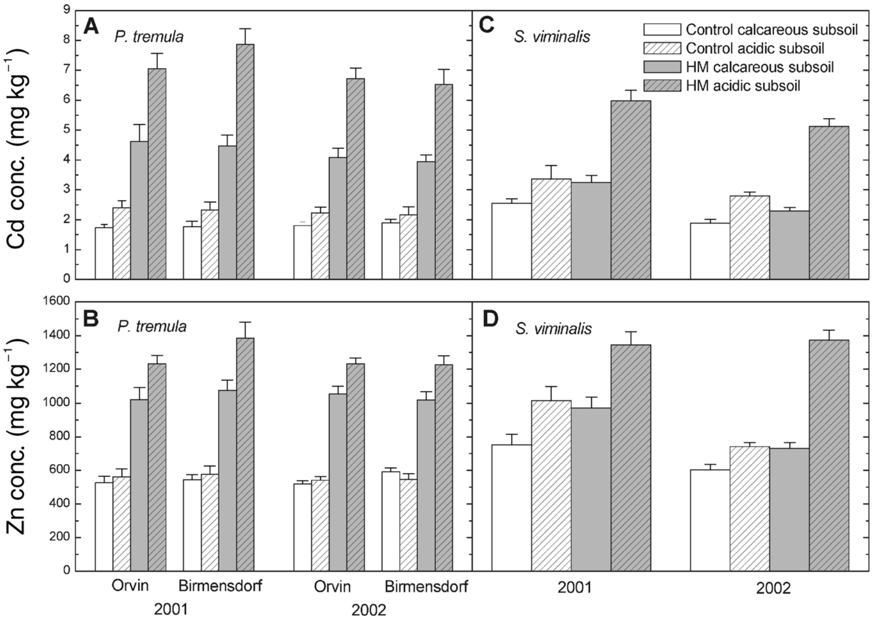


Figure 1. Foliar heavy metal concentrations (for statistical significance, see Table 1). (A) Cadmium (Cd) and (B) zinc (Zn) concentrations in the foliage of *Populus tremula* provenances Orvin and Birmensdorf in 2001 and 2002 in relation to the heavy metal treatment and subsoil type. Concentrations of (C) Cd and (D) Zn in the foliage of *Salix viminalis* in 2001 and 2002 in relation to the heavy-metal treatment and subsoil type. Values are means + SE; $n = 8$ trees.

tive of the year and treatment. In 2002, however, the foliar Cu concentration was enhanced ($P < 0.0144$) in heavy metal treated *P. tremula* trees compared with control trees (means ± SE: 9.4 ± 0.08 mg kg^{-1} in HM versus 8.6 ± 0.07 mg kg^{-1} in control, $n = 32$) in the absence of a significant influence of subsoil, provenance or acid rain. The heavy metal treatment did not increase the foliar Pb concentration above the detection limit of 3 mg kg^{-1}.

Leaf injury

Early macroscopic symptoms (whitish adaxial stippling evenly scattered across the leaf blade) appeared in heavy metal treated leaves of *P. tremula* about 20 to 30 days after bud break, shortly after the brownish coloration caused by carotenoids in the developing leaves faded. Necrotic (and expanding) spots developed later in the season in parallel with leaf bleaching (Figure 2B). The symptoms were most pronounced in leaves near the shoot base (Figure 2A). Younger leaves near the apex of the intermediate coppice shoots remained asymptomatic.

Development of heavy metal induced leaf injury was independent of shading. Symptoms appeared on the first-formed leaves within 1 month of bud break. Symptoms developed more slowly in later-formed leaves. Differences in visible symptoms of leaf injury between heavy metal treated and con-

Table 1. The statistical significance, according to the analysis of variance, of the effects of treatment (heavy metal (HM) and acid rain), provenance, year, subsoil type and the interaction of HMs and subsoil type on the foliar zinc (Zn) and cadmium (Cd) concentrations and visible symptoms of leaf injury in *Populus tremula* and *Salix viminalis* (ns indicates $P \geq 0.05$).

	Year	HM	Subsoil type	HM × subsoil	Acid rain	Provenance	Comparison of years
Zn concentration							
P. tremula	2001	0.0001	0.0187	ns	ns	ns	
	2002	< 0.0001	0.0007	0.0002	ns	ns	ns
S. viminalis	2001	0.0005	0.0001	ns	ns		
	2002	< 0.0001	< 0.0001	< 0.0001	0.0074		0.0003
Cd concentration							
P. tremula	2001	0.0001	0.0001	0.0036	ns	ns	
	2002	< 0.0001	< 0.0001	< 0.0001	ns	ns	0.0484
S. viminalis	2001	0.0001	0.0001	0.0090	ns		
	2002	< 0.0001	< 0.0001	< 0.0010	0.0024		0.0001
Visible symptoms of injury							
P. tremula	2001	0.0001	ns	ns	ns	0.0001	
	2002	< 0.0001	ns	ns	ns	< 0.0001	0.004
P. tremula	2003	0.0007	0.0417	0.049	ns	0.0004	ns[1]

[1] Year 2003 versus Year 2002 or 2001.

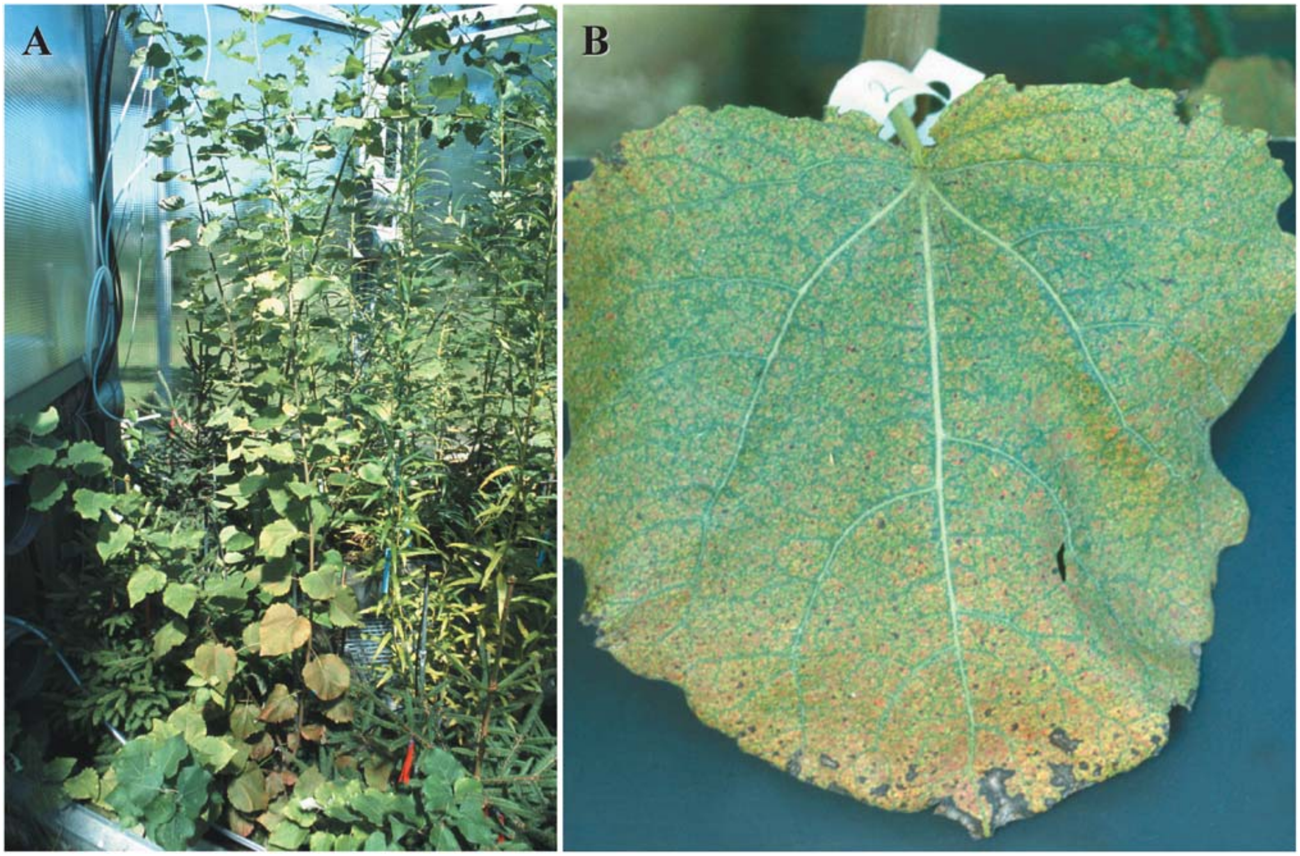


Figure 2. Visible symptoms of heavy metal injury on yearly coppiced *Populus tremula*. (A) Symptomatic tree 133 days after bud break. Leaf injury decreased acropetally independent of light exposure. (B) Leaf symptoms (Class 2). Uniformly distributed stippling developed on the adaxial leaf surface followed by necrotic spots, mainly at the leaf margin.

trol trees and between the two provenances were significant throughout the 3-year study (Figure 3, Table 1). Trees on acidic subsoil tended to develop more extensive symptoms than trees on calcareous subsoil (the difference was significant only in 2003; Table 1). The acid rain treatment slightly promoted the development of adaxial leaf stippling, although the increase was not statistically significant. The extent of leaf injury varied from year to year (less in 2001 than in 2002) but showed no trend over the 3-year study.

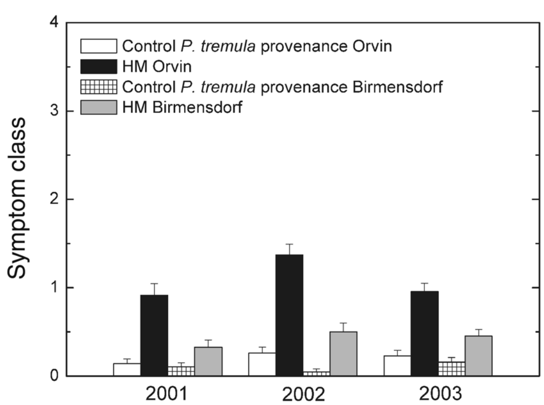


Figure 3. Effects of the heavy metal (HM) treatment on visible symptoms of injury averaged at the end of July 2001, 2002 and 2003 for both provenances of *Populus tremula*. Values are means + SE; $n = 32$ trees. For statistical significance, see Table 1.

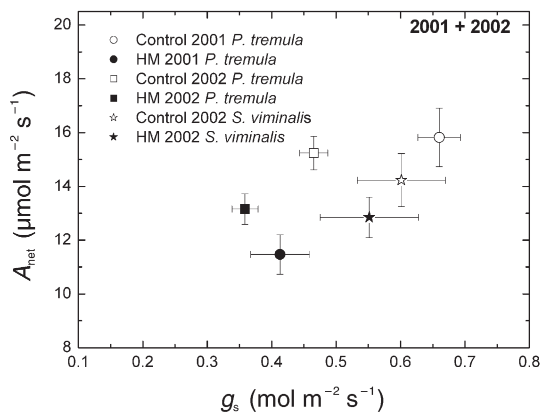


Figure 4. Effects of the heavy metal (HM) treatment on net CO_2 assimilation rate (A_{net}) relative to stomatal conductance (g_s) for water vapor in *Populus tremula* (July 2001 and July + August 2002, both provenances) and *Salix viminalis* (July 2002). Mean climatic conditions were similar in both years. Values are means ± SE; $n = 8$ and 46 trees for *P. tremula* in 2001 and 2002, respectively, and $n = 12$ trees for *S. viminalis* in 2002. For statistical significance, see Table 2.

Table 2. The statistical significance, according to the analysis of variance, of the effects of treatment (heavy metal (HM) and acid rain), provenance, year, subsoil type and the interaction of HMs and subsoil type on the net CO_2 assimilation rate (A_{net}) and stomatal conductance (g_s) of *Populus tremula* and *Salix viminalis* (ns indicates $P \geq 0.05$).

	Year	HM	Subsoil type	HM × subsoil	Acid rain	Provenance	Month
A_{net}							
P. tremula	2001	0.0072	ns	ns	ns		
	2002	0.0164	ns	ns	ns	ns	0.0013
	2003	ns	0.043	ns	ns		
S. viminalis	2002	ns	ns	ns	ns		
	2003	0.0001	ns	0.0005	ns		
g_s							
P. tremula	2001	0.0007	ns	ns	ns		
	2002	0.0005	ns	ns	ns	ns	ns
	2003	ns	0.0465	ns	0.0147		
S. viminalis	2002	ns	ns	ns	ns		
	2003	< 0.0001	ns	< 0.0001	ns		

Leaf gas exchange

The heavy metal treatment significantly reduced instantaneous net CO_2 assimilation rate (A_{net}) and g_s in *P. tremula* in 2001 and 2002 (Figure 4, Table 2). The declining trend in both parameters was linked with other environmental constraints, irrespective of species or year. Stomatal conductance was higher in *S. viminalis* than in *P. tremula* in 2002, regardless of the heavy metal treatment. Neither rainwater acidity nor subsoil type significantly influenced the relationships shown in Figure 4 (Table 2), although foliar Cd and Zn concentrations were significantly affected by subsoil type in *P. tremula* (Table 1).

Irrespective of rain acidity or subsoil type, heavy metal contamination significantly decreased A_{net} (Figure 5A) and WUE (Figure 5B) in *P. tremula* across the entire PPF range. As a consequence, the intercellular CO_2 concentration (c_i) in leaves increased (Figure 5C, Table 3). Increased c_i reflects a greater proportional decline in A_{net} than in g_s (Figure 4). The light response of photosynthesis reflected impairment of the apparent quantum yield of CO_2 uptake at low PPF and significant depression at light saturation (> 500 PPF; Figure 5). Maximum WUE was reached at a PPF of about 500 μmol m^{-2} s^{-1} in both treatments, but maximum WUE was 31% lower in the heavy metal treatment. Measurements in 2002 (not shown) were consistent with those in 2001.

The provenances of *P. tremula* were compared in 2002 for differences in leaf gas exchange in response to the heavy metal treatment (Figure 6, Table 2). Irrespective of treatment and provenance, A_{net} was reduced in August compared with July, whereas g_s remained unchanged. In July, the heavy metal treatment decreased g_s in Orvin trees, whereas in August, it caused

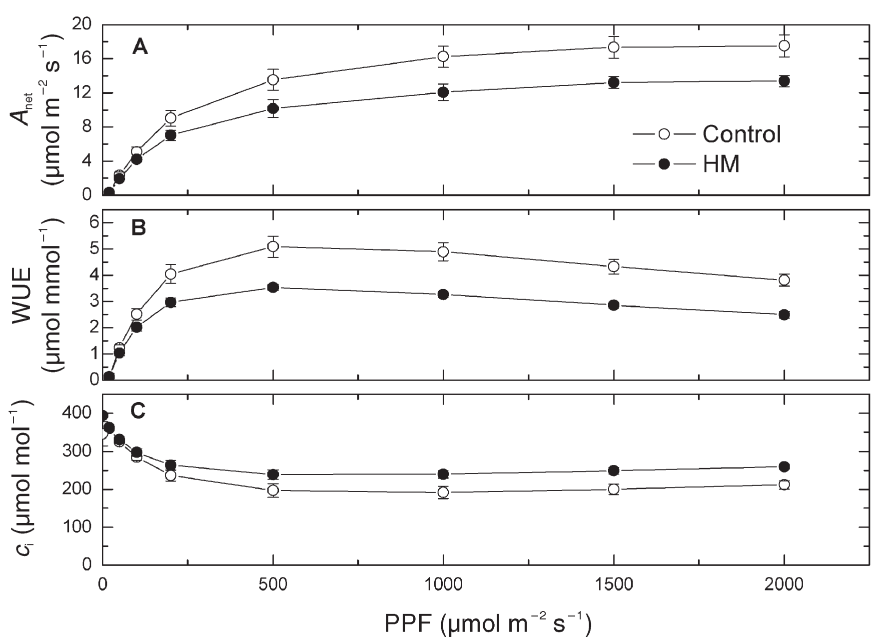


Figure 5. Effects of the heavy metal (HM) treatment on leaf gas exchange parameters in relation to the photosynthetic photon flux (PPF) in both provenances (Orvin and Birmensdorf) of *Populus tremula* in 2001. (A) Light response curve of net CO_2 assimilation rate (A_{net}), (B) water-use efficiency (WUE) and (C) intercellular CO_2 concentration (c_i). Values are means ± SE; n = 4 trees. For statistical significance, see Table 3.

Table 3. Statistical significance, according to the analysis of variance, of the heavy metal (HM) treatment on the light response of net CO_2 assimilation rate (A_{net}) and intercellular CO_2 concentration (c_i) in *Populus tremula*. Effects of subsoil type and interaction of HM × subsoil type were not significant (ns).

PPF	A_{net}	WUE	C_i
0	ns	ns	ns
20	ns	ns	ns
50	ns	ns	ns
100	ns	ns	ns
200	ns	0.0339	ns
500	ns	0.0101	ns
1000	0.0379	0.0041	ns
1500	0.0291	0.0029	0.0505
2000	0.0296	0.0023	0.0303

reductions in both A_{net} (stronger in the Orvin than in the Birmensdorf provenance) and g_s in both provenances. Despite the photosynthetic decline and more severe leaf injury in provenance Orvin (Figure 3), dark respiration was not significantly affected by the heavy metal treatment (Figure 7). Dark respiration rate declined with increasing leaf age toward the stem base and tended to be higher in 2003 than in 2002.

The hot and dry summer of July and August 2003 lowered A_{net} and g_s in *P. tremula* and *S. viminalis* trees in the control treatment relative to values measured during the two preceding years, whereas the heavy metal treated plants showed no further reduction in gas exchange responses to the additional stress of the summer drought (cf. Figure 8 with Figures 4 and 6). Consequently, in July 2003, A_{net} and g_s were higher in the heavy metal treatment than in the control treatment in both *P. tremula* Orvin and *S. viminalis* (Figure 8), whereas the treatment differences in *P. tremula* Birmensdorf were not significant (Figure 8). In August 2003, gas exchange rates were at their minimum during the 3-year study (cf. Figure 8 with Figures 4 and 6).

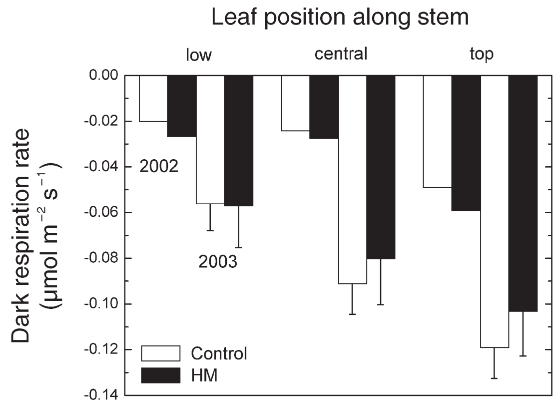


Figure 7. Effect of the heavy metal (HM) treatment on dark respiration rate of *Populus tremula* provenance Orvin in 2002 (n = 1) and 2003 (n = 10). Values for 2003 are means + SE. Measurements were performed at three successive leaf positions along the stem and thus along a leaf-age gradient. Differences between the heavy-metal and control treatment were not significant ($P \geq 0.05$).

Changes in leaf structure

The most typical changes in *P. tremula* Orvin leaves in response to the heavy metal treatment occurred in the lower leaf blade, especially in the abaxial epidermis. Here, groups of dead and collapsed epidermal cells, sometimes including stomata, were scattered across the leaf lamina, particularly next to veins (Figures 9B and 9D versus 9A and 9C). The cell wall chemistry was modified, especially in the outer cell wall layer (Figure 9D versus 9C), with deposits of lignin-like material (Figure 9D) and pectins (not shown). Similar changes were observed in spongy parenchyma close to necrotic sections of the lower epidermis (Figure 9D). Based on TEM, the cell wall appeared to be thickened in symptom classes ≥ 1 (Figures 9E, 10B and 10C). Other injuries underlying the reduction in gas exchange occurred in the mesophyll (Figure 9B versus 9A) in the form of discrete groups of necrotic cells sur-

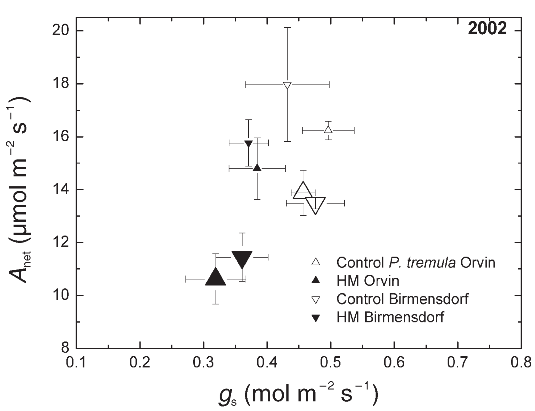


Figure 6. Effects of the heavy metal (HM) treatment on net CO_2 assimilation rate (A_{net}) relative to stomatal conductance (g_s) in both provenances (Orvin and Birmensdorf) of *Populus tremula* in July (small symbols) and August (large symbols) 2002. Values are means ± SE; n = 12 trees. For statistical significance, see Table 2.

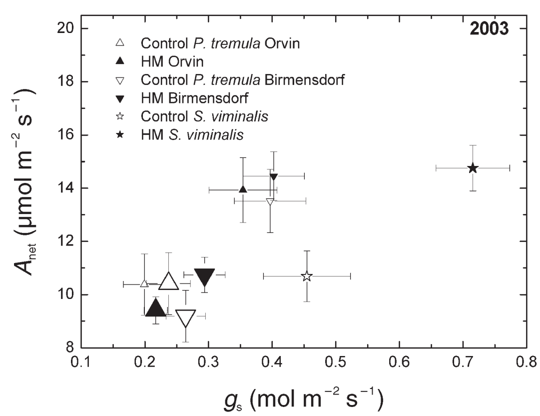


Figure 8. Effects of the heavy metal (HM) treatment on net CO_2 assimilation rate (A_{net}) relative to stomatal conductance (g_s) in *Populus tremula* and *Salix viminalis* in July (small symbols) and August (large symbols) of the exceptionally hot and dry summer of 2003. Values are means ± SE; n = 12 trees. For statistical significance, see Table 2.

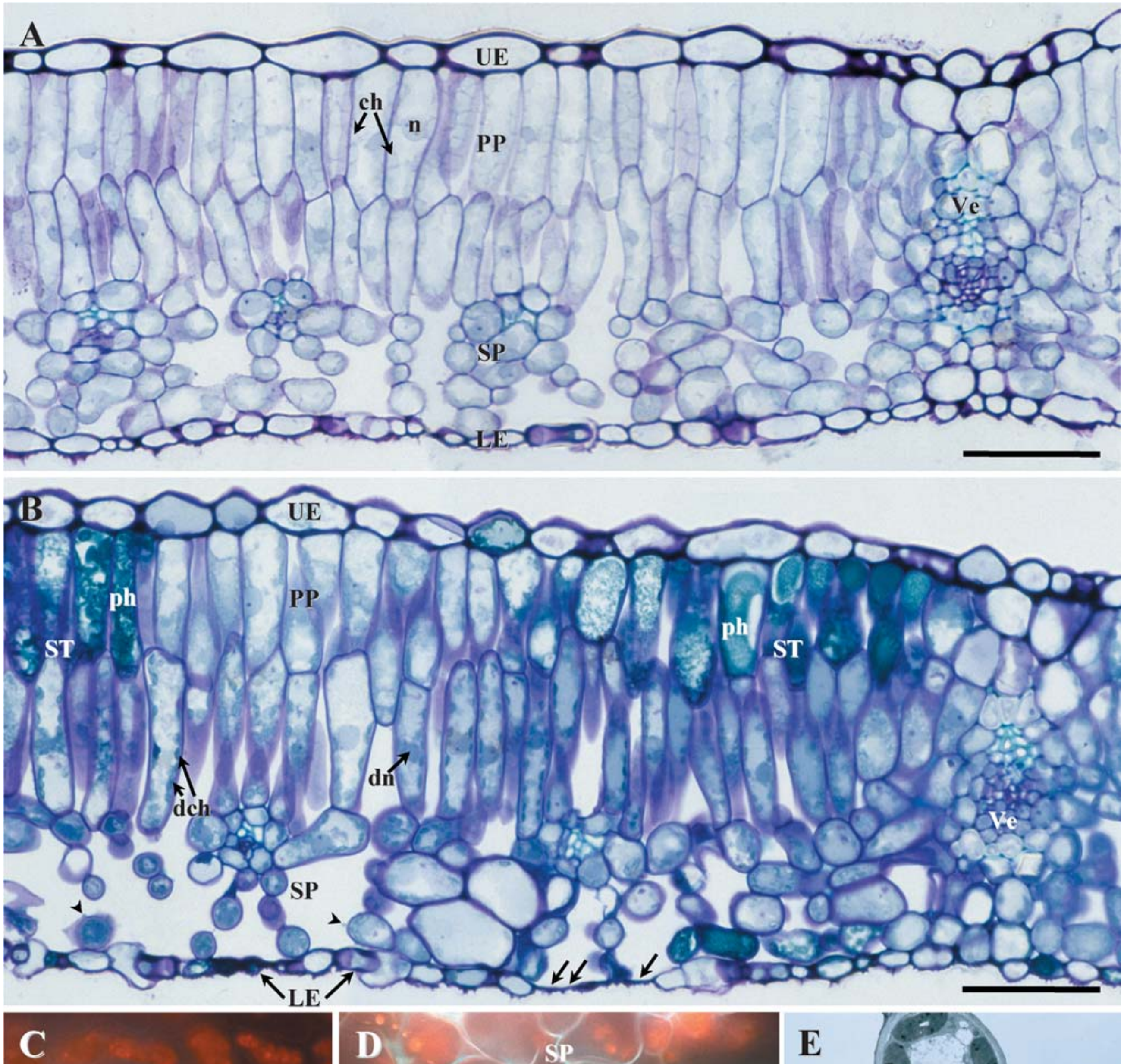


Figure 9. Histological changes in *Populus tremula* provenance Orvin control leaf blade tissue (A) and leaf blade tissue (Symptom Class 4) from trees in the heavy metal treatment. Tissues showed injuries of varying severity, whereas cell vitality was uniformly retained in control leaves. The greatest variability was found in the palisade parenchyma (PP) in zones with or without stippling (ST). Cells in stipples showed disrupted cell content with barely recognizable organelles and the accumulation of phenolic (ph) material. Cells showed increasingly normal cell structure with increasing distance from stipples, as shown by large and light (= less condensed) chloroplasts (ch). Spongy parenchyma (SP) showed more uniform structural changes than palisade parenchyma with thickened cell walls, sometimes including pectinic wart-like droplets (arrowheads) and both dense chloroplasts (dch) and nuclei (dn). The lower epidermis (LE) was more affected than the upper epidermis (UE). Entire cell sections collapsed (arrows), especially near veins. Vacuoles accumulated phenolic material. Changes in the lower leaf blade epidermis are shown in C–E. In control leaves (C), only the chlorophyll autofluoresced (red). In heavy-metal-injured samples (D), cell walls showed autofluorescence (blue) in the collapsed lower epidermis and spongy parenchyma, indicating the inlay of lignin-like material. In transmission electron microscopy sections (E), other cell wall deposits stained darkly, especially in the pectin-rich outer cell-wall layers (arrowhead: pectinic wart-like droplet). Abbreviations: Ve, veinlet; st, stomata; ch, chloroplast; and n, nucleus. Symptom class: 0 (A, C); 1 (D); 2 (E); and 4 (B). Bars: 50 µm (A, B); 25 µm (C, D); and 5 µm (E). Staining was with toluidine blue and *p*-phenylenediamine (A, B); autofluorescence of fresh tissues was observed under UV excitation at 340–380 nm (C, D); and staining and contrasting was as in Figure 11 (E).

rounded by senescent cells. As in the case of the necrotic lower epidermis sections, these injuries were irregularly scattered throughout the leaf blade, but preferentially found next to a vein. The discrete groups of necrotic cells (Figure 9B) were responsible for leaf stippling (tiny whitish, later brown dots) visible on the leaf adaxial side (Figure 2B). Cells with the most severe symptoms often belonged to the lower palisade layer and showed features characteristic of hypersensitive-response-like (HR-like) processes.

Light microscopy revealed the disruption of cell contents, condensation of cell remnants, thickening of cell walls and partial cell collapse (Figure 9). Adjacent to necrotic stipples, mesophyll cells displayed markers of accelerated cell senescence (ACS). Changes in palisade cells observed by light microscopy (Figures 11B–D versus control A) included progressive cell wall thickening, condensation of the nuclei and occasional accumulation of vacuolar phenolics. The most conspicuous structural changes occurred in the chloroplasts. Their size decreased, lipids were accumulated (as indicated by dark chloroplast contents), plastoglobuli became visible in the form of black dots, and the size and frequency of starch grains increased. During the final stages of ACS, chloroplasts disintegrated and eventually disappeared (Figure 11D). The TEM revealed ultrastructural injuries to the chloroplasts (cf. Figures 10B and 10C and Figures 10E and 10F with Figures 10A and 10D, respectively). The chloroplast envelope was frequently deformed (Figures 10C and 10E), suggesting budding and extrusion of waste material into the cytoplasm. Grana and thylakoids became increasingly difficult to discern in samples with advanced macroscopic injury, which could be due to the repeated membrane breakages (Figures 10C and 10F). The peroxysomal microbodies associated with chloroplasts in healthy tissues (Figure 10A) were seldom detected in samples of symptom classes ≥ 1 (cf. Figure 10B and 10C with 10A). Crystalline structures inside peroxysomes (Figures 10A and 10D), similar in appearance to catalase crystals (Gunning and Steer 1996), were detected in control samples only. The heavy metal treatment had no effect on the structure and frequency of mitochondria. The extent of ACS symptoms varied across the leaf blade, but the spongy parenchyma was more uniformly senescent than the palisade tissue, consistent with the overall prevalence of structural injury in abaxial tissues inside the leaf blade.

Discussion

Tree responses to heavy metal treatment

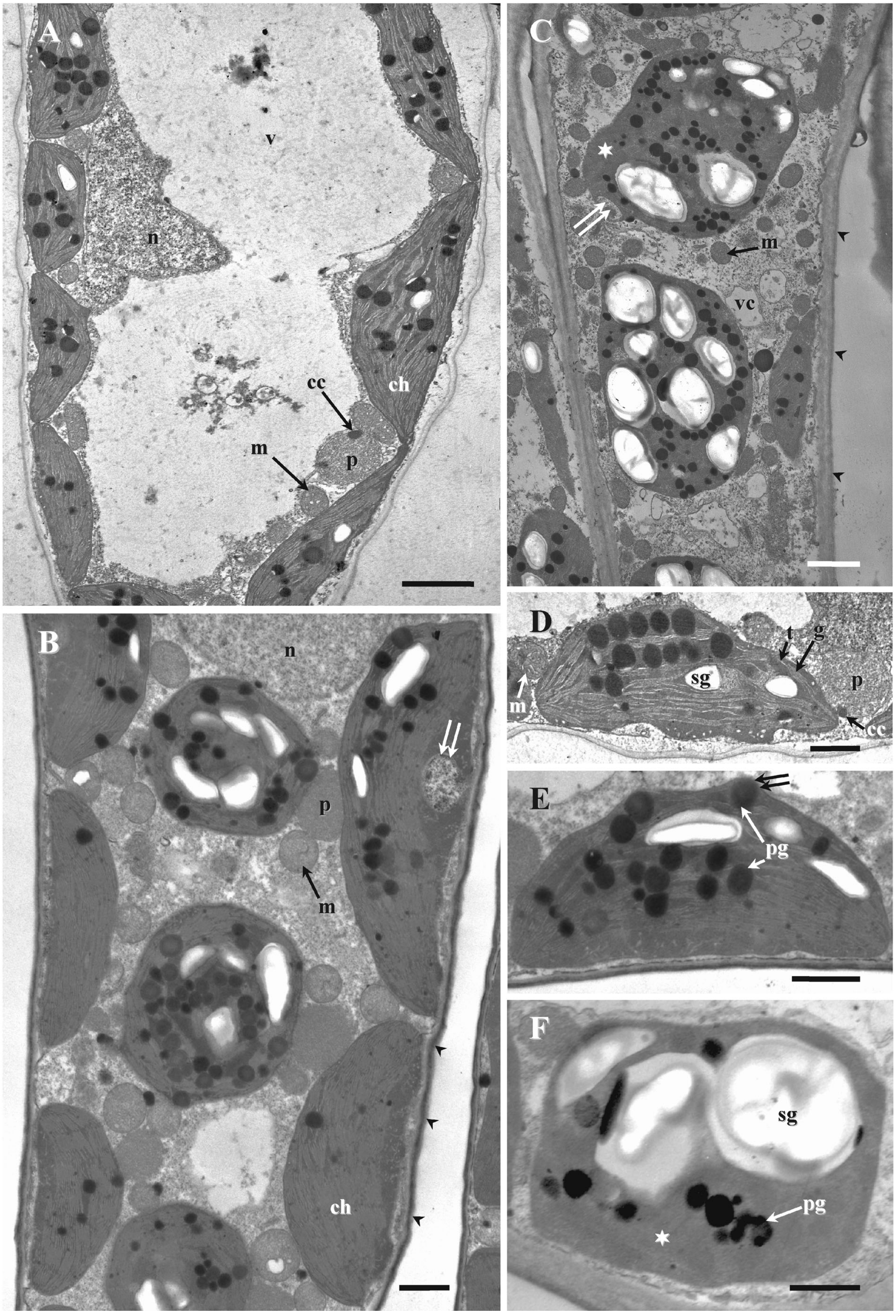
According to Kabata-Pendias and Pendias (2001), heavy metal toxicity in crop plants occurs when the heavy metal concentration in foliage exceeds 5–10 ppm Cd, 150–500 ppm Zn or 15–20 ppm Cu. No comparable data exist for trees. In our study, the distribution of visible symptoms of foliar injury, where the injury is assumed to be caused, at least partly, by the direct impact of heavy metal toxicity, was not randomly distributed across the crowns of *Populus tremula*, but occurred predominantly in the first-formed leaves located near the base of each shoot. Similar patterns of injury have been found in Cu, nickel (Ni) and Pb toxicity in *Empetrum nigrum* L. (Uhlig et al. 2001), cobalt (Co) in tomatoes (Rajeev et al. 2003) and Pb and Zn in *Typha latifolia* L. (Chen et al. 1993). Because the concentrations of Pb and Cu in the foliage of *P. tremula* did not reach the threshold for toxicity, or in the case of Cd, barely exceeded it, heavy metal induced leaf injury appeared to be attributable primarily to Zn, which reached leaf concentrations up to three times the threshold for toxicity (Kabata-Pendias and Pendias 2001).

Our findings of heavy metal accumulation in leaves, altered leaf structure and reduced gas exchange corroborated Hypothesis 1. However, Hypothesis 2 was not supported because we obtained no evidence that heavy metal effects were promoted by acid rain and low soil pH. The enhanced heavy metal uptake found on acidic subsoil was related to shallow rooting (Menon et al. 2005) rather than to subsoil pH. No conclusive evidence was obtained for Hypothesis 4; namely, that *Salix viminalis* is less affected by heavy metals than *P. tremula*. Therefore this hypothesis has to be rejected.

Irrespective of the heavy metal treatment, *S. viminalis* shed its senescent and presumably highly contaminated leaves at the stem base early (mobile elements like Zn and Cd accumulate predominantly with leaf age; Pulford and Watson 2003, Laureysens et al. 2004, Cosio et al. 2006). Conversely, *P. tremula* retained its leaves despite large heavy metal induced necroses. Lower heavy metal concentrations in the remaining leaves of *S. viminalis* were related to a minor influence of heavy metals on stomatal conductance and biomass production compared with *P. tremula* (Hermle et al. 2006). Differences in heavy metal accumulation and leaf gas exchange between *P. tremula* and *S. viminalis* remained small, however, so that both species confirmed their capacity for heavy metal accumulation and their potential value in phytoremediation assuming all aboveground biomass is regularly harvested. The advantages of these pioneer trees include rapid biomass formation (Hermle et al. 2006) and accumulation of heavy metals in aging leaves while sustaining shoot growth until the end of the growing season.

Leaf gas exchange under heavy metal stress

The declining trend in A_{net} and g_s in response to the heavy metal treatment was linked in both species and years (irrespective of rain or subsoil acidity), as shown for several species grown in the presence of various environmental constraints (Schulze and Hall 1982, Barcelò and Poschenrieder 1990). The light response curves reflected the impairment of photosynthesis by heavy metal stress. Water-use efficiency, which was associated with enhanced c_i, decreased above a PPF of 200 μmol m^{-2} s^{-1}. The decrease in WUE was the result of a greater decline in photosynthesis in response to the heavy metal treatment than in g_s. Thus, the heavy metal treatment appeared to cause a reduction in carboxylation efficiency, which limited CO_2 gain, as shown by Kitao et al. (1997) for other broad-leaved trees grown with excess manganese and by Schlegel et al. (1987) for *Picea abies* (L.) Karst. grown with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excess Zn, Cd or mercury (Hg).

Hypothesis 3 was rejected because dark respiration rate was unaffected by the heavy metal treatment. This is similar to the findings for *P. abies* (Schlegel et al. 1987), but in contrast to findings for pea, barley, maize, Scots pine and bilberry (Yarmishko et al. 1995, Romanowska et al. 2002). Increased respiration may be an indicator of repair, and old leaves are reported to have lower respiration rates and repair/defence capacities (Di Baccio et al. 2003). During the hot dry summer of 2003, dark respiration tended to increase at all crown heights compared with 2002, irrespective of heavy metal exposure. Unlike in 2002, in 2003, respiratory demand, triggered by heat stress especially in the control trees, may have masked heavy metal related effects, as suggested by the increased dark respiration at all crown levels. Respiratory activity decreased in *P. tremula* as leaf age increased, as found in other studies (Larcher 1994, Villar et al. 1995, Maurer and Matyssek 1997, Rajendrudu and Naidu 1997, Shirke 2001).

Effects of summer drought

The 2003 summer was the warmest and driest in central Europe for more than 500 years (Luterbacher et al. 2004, Ciais et al. 2005), with the mean monthly air temperatures from June to August exceeding the long-term means in Switzerland (1864–2003) by 4.0–5.5 °C. Despite ventilation, the OTCs were somewhat warmer than the ambient air during this exceptionally warm summer. On hot and dry days during summer 2003, photosynthesis and transpiration steadily declined in the morning and reached values below the detection limits toward noon and throughout the afternoon. According to measurements taken in the morning, the control trees of both *P. tremula* and *S. viminalis* suffered severe reductions in gas exchange, whereas heavy metal treatment hardly affected gas exchange. Soil water potentials remained higher in heavy metal contaminated topsoils than in uncontaminated topsoils (Menon et al. 2005), suggesting that the water supply was less rapidly exhausted by the heavy metal injured foliage because of decreased g_s at the leaf level and reduced foliage production (Hermle et al. 2006) at the whole-tree level. Similarly, Barcelò and Poschenrieder (1990) argued, in a review based mainly on results with crop plants, that heavy metal contamination limits root growth (limiting water uptake) and increases the degree of root suberization and lignification (increased resistance to water flow) as well as leaf senescence and abscission. These processes seem to favor water conservation within the plant.

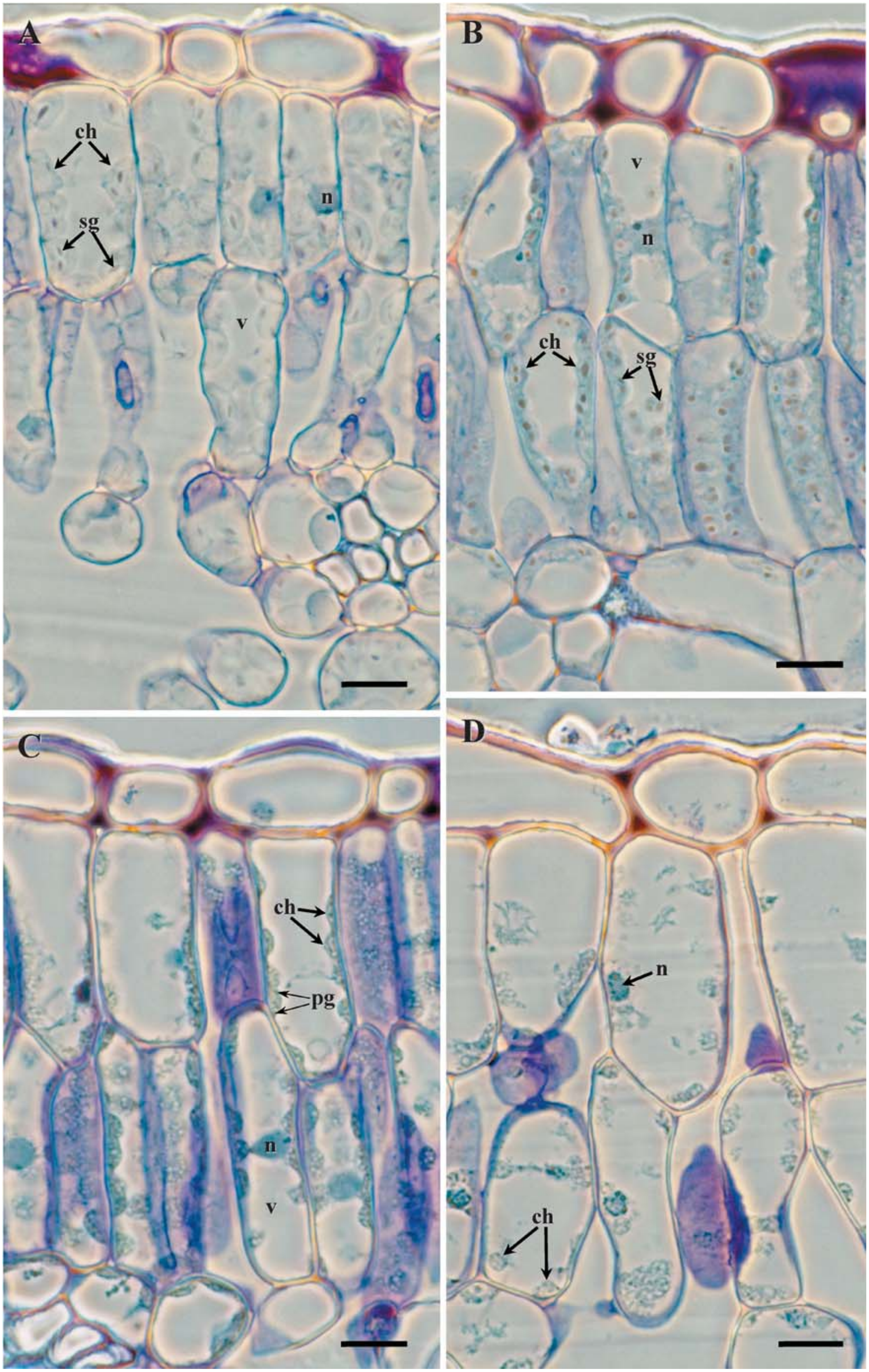
Because both the control and heavy metal exposed trees were affected by the unexpected drought and heat, the possible interactions between these factors and the experimental exposure to heavy metals cannot be assessed from our data. Without the irrigation, however, the interaction between the heavy metal treatment and heat and drought stress would have been different, and some trees might have died.

Macro- and microscopic stress symptoms

Visible symptoms of heavy metal stress were closely related to changes in mesophyll structure. The HR-like processes were responsible for the early occurring whitish, and later brownish, stipples. Leaf bleaching, however, indicated progressive injury caused by ACS in the mesophyll (Vollenweider and Günthardt-Goerg 2006). This suggests that HR-like processes initiate photosynthetic decline during spring, whereas ACS progressively increases the decline in photosynthetic activity during summer. The Orvin and Birmensdorf provenances of *P. tremula* differed in the extent of visible leaf injury by heavy metals. In contrast, foliar concentrations of heavy metals, as well as mean leaf gas exchange rates, were similar for both provenances, suggesting that the Birmensdorf provenance is more tolerant of heavy metal stress than the Orvin provenance.

The heavy metal effects on chloroplasts and associated peroxysomes resembled processes observed during ACS in Norway spruce in response to ozone pollution (Sutinen et al. 1990), which is another abiotic elicitor of plant defense reactions (Sandermann et al. 1998). Structural changes in mitochondria, as found after advanced degeneration of chloroplasts and peroxysomes in response to ozone, were not detected in our study. Electron-dense stroma in chloroplasts probably indicate chloroplast aging (Pääkkönen et al. 1995). Budding and extrusion of chloroplast material were similar to plastoglobuli development in senescent or ozone-treated beech leaves (Mikkelsen and Heide-Jorgensen 1996), but smaller in size. These processes probably contributed to the observed reduction in chloroplast size. To our knowledge, the progressive disruption of thylakoid and grana structures in response to heavy metal treatment constitutes an unknown type of chloroplast injury (Sutinen and Koivisto 1995, Kukkola et al. 1997) both in view of heavy metal and other abiotic stress. This kind of chloroplast decline may result from terminal lipid peroxidation after months of heavy metal exposure (Chaoui et al. 1997, Dietz et al. 1999). Catalase-like crystals observed in peroxysomes may represent catalase pools (Gunning and Steer

Figure 10 (Facing page). Cytological changes in the structure of assimilative cells inside the leaf mesophyll of *Populus tremula* provenance Orvin induced by heavy metal treatment. Compared with control tissue (A), palisade cells in the heavy metal treatment (B, C) showed thickened cell walls with darker outer layers (arrowheads), a vacuolated cytoplasm (vc) and fewer peroxysomes (p) without catalase-like crystals (cc). Compared with chloroplasts from control tissue (D), chloroplasts in the heavy metal treatment (E, F) showed stress reactions including a size reduction and an increased frequency of starch grains (sg) and osmiophilic plastoglobuli (pg). Chloroplast shape became irregular following budding-like reactions (double arrows), apparently associated with the extrusion of plastoglobuli (E). Grana (g) and thylakoid (t) structures became increasingly difficult to discern in injured chloroplasts (✶). Stroma was dark, even in control samples. Abbreviations: ch, chloroplasts; m, mitochondria; n, nucleus; and v, vacuole. Symptom class: 0 (A, D); 1 (B, E); 3 (F); and 4 (C). Bars: 2 μm (A–C); and 1 μm (D–F). Samples were post-fixed in OsO_4 and contrasted with uranyl acetate and lead citrate. Sections were examined in a JEOL JEM-1010 transmission electron microscope operated at 80 keV.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1996) involved in antioxidant defence (Polle 1997). The disappearance of such crystals, together with other microscopic changes in peroxysomes and chloroplasts, indicate oxidative stress, which is a driving force for structural injury in the symplast of mesophyll cells.

Oxidative stress in mesophyll cells is commonly observed in response to toxic concentrations of heavy metals (Dietz et al. 1999, Vollenweider et al. 2006) and other biotic and abiotic factors (Vollenweider et al. 2003, Günthardt-Goerg and Vollenweider 2006). Starch accumulation in chloroplasts suggests that the translocation of assimilates from leaves was impaired, as observed in response to ozone pollution (Matyssek et al. 1992, Günthardt-Goerg et al. 1993, Matyssek and Sandermann 2003). A disruption in phloem structure in response to heavy metal treatment (data not shown) might have impeded assimilate transport and indirectly reduced photosynthetic capacity by end product inhibition.

Alterations in leaf gas exchange may have resulted from structural injuries in the lower epidermis, including: (1) cell collapse and necrosis of tissue sections; (2) thickening of cell walls; and (3) inlay of lignin-like material modifying cell wall plasticity and permeability. The apoplastic thickening by pectins was less important, although it is indicative of oxidative stress (Günthardt-Goerg et al. 1997, Vollenweider et al. 2003). Zinc, which frequently accumulates in the lower epidermis (Frey et al. 2000 and authors' observations) contributed to the above-mentioned structural injuries, probably by locally increasing oxidative stress. Injuries developed irrespective of cell type and function, but caused no macroscopic symptoms on the lower leaf side. Their proximity to veins, and thus the translocation routes of heavy metals, suggests the occurrence of Zn gradients within the leaf blade. Oxidative stress reactions contiguous to veins thus provide a sensitive bio-indication of heavy metal stress, which is potentially useful in diagnosis (Vollenweider and Günthardt-Goerg 2006).

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Figure 11 (Facing page). Four different stages of accelerated cell senescence induced by heavy metal treatment in palisade cells outside stipples in leaves of *Populus tremula* provenance Orvin as visible in September. (A) Control cells: cell content not condensed in the aging tissues, except for the nucleus (n), a limited part of the cell volume is filled with one or several vacuoles (v), cells have thin walls, large chloroplasts (ch) with little staining and contain few but large starch grains (sg). (B) Slightly injured cells: the cell content is slightly condensed, vacuoles occupy a considerable portion of the cell volume, slightly thickened cell walls, smaller and denser chloroplasts containing proportionally more starch than those in control leaves. (C) Severely damaged cells: a single vacuole fills most of the cell volume, the nucleus is condensed and the cell walls regularly thickened. Cells contain fewer chloroplasts than in control leaves, and the chloroplast stroma stained dark gray, indicating lipid accumulations in the form of plastoglobuli (pg). (D) Senescent cells: with strongly condensed nuclei and only a few small and condensed or disintegrating chloroplasts or none at all. Symptom Class: 0 (A); 1 (B); 2 (C); and 4 (D). Bars: 10 µm. Samples were stained with toluidine blue and *p*-phenylenediamine and viewed by phase contrast microscopy.

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