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Localization and effects of cadmium in leaves of a cadmium-tolerant willow (Salix viminalis L.)

I. Macrolocalization and phytotoxic effects of cadmium

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Abstract

Willows have been shown to be promising for Cd phytoextraction. Nevertheless, plant responses to Cd are still not clearly understood. We investigated the effects of Cd accumulation on morphological parameters, and Cd allocation in leaves by autoradiography in Salix viminalis grown in hydroponics with increasing concentrations of Cd (0–200 μM) to assess the effect of Cd localization on plant performance.

Willow was highly tolerant to Cd, with only an 18% shoot and no significant root biomass reduction at 20 μM Cd, although Cd concentration in shoots exceeded 100 mg kg−1. At 50 μM significant reduction in root and shoot biomass and total root length, as well as change in root architecture was observed. At 100 μM, S. viminalis exhibited strong stress symptoms, whereas 200 μM impaired survival. Cadmium accumulation and the intensity of visible phytotoxicity symptoms on the leaves increased from 3 to 50 μM and then decreased at higher concentrations.

At all Cd concentration exposure Cd was localized mainly in the tips and edges of the younger leaves, whereas it was mainly located at the base of the older leaves. This localization coincided with visible necrotic spots and indicated that Cd tolerance had been exceeded.

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Keywords: Autoradiography; Cadmium (Cd); Metal allocation; Phytoextraction; Salix viminalis; Tolerance; Toxicity

1. Introduction

Cadmium (Cd) is one of the most widespread and toxic metals, and cost-effective means to remove it from the soil are needed. Since Cd tends to adsorb to topsoil, phytoextraction has been proposed as a low-cost technique. Willows have been shown to be promising for Cd phytoextraction (Granel et al., 2002; Hammer et al., 2003; Klang-Westin and Eriksson, 2003; Punshon et al., 1996; Pulford and Watson, 2003). Punshon and Dickinson (1997) observed that acclimatizing willows to toxic metals could be achieved by gradually increasing the heavy metal concentration in the nutrient solution. Additionally the authors reported that leaf morphological alteration occurred within the lifetime of a clone according to the exposure to Cd, highlighting the capacity of willows, as a pioneer species, to adapt to their environment. Hammer et al. (2003) found that in the field leaves represented 15–19% of the total biomass produced in mature Salix viminalis plants but yielded 34–37% of the total Cd extracted per year, confirming the importance of Cd storage in leaves. However, knowledge about Cd allocation in S. viminalis according to leaf position, within leaf tissue or at the cellular level is missing. More generally, the mechanisms inducing tolerance to Cd and the factors limiting phytoextraction efficiency are still not clearly understood, although metal distribution within plant organs and tissues may be good indicators of detoxification and tolerance mechanisms.
The link between the visible toxicity symptoms and the presence of Cd in the organ has only been reported in a few studies because of low Cd concentrations usually found in plants and methodological difficulties. For example, a correspondence between Cd distribution and chlorosis or necrosis in leaves has been observed in Brassica juncea (Salt et al., 1995) and in Thlaspi caerulescens (Cosio et al., 2005). Additionally, results have usually been obtained either on hyper-accumulating plants, or on crop species following short-term exposure to elevated external levels of metals, not comparable to chronic ‘natural-field-condition’ stress (di Toppi and Gabbrilli, 1999). Besides, the techniques used for other metals are not always applicable to Cd. For example energy dispersive X-ray micro-analysis (EDXMA), electron spectroscopic imaging (ESI) and electron energy loss spectroscopy (EELS) have been used to plot the subcellular distribution of metals (Bringezu et al., 1999; Frey et al., 2000; Küpper et al., 1999; Nassiri et al., 1997; Wójcik et al., 2005; Zhao et al., 2000), but their poor sensitivity and interference with other cations (e.g. K) limit Cd detection, and do not allow visualization of Cd distribution over the whole leaf surface. Physical methods such as atomic absorption spectroscopy (AAS) after tissue disruption (Brown et al., 1995; Chardon- nens et al., 1998), secondary ion mass spectroscopy (SIMS) imaging (Lazof et al., 1996), or short-term desorption with radiotracer (Blaudez et al., 2000; Lasat et al., 1996) are appropriate to find the approximate metal storage location, but not to localize metal allocation precisely. There is thus a need for a simple low-cost method without extensive sample preparation, which enables direct visualization of metals in leaves, cells and subcellular organelles in situ. Autoradiography has been used to localize metal accumulation in vertebrate (Takeda et al., 2000) and in plant tissues (Crafts and Yamaguchi, 1964; Salt et al., 1995; van Balen et al., 1980). The technique is very precise because the radioactive emission enables detection of low metal concentrations (Gahan, 1972) and is therefore highly useful for Cd detection in plants.

The aims in this study were to investigate in a Cd tolerant and accumulating clone of S. viminalis (clone no. 78198) which accumulates large amounts of Zn and Cd in its shoots (Landberg and Greger, 1996). Long stem cuttings (10 cm) were rooted and grown in 100 mL pots (25 cm height, 3 cm diameter, one plant per pot) filled with modified quarter-strength Hoagland’s nutrient solution (Sigma, St. Louis, USA) supplemented with 20 µM Fe–HBED (Strem chemical, Newburyport, USA). Fe(III)-HBED was prepared as described by Chaney et al. (1998). Plant culture was performed in a climate chamber (day/night period 16/8 h, day/night temperatures 20 °C/16 °C, and a light intensity of 5001x). Plants were allowed to develop roots and grow 2 weeks in hydroponics before the Cd treatment was started. The nutrient solution was renewed from twice a week at the beginning to every 2 days at the end of the exposure. Plants treated with 3–50 µM Cd had a higher requirement of nutrient solution than control plants, whereas those grown in 100 and 200 µM Cd a lower.

To determine Cd tolerance and accumulation in the plants, an initial experiment with four different treatments (0, 3, 10 and 20 µM Cd) was performed during 6 weeks. After observing the high Cd tolerance of the selected clone, it was tested with 50, 100 and 200 µM Cd in a second 6 weeks experiment. Finally a third experiment with a longer growth period of 15 weeks and with 0, 10, 50 and 200 µM Cd was carried out. All treatments were performed in four replicates.

For autoradiography of Cd in plants five different treatments were applied (0, 5, 10, 50 and 200 µM Cd) and autoradiographs were performed after 3 days, 2, 4 and 6 weeks of exposure to the metal. All nutrient solutions were spiked with 0.1 kBq mL⁻¹ (2.2 × 10⁻³ µM) of ¹⁰⁹CdCl₂ (NEN Life Science Products, Boston, USA).

2.2. Assessment of the plant response to increasing Cd concentrations

Any visible symptoms of Cd toxicity in the leaves were noted. The shoot and root dry mass was determined for each experiment. The total root length, the distribution of root diameter (16 classes from 100 to 3000 µm) and the number of root bifurcations were measured with the software winRHIZOPro 5.0 (Régent Instruments, Quebec, Canada) coupled with a STD1600 desktop scanner (Epson) provided by Régent Instruments. All measurements were carried out at a resolution of 400 dpi. Root parameters were quantified only in two 6-week-old plants previously used for autoradiography.
2.3. Cadmium concentration in plants

Total Cd concentrations in leaves and roots of *S. viminalis* were measured for the three experiments. Shoots and roots were washed thoroughly with deionized water. To remove cations slightly adsorbed on the root surface, the roots from the first trial (3–20 μM Cd, 6 weeks) were washed 5 min with 20 mM Na ethylenediamine tetra-acetic acid (EDTA), followed by a quick rinse with deionized water (Keller et al., 2003). Roots and shoots were dried at 80 °C until a constant weight was reached. Shoots were divided into leaves and stems that were individually ground in a tungsten Retsch mill (Haan, Germany). Finely ground samples (0.5 g) were digested in 8 mL HNO₃ 65% supra pure (Fluka, Buchs, Switzerland) and evaporated. The residue was re-dissolved in 1 mL HClO₄ 70% pro analysis (Fluka, Buchs, Switzerland) and heated 1 h to 235 °C before dilution to 20 mL with purified water. The root biomass of the plants grown with 200 μM Cd was low and the roots of the four plants were thus pooled before digestion. The EDTA solution from root washing was recovered, filtered and the concentration of Cd desorbed was measured. Cadmium concentrations were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES; Plasma 2000; Perkin-Elmer, Wellesley, USA) or by graphite furnace atomic absorption spectrometry (GF-AAS; 5100PC atomic absorption spectrometer equipped with a HGA 600 graphite furnace, a Zeeman-corrected furnace module, and a AS-60 Furnace autosampler; Perkin-Elmer, Wellesley, USA) when needed. In house reference plant material was used to assess the accuracy of the measurements. For homogeneity all root Cd concentrations presented in the tables of this paper are concentrations of roots not washed with EDTA. Data of roots washed with EDTA are presented in the text only.

2.4. Autoradiography of Cd in plants

Shoots and roots were collected separately, quickly rinsed with deionized water and blotted dry. The samples were arranged as flat as possible and wrapped in a single layer of thin PE film. To obtain autoradiographs of adaxial and abaxial sides, samples were sandwiched between two X-OMAT AR-5 autoradiography films (Kodak, Rochester, USA). In order to obtain an image on films, leaves harvested after plants had grown 3 days in Cd solution were exposed 6 weeks, whereas leaves from all the other treatments were exposed 48 h at room temperature. In all cases control plants grown without ¹⁰⁹Cd were processed in parallel with radioactive samples to detect possible artifacts. Autoradiographs were developed with an automatic film-processor SRX-101A (Konica, Tokyo, Japan) and numerized on a Tango digitalizator (Linotype-Hell Heidelberg, Kiel, Germany). Subsequently, for each concentration tested, leaves of one single plant were individually dried at 80 °C, weighed, digested in HNO₃ 65% sp. (Fluka, Buchs, Switzerland) 1 h at 90 °C and analyzed for Cd concentration as described above.

3. Results

3.1. Assessment of the plant response to increasing Cd concentrations

The most obvious symptom of Cd toxicity was the reduction of plant growth (see below). Besides the biomass reduction, the Cd-treated willows developed even gradients of visible symptoms, which progressively increased with the leaf age. They included a patchy chlorosis and necroses (Fig. 1). The chlorosis developed preferentially at the base of the leaf. The necroses were already detected after 3-week exposure to Cd as small dots at the adaxial side of the leaf margins, frequently crossing the veinlets (Fig. 1 c). While chlorosis was observed in all Cd treatments (Table 1), necrosis was restricted to the 10 and 50 μM Cd treatments. Before Table 1

<table>
<thead>
<tr>
<th>Cd in nutrient solution (μM)</th>
<th>Visible symptoms Shoot growth reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No symptoms 0</td>
</tr>
<tr>
<td>5</td>
<td>Chlorosis (+) 2(0)</td>
</tr>
<tr>
<td>10</td>
<td>Chlorosis (++), necrosis (+) 8(1)</td>
</tr>
<tr>
<td>50</td>
<td>Chlorosis (+++), necrosis (++), leaf rolling 45(17)</td>
</tr>
<tr>
<td>200</td>
<td>Slight chlorosis 90(2)</td>
</tr>
</tbody>
</table>

Symptoms were displayed as even gradients. Relative symptom intensity is given in brackets. Percent of growth reduction are given to the 0 μM Cd treatment (n = 4, S.D. in parentheses).
the 15th week of growth, symptoms in plants exposed to Cd had further developed into well visible necrotic flecks (Fig. 1e). Additionally the leaves in the upper portion of the shoot tended to dry out at the tip. Another visible phytotoxic symptom restricted to the 10 and 50 \( \mu \)M Cd treatments was leaf rolling (not shown), which occurred on 6- as well as on 15-week-old willows excluding a pathogenic origin. The plants treated with 200 \( \mu \)M Cd showed fewer leaf symptoms (but major growth reduction) than the plants grown at lower concentrations and no necroses were detected. Roots turned brownish with increasing Cd concentration.

### 3.2. Biomass production

Tolerance of *S. viminalis* to Cd (Table 1, Fig. 2) was confirmed with only an 18 ± 14% shoot biomass reduction at 20 \( \mu \)M Cd (versus the control, Student’s \( t \)-test \( P < 0.05 \)) after 6-week growth and no significant root biomass reduction. At 50 \( \mu \)M Cd we observed a 45 ± 17% shoot biomass reduction \( (P < 0.01) \), whereas at 200 \( \mu \)M Cd there was a 90 ± 2% shoot biomass reduction \( (P < 0.001) \) and a severe 96 ± 1% root biomass reduction \( (P < 0.001) \). At 100 and 200 \( \mu \)M Cd the shoot:root biomass ratio increased from 4.6 to 5.6 for the lower treatment concentrations to 8.3 for 100 \( \mu \)M Cd \( (P < 0.05) \), and 12.9 for 200 \( \mu \)M Cd \( (P < 0.01) \) (Table 2).

Total biomass was larger at 15 weeks than after 6-week exposure (Fig. 2). The overall biomass decreased more after 15 weeks than after 6 weeks when the Cd-treated and the control plants were compared. For example at 50 \( \mu \)M Cd the shoot biomass reduction was 45 ± 17% at 6 weeks, but 61 ± 5% at 15 weeks, with a decrease in root biomass of 46 ± 19 and 72 ± 1%, respectively. If 200 \( \mu \)M Cd was excluded from the calculations, the average shoot:root biomass ratio was 5.6 after 6 weeks, but 2.7 after 15 weeks, reflecting the observed proportionally greater development of the root system during the second and third months of growth (Table 2).

### Table 2

<table>
<thead>
<tr>
<th>Cd in nutrient solution (( \mu )M)</th>
<th>Shoot:root biomass ratio</th>
<th>Cd (mg kg(^{-1}))</th>
<th>Leaf:root Cd ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leaves</td>
<td>Roots</td>
</tr>
<tr>
<td>(a)</td>
<td></td>
<td>0.9 (0.5)a</td>
<td>0.4 (0.3)a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39 (13)b</td>
<td>313 (96)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90 (30)c</td>
<td>706 (306)c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>201 (21)d</td>
<td>1197 (159)d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>519 (105)e</td>
<td>4048 (946)e</td>
</tr>
<tr>
<td></td>
<td></td>
<td>260 (90)f</td>
<td>798 (289)f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52 (21)bc</td>
<td>1310</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td>2.25 (0.38)a</td>
<td>1 (0.5)a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>153 (32)b</td>
<td>520 (57)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>584 (159)b</td>
<td>3554 (534)c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>181 (79)c</td>
<td>13114</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences between the means with \( P < 0.05 \). \( (n=4, \) S.D. in parentheses.\)

\( ^a \) Roots were pooled prior to analysis.
3.3. Root length and diameter

As with the root mass, total root length was reduced by 65 ± 6% at 50 μM Cd, and 96 ± 3% at 200 μM Cd versus the control after 6-week treatment (Table 3). Fig. 3 shows the distribution of root diameter classes in percent of the total root length for the different Cd treatments. In all treatments most of the roots were found in the smaller diameter classes (0–100 and 100–200 μm). Control plants exhibited a sharp decrease of root length in the classes between 200 and 800 μm and an almost negligible proportion of roots with a diameter above 900 μm. In the Cd-treated plants, the sharp decrease was shifted to the classes between 200 and 500 μm, whereas the proportion of the 500–900 μm classes was increased. The summed root lengths of the classes between 0 and 200 μm were compared with that of 500–900 μm (Table 3). Compared to the control, the length of finer roots diminished by 18 ± 5% at 5 μM Cd, whereas the length of the 500–900 μm classes increased by 49 ± 14%. At higher Cd concentrations (50 and 200 μM Cd) root growth of both classes was inhibited compared to the control, although finer roots were more affected than the 500–900 μm classes. With increasing Cd concentrations there was therefore less development of lateral roots, although the bifurcation number per root cm remained between 3.8 and 4.9 (Table 3). Finally, this indicated that the root growth, elongation and absorption zones were restricted by exposure to Cd.

3.4. Cadmium concentration in whole plants

Cadmium concentrations were always larger in roots than in leaves (Table 2). After the 6-week treatment, Cd concentration in roots and leaves increased with increasing Cd treatment (3–50 μM Cd in solution), but was smaller in the 100 μM treatment. In the 200 μM treatment the Cd concentration in leaves was similar to that in the 3 and 10 μM Cd treatments (Table 2). Cadmium concentrations in leaves were linearly related to Cd concentrations in roots (Pearson correlation $R = 0.956$; significance 2α < 0.001), when the 200 μM Cd treatment, that also exhibited a smaller leaf:root Cd ratio, was excluded from the calculations. When Cd adsorbed onto the roots was removed by EDTA washing, about half (46 ± 2%) of Cd was removed from the roots of 6-week-old willows grown with 3, 10 or 20 μM Cd, but the linear correlation between root and shoot concentrations remained ($R = 0.986$; sign. 2α < 0.001). However, the mean corrected leaf:root concentration ratio reached 0.54 ± 0.11, indicating that Cd concentrations in roots were twice as high as leaf concentrations.

Except for the 200 μM Cd treatment, Cd concentrations measured in leaves and roots after the 15-week exposure were of the same order of magnitude as those found after the 6-week exposure (Table 2) although the biomass had increased (Fig. 2). At 200 μM Cd, Cd concentrations increased by respectively, 3.5- and 10-fold in leaves and roots after 15-week compared to 6-week exposure even though the biomass and shoot:root biomass ratio remained similar.

3.5. Visualization of Cd in plants

The technique of autoradiography using labelled Cd could not discriminate between metal adsorbed onto and located into the roots (data not shown), but it was very efficient at visualizing Cd distribution in leaves. Cadmium allocation followed gradients at the shoot and leaf levels (Fig. 4). The shoot gradients were primarily determined by the leaf age and leaf position. In younger leaves, $^{109}$Cd gave a diffuse and homogeneous background signal over the entire leaf area and was principally accumulated in the leaf tip, and along the leaf edges. The homogeneous signal tended to disappear with increasing leaf age. It was replaced by a point-like accumulation spreading from the leaf top to the whole limb and

### Table 3

<table>
<thead>
<tr>
<th>Cd concentration in nutrient solution (μM)</th>
<th>Total root length (m)</th>
<th>Forks (cm$^{-1}$)</th>
<th>Root length (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fine roots</td>
<td>Coarse roots</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.64</td>
<td>3.77</td>
<td>1.60</td>
</tr>
<tr>
<td>5</td>
<td>2.42</td>
<td>4.02</td>
<td>1.32</td>
</tr>
<tr>
<td>10</td>
<td>2.16</td>
<td>3.76</td>
<td>1.26</td>
</tr>
<tr>
<td>50</td>
<td>0.90</td>
<td>4.14</td>
<td>0.38</td>
</tr>
<tr>
<td>200</td>
<td>0.08</td>
<td>4.88</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of Cd on root diameter: proportion of the various root diameter classes accounting for the total root length in 6-week-old *S. viminalis* grown in hydroponics with or without Cd. Values represent means of two plants. Symbols are placed at the end of each class.
Fig. 4. Cadmium visualization in leaves: autoradiographs of leaves of 6-week-old *S. viminalis* grown in hydroponics with 2.2 × 10⁻³ μM of ¹⁰⁹Cd and 10 μM Cd spiked with 2.2 × 10⁻³ μM of ¹⁰⁹Cd. Leaves are ordered according to their position on the shoot. The youngest leaf is at the top and the oldest at the bottom (film exposure: 48 h). Bar: 1 cm.

### Table 4

<table>
<thead>
<tr>
<th>Cd in nutrient solution (μM)</th>
<th>Number of leaves</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>0.958⁻¹</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>0.911⁻¹</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>0.782⁻¹</td>
</tr>
<tr>
<td>200</td>
<td>7</td>
<td>0.306</td>
</tr>
</tbody>
</table>

One single plant was analyzed for each concentration tested. (n.d.: not determined.)

⁻¹ Significance 2α < 0.001.

by a growing vein signal with increasing leaf age. After 3 days of exposure to Cd, concentration of ¹⁰⁹Cd was too low to be clearly visible on the autoradiographs. However, the accumulation in the central vein and the difference between younger and older leaves were already visible (autoradiographs not shown). Two, four or 6 weeks of exposure to Cd did not modify the Cd distribution patterns in the leaves, although Cd concentration seemed to increase as reflected by the increasing darkness of the autoradiographs (data not shown). The same Cd distribution pattern was observed at all concentrations (5–200 μM Cd, data available in Cosio, 2004). The autoradiographs of the adaxial and abaxial sides of the leaves were almost identical, with only rare and not significant differences (data not shown).

### 3.6. Biomass and Cd accumulation in individual leaves

To assess if differences between younger and older leaves seen on the autoradiographs were reflected by different Cd concentrations, we measured Cd concentration in individual leaves according to their insertion height on the shoot. In the 200 μM Cd treatment, differences in the Cd concentration and biomass according to the leaf position were very small (Fig. 5). For the plants selected from the 5, 10 and 50 μM Cd treatments, the youngest leaves had always the lowest Cd concentrations (Fig. 5). The Cd concentration in older leaves irregularly increased according to a treatment- and plant-specific trend. Cadmium concentration in individual leaves was significantly correlated with leaf dry matter (Table 4) indicating that more Cd was allocated to leaves with a greater physiological activity of water uptake, as indicated by their larger biomass.

### 4. Discussion

#### 4.1. Toxic symptoms on leaves

The whole complex of visible symptoms in the foliage was typical for abiotic stress and the observed necroses formed the most specific visible symptom of Cd stress. Symptoms were not limited to those usually described, namely chlorosis, stunting and leaf rolling (Das et al., 1997), but also
Fig. 5. Cadmium effect on leaf Cd concentration (mg kg$^{-1}$) and biomass (mg) of *S. viminalis* grown 6 weeks in hydroponics with (a) 5 $\mu$M, (b) 10 $\mu$M, (c) 50 $\mu$M and (d) 200 $\mu$M Cd according to their order on the stem from the top, the first clearly identifiable leaf from the top of the stem counting as no. 1. One single plant was analyzed per treatment.
included characteristic necroses and partial leaf desiccation. Salt et al. (1995) similarly found that Cd toxicity produced chlorosis in young leaves of B. juncea, where Cd was preferentially accumulated. Cosio et al. (2005) also reported that a 12-week 50 μM Cd treatment produced necrotic dots on leaves of the Prayon ecotypes of T. caerulescens. However, in this later case necroses were evenly distributed over the leaf surface. Necroses along edges have also been found with Zn (Günhardt-Goerg and Vollenweider, 2003) and frequently indicate different macronutrient deficiencies (Hartmann et al., 1995). Together with the chlorosis, they might also indicate disturbances in the whole mineral nutrition (Siedlecka, 1995). They differed from the necrotic stiples (not observed here), dots and flecks induced by other abiotic stress factors. For example with ozone stress, stiples are always interveinal (Vollenweider et al., 2003) whereas drought does not cause stippling (Hartmann et al., 1995).

4.2. Tolerance of S. viminalis: effect of Cd on biomass

A reduction in biomass and leaf size was observed with increasing Cd concentrations. Salix viminalis was nevertheless surprisingly tolerant to Cd with a shoot biomass reduction of 18% versus the control in the 20 μM treatment and a significant root reduction at 50 μM only. For comparison, Landberg and Greger (2002) observed that the most resistant clone of S. viminalis tested in a 20-day assay had a 19% decrease in biomass shoots after a 7 μM Cd treatment. Punshon and Dickinson (1999) obtained a 44% reduction in biomass when they grew a clone of S. viminalis with 10 μM Cd during 4 weeks. These values are larger than the values reported here for S. viminalis clone no. 78198 and thus highlight the exceptional tolerance of this clone to Cd. Nevertheless, since the effects on the biomass were enhanced by the duration of the exposure, long-term tolerance is certainly less than short-term tolerance. It would thus be necessary to test Cd tolerance of S. viminalis during at least one growing season.

Similar to the results of this study for the lower concentrations tested (5–20 μM Cd), Cieslinski et al. (1996) found that in soil grown plants, Cd affected the leaf dry mass more than the root dry mass of strawberry plants, although Cd was mainly accumulated in roots. The authors concluded that leaf dry mass was the best indicator of Cd toxicity. However, in agreement with other authors (Shukla et al., 2003; Sottnikova et al., 2003) we found that at high Cd concentrations (100 and 200 μM Cd) roots clearly suffered more than shoots from Cd toxicity. The change in shoot:root biomass ratio indicated that Cd altered biomass allocation. However, the length of roots of smaller diameter (0–100 and 100–200 μm) started decreasing at lower Cd concentration in solution than root biomass, indicating that root growth inhibition caused by Cd stress may have been compensated for by an increased proportion of roots with a larger diameter (500–900 μm) with increasing Cd concentrations.

There was no effect on root branching, as has also been reported in maize by Seregin and Ivanov (1997). This further shows that the thicker roots were less affected than the finer ones by exposure to Cd. Indeed, Cd failed to prevent root branching, although the main root growth was strongly inhibited. Another root toxicity symptom observed here was root browning. It has been reported that root browning was due to an enhanced suberization or lignification of roots tips that consequently lost their capacity for nutrient uptake (Hagemeyer and Breckle, 1996; Kahle, 1993; Schützendübel and Polle, 2002). Although Cd probably has a significant effect on root hairs (Gussarson, 1994), this was not observed here as a consequence of missing root hairs in hydroponics (Hagemeyer and Breckle, 1996).

In summary, the Cd treatments from 0 to 200 μM covered the whole tolerance range of the tolerant clone no. 78198 of S. viminalis grown in hydroponics. From 0 to 20 μM we observed simultaneously an increase in Cd concentrations, a small loss in root biomass and length compensated by bigger diameter roots, a small loss in leaves biomass, a decrease in water stress tolerance, a patchy chlorosis and more or less developed necroses. These symptoms are likely to also be observed in contaminated soils if Cd is the only contaminant. At 50 μM a significant reduction in root and shoot biomass and total root length, as well as change in root architecture was observed. Cadmium stress exceeding the tolerance limits of this willow clone appeared at 100 and 200 μM Cd. Symptoms included a combination of a decrease in Cd concentration, an increase in shoot:root biomass ratio, a significant decrease in shoot biomass, in root biomass, in total root length and a reduction of water uptake. These symptoms are similar to those reported for other plants exposed to Cd concentration above the critical toxicity level (Barcelo and Poschenrieder, 1990; Poschenrieder and Barcelo, 1999; di Toppi and Gabbrielli, 1999).

4.3. Cadmium concentration in whole plants

Cadmium concentration after 6 weeks of growth followed a similar trend in leaves and roots, increasing with the Cd concentration applied in the nutrient solution up to 50 μM Cd, but decreasing at 100 and 200 μM Cd. Cadmium accumulation was not correlated to biomass production, since biomass decreased with increasing Cd concentration in the nutrient medium.

Cadmium concentrations found in S. viminalis clone no. 78198 were higher in roots than in leaves when grown in hydroponics, whereas in soils, Cd concentrations were higher in the leaves (Hammer and Keller, 2002; Rosselli et al., 2003). Therefore the larger root concentrations observed here were probably artifacts due to hydroponics.

Cadmium concentration in leaves of S. viminalis reached 100 mg kg⁻¹ in the 10 μM Cd treatment without strong growth reduction. This concentration is the threshold that defines Cd hyperaccumulation in natural environment (Brown et al., 1994). Punshon and Dickinson (1997) reported
Cd concentrations in the same range in leaves of willows grown in hydroponics with Cd concentrations up to 13 μM Cd. However, 10 μM Cd (1.12 mg Cd L\(^{-1}\)) is already an elevated concentration for a soil solution (Knight et al., 1997; Lombi et al., 2001; Wagner, 1993) and plants in solution culture usually accumulate more Cd than those in soil (Dickinson, in press; Grant et al., 1998). Cadmium concentrations in leaves of S. viminalis grown in the field may therefore be in the range of 10 mg kg\(^{-1}\) rather than above 100 mg kg\(^{-1}\) (Dickinson, in press).

Studying plants at higher concentrations may allow the extent of S. viminalis tolerance to Cd to be assessed. For example, the pattern of Cd uptake could indicate that the toxicity threshold has not been exceeded after 6 weeks of exposure even at high Cd supply, since the metabolic control of Cd uptake was not lost (Dan et al., 2002). Cadmium concentrations found in leaves in tobacco plants and in geranium were related to Cd concentrations in roots, indicating a tight control of Cd translocation in plants (Grant et al., 1998; Dan et al., 2002). In the case of willow a similar scenario may exist, because metal concentration in the leaves and roots were correlated. Moreover, after 13 weeks at 200 μM Cd the strong increase of Cd concentration in roots may reflect the loss of metabolic control. Possibly, as a result of Cd toxicity, roots lost not only their capacity for nutrient uptake, leading to plant growth inhibition, but also their capacity to limit metal accumulation in roots (Schützendübel and Polle, 2002), whereas plant growth inhibition resulting from the 200 μM Cd treatment led to reduction of Cd translocation to the shoots.

4.4. Cadmium localization in leaves

The accumulation of Cd was uneven at the leaf surface. There was some correspondence between the Cd spots observed on autoradiographs and the necrotic dots observed on the margin of the leaves. The structural connection between Cd accumulation and necrosis was also found at the microscopic level (Vollenweider et al., 2006). To our knowledge, this is the first direct evidence of Cd distribution in leaves of willow. Cosio et al. (2005) also localized Cd by autoradiography in two ecotypes of T. caerulescens and reported that Cd was found mainly at the edge of the leaves but also in points of higher concentration spread over the whole leaf surface. This localization similarly corresponded to some necrotic dots observed on the leaves of the Prayon ecotype. Also using autoradiography Cunningham et al. (1975) observed prominent Cd allocation in the stems and leaf veins of young sprouts of soybean together with leaf age-driven gradients. On the contrary, Salt et al. (1995) using autoradiography found an even distribution of points of higher concentration of Cd at the leaf surface of Indian mustard and clearly showed that Cd was accumulated preferentially in trichomes. In a number of other annual plants sequestration of metals in trichomes has been reported (Ager et al., 2002; Choi et al., 2001; Küpper et al., 1999). Trichomes are ideal for allocation of heavy metals because of their localization at the leaf periphery. Although S. viminalis possesses trichomes they did not accumulate Cd, as also observed by Vollenweider et al. (2006). Cadmium distribution in the plant foliage is thus clearly species-specific and its visualization is needed to understand patterns of accumulation and tolerance of metal-treated plants. Cadmium concentration also depended on leaf age. In S. viminalis, we found that the smallest concentrations were always in younger leaves whereas the location of the largest ones depended on Cd concentration in the medium. On the contrary Cd accumulated preferentially in the youngest leaves of both B. juncea and T. caerulescens (Salt et al., 1995), whereas it was found at higher concentrations in the older leaves in Silene vulgaris (Chardonnens et al., 1999), Empetrum nigrum (Uhlig et al., 2001) and Armeria maritima ssp. halleri (Dahmani-Muller et al., 2000). As consequence Dahmani-Muller et al. (2000) proposed leaf-fall as a metal detoxification mechanism in the case of A. maritima. Leaf age dependence of Cd allocation is thus also species specific and certainly relates to the plant structure and growth habit.

In contrast to the visible leaf toxicity symptoms, where intensity varied according to Cd concentration in the leaves, the localization pattern of \(^{109}\)Cd in leaves was similar in the different Cd treatments. Cadmium localization in young and old leaves was different, also in plants that had formed all leaves before Cd treatments were imposed. The treated willows showed an unfinished growth pattern and had not set any bud primordia by the final harvests, as generally observed at least in regularly coppiced S. viminalis (authors observation) and as typically observed for pioneer species. Such a growth habit, heavily relying on new growth and rapidly discarding older leaves, certainly influenced the Cd allocation patterns. It could probably explain why some Cd was always found in the tips and at the edges of the youngest leaf. Young growing leaves also have a higher demand for nutrients than older ones (Yoshida, 2003). Cd localization in the youngest leaves can in addition be explained by increased transpiration driving Cd to the terminal sites of the transpiration stream as observed with other ions (Marschner, 1995). On the contrary in older leaves the transpiration stream into leaves is reduced (Patrick, 1988), and Cd did not reach the edges anymore. Such a difference in flow has been demonstrated in poplar by transpiration measurement between young and old leaves (Siebrecht et al., 2003). On the other hand, a reallocation of Cd with leaf aging could not be ruled out because the localization pattern of \(^{109}\)Cd in leaves was similar in the different Cd treatments irrespective of the sampling time. It therefore means that Cd spots on the edges of young leaves were later reallocated to other places when leaves got older. Vollenweider et al. (2006) documented microscopically that leaf veins in older leaves play an important storage role, certainly as a consequence of their important biomass increase. They also showed that the detected reallocation occurred, probably through the phloem as already shown for other cations (e.g. Ca) (Marschner, 1995; Patrick, 1988).
5. Conclusion

Observations performed at various Cd concentrations and times of exposure gave information on the dynamics of Cd accumulation in the plant following the physiological changes occurring with leaf maturation and senescence, and also highlighted the large potential of S. viminalis (clone no. 78198) for Cd decontamination. These dynamics are probably responsible for the ability of this clone to cope with high Cd concentrations and its overall high tolerance to Cd. Indeed Cd tolerance was not exceeded until 100 and 200 µM Cd, levels of contamination that would be found only in experimental conditions. Additionally, the large Cd concentrations found in leaves indicated that the harvest of all leaves would be valuable when S. viminalis is grown on contaminated substrates. Finally, the large root concentrations observed here, although probably partly due to hydroponic conditions, indicated that removal of roots at the end of the remediation process might allow extraction of an additional amount of Cd.

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