Localization and Toxic Effects of Cadmium, Copper, and Uranium in *Azolla*

Received for publication June 16, 1987 and in revised form March 25, 1988

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ABSTRACT

The storage and distribution of copper, cadmium, and uranium and their effects on ionic contents in roots and shoots of *Azolla filiculoides* has been studied by x-ray microanalysis. The relative content of copper was eightfold higher in the root than in the shoot, suggesting low mobility of this metal in *Azolla* plant. Cadmium relative content in the shoot was similar to its content in the root, hence its mobility was relatively high. The absence of significant uranium quantities in the shoot and its relative high content in the root suggest the immobility of this metal from *Azolla* root. Cadmium formed precipitates with phosphate and calcium in xylem cells of the shoot bundle and caused a two-to-threefold increase in the content of phosphate in the root. Uranium in roots and cadmium in shoots were associated with calcium. All three treatments caused losses of potassium, chloride, and magnesium from *Azolla* roots. Accumulation of heavy metals in *Azolla* and their mobility from the root to the shoot can be correlated with damage caused by the loss of essential nutrients.

*Azolla filiculoides* has recently been found to take up and accumulate heavy metal ions, demonstrating a high binding capacity (M Sela, J Garty, E Tel-Or, submitted for publication). The contents of cadmium, copper, and uranium for the *Azolla* whole plant grown in the presence of 10 ppm metal were 6,021, 5,365, and 5,082 ppm, respectively, as detected by atomic absorption of digested plants. Cadmium and copper contents in the roots of these plants were 14,328 and 23,941 ppm, respectively, and their contents in the shoot were 4,857 and 2,748 ppm, respectively.

The aim of this investigation was to analyze the localization of copper, cadmium, and uranium in shoot and root tissue of *Azolla* and in *Anabaena* cells. Furthermore, the effect of these heavy metals on the contents of chloride, phosphorus, potassium, sodium, magnesium, calcium, and iron in *Azolla* tissues and in *Anabaena* cells was studied. This may be important for an understanding of the action of heavy metals in plant tissues. In order to prevent the loss of water soluble ions, this study used a recent improvement in tissue fixation technique (6) that has been successfully used for localization of water-soluble material, e.g. sugars (23), assimilates (5-7), and ions (8, 29). Difficulties encountered with the plastic infiltration of freeze-dried plant tissue have been largely overcome by utilizing pressure during infiltration (6).

**MATERIALS AND METHODS**

*Azolla Cultures.* *Azolla filiculoides* (Lamarck) was cultured in the phytontron of the Department of Agricultural Botany of the Hebrew University of Jerusalem, in diluted (1:40) Hoagland medium. Plants (20 g fresh weight) were transferred to containers with 10 ppm of either CuSO4, Cd(NO3)2, or UO2(NO3)2; in tap water and were collected as soon as toxic damages were visible (after 1, 2, or 4 d for copper, cadmium, and uranium, respectively). Toxicity was characterized by changes in frond pigmentation and loss of rigidity.

**Sample Processing.** Sample preparations were based on the method described in detail by Fritz (8), therefore only a brief description is given here.

Fronds were shock-frozen in isopentane at $-176^\circ$C, freezedried at $-45^\circ$C, and fixed with vapors of paraformaldehyd. The freeze-dried samples were infiltrated with plastic (Lowicryl HM 20, Chemische Werke, Lowi, Waldkraburg, FRG) under vacuum ($3 \times 10^{-3}$ mbar) and pressure (the pressure chamber, containing the tissue sample, was completely filled with the plastic-mixture, closed at 23°C, and warmed to 27°C). Sections of 1 μm thickness were cut with a dry glass knife on an ultramicrotome, mounted on folding grids, and coated with carbon. Nickel grids were used for copper-containing samples and copper-grids for control, cadmium- and uranium-containing samples.

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1 Supported by a research grant from the State of Niedersachsen, FRG.
X-ray Microanalysis. Samples were analyzed with a Phillips EM 420 electron microscope with EDAX 9100 energy dispersive system, at a voltage of 120 kV and an electron beam spot diameter of 200 to 250 nm. The element content in the analyzed areas was expressed in RCU\(^2\) (ratio between the specific emission intensity of the element above background and the background nonspecific emission intensity). As the measuring parameters (section thickness, beam emission current, spot diameter, tilt angle) were kept constant throughout, the RCU values may be compared between different locations in one section and in different sections.

Cross-sections of roots and shoots were viewed and divided into anatomical and functional regions. Six regions were selected in the root cross-section (Fig. 1, A and B): (a) outer cell walls of epidermis, (b) inner cell walls of epidermis, (c) tangential cell walls of parenchyma in cortex, (d) radial cell walls of parenchyma in cortex, (e) cells of vascular bundle, (f) cytoplasm in cells throughout the section. Shoot cross-sections were divided into seven regions (Fig. 2, A and B): (a) outer cell walls of epidermis, (b) cell walls of lower stem, (c) cell walls of upper stem, (d) cell walls of leaf, (e) cell walls of vascular bundle in stem, (f) cytoplasm throughout the section, (g) Anabaena in the leaf cavity. The element content in each region was determined from three to twelve measurements in different cells. Two specimens for each root and one specimen for each shoot were analyzed.

Data Analysis. The layout of the experiment for element content was completely randomized, with an unequal number of replicates. The data were statistically analyzed; the significance of the differences between the treatments of the three metals, tested against the control of each region, were tested by Duncan’s multiple range test ($P = 0.05$).

RESULTS

Distribution of Heavy Metals in Root and Shoot. The average copper content in roots of plants grown in the presence of 10 ppm copper was 19 RCU in outer and inner cell walls of epidermis and tangential and radial cell walls of parenchyma, while copper content in the bundle cells and the cytoplasm was lower (Fig. 3). The average content of cadmium in the root grown in the presence of 10 ppm cadmium was 2.8 RCU except for the outer cell wall of the epidermis, which was significantly higher (Fig. 3). The root of a plant grown in the presence of 10 ppm uranium contained an average of 6.7 RCU of uranium in outer and inner cells of epidermis and tangential and radial cells of parenchyma. The uranium content was low in the bundle cells and high in the cytoplasm (Fig. 3). In the latter, uranium was detected as clusters of little black dots containing 11.0 RCU uranium.

Copper content in copper-grown Azolla shoot was low, below 3 RCU, and no significant difference was detected between the areas of the cross sections (Fig. 4). The content of cadmium in the cadmium-grown Azolla shoot was very similar to the content observed in the root, approaching 2.5 RCU (Fig. 4). The analysis of the cadmium content in the shoot demonstrated one exceptional phenomenon: the appearance of dark grains, about 0.3 $\mu$m in diameter which were mainly located in the xylem cells of the bundle and in a few cells in the lower part of the stem (Fig. 5, A and B). The content of cadmium in these grains was extremely high, 18.2 RCU, and was accompanied by phosphorus and calcium at relatively high content as shown in Figure 6, A and B. High cadmium content was also observed in black oval bodies, about 0.2 $\mu$m length, which were located within the cell walls of most of the bundle cells, Figure 7, and contained high phosphorus and calcium. The black oval bodies were also observed at low frequency in other regions including the Anabaena cells.

No detectable uranium was observed in shoots of uranium-grown Azolla (Fig. 4). This demonstration of the immobility of uranium from the root to the shoot may explain the low toxicity of uranium to the Azolla plant (M Sela, E Fritz, A Huttermann, E Tel-Or, in preparation).

Amounts of copper and cadmium detected in Anabaena cells in the leaf cavity were similar to their content in other regions of the shoot. This indicates that the metals are mobile to the leaf cavity and that the cyanobacterial cells do not differ in their metal content from the Azolla cells (Fig. 4).

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\(^2\) Abbreviation: RCU, relative content units.

**Fig. 1.** Azolla root cross-section. (A) Microscopic photograph (bar = 30 $\mu$m); (B) schematic presentation. OCE = outer cell wall of epidermis, ICE = inner cell wall of epidermis, TCP = tangential cell wall of parenchyma in cortex, RCP = radial cell wall of parenchyma in cortex, BU = bundle cell, Cyt = cytoplasm throughout the section.
Effect of Heavy Metals on Content of Calcium, Potassium, Chloride, Phosphate, Magnesium, and Iron. The content of calcium in an untreated control root cross-section is low in all regions except for a high content in the cell wall of the epidermis (Fig. 8). Calcium content in roots of plants grown in the presence of uranium increased to a value of 10 to 14 RCU in all regions. The growth of Azolla in the presence of copper or cadmium did not affect significantly the content of calcium in the root, except for decreases in the calcium content in the outer cell wall of the epidermis.

The content of calcium in the shoots of plants grown in the presence of the uranium was 0.8 to 1.1 RCU in all regions, and in the presence of copper it was 2.5 to 3.0 RCU in all regions. Calcium content in the presence of cadmium was 2 to 3 RCU in all regions, except for the dark grains where calcium content was 5.6 RCU.

The content of potassium in the root cross-section was affected by all three treatments (Fig. 9). Azolla grown in the presence of uranium exhibited a decrease in potassium content in all regions of the root cross-section. Copper, too, caused a decrease in potassium content in all regions, except for the radial parenchyma cell walls, and cadmium caused a decrease in the content of potassium in the outer cell wall of the epidermis and the cytoplasm.

The potassium content in the shoots of the copper-grown Azolla was two- to threefold higher in all regions as compared to the control Azolla shoot. It seems that copper may enhance the translocation of potassium from the root to the shoot. The effect of uranium on potassium content in the shoot of Azolla was not significant, when compared to the control shoot. Hence, the decrease in potassium content of uranium-treated root could involve leakage to the medium, rather than movement of potassium to the shoot. Cadmium, too, had no effect on potassium content in the shoot.

The analysis of chloride in Azolla root (Fig. 10), demonstrated significant decreases in chloride content in the presence of uranium and copper and a moderate decrease in the presence of cadmium, the exception being an increase in chloride in bundle cells of cadmium treated plants. The content of chloride in the shoots was not significantly affected by any of the tested heavy metals. These results suggest that uranium and copper caused a leakage of chloride from the root to the medium.

A two- to three fold increase of phosphate content in roots of Azolla grown in the presence of cadmium was demonstrated in all regions, while copper and uranium had no effect (data not shown). The high content of phosphate observed in the large black grains in the shoot sample of the cadmium-grown fronds, shown earlier in Figure 6, suggests that the phosphate was complexed or precipitated with cadmium. However, phosphate content in the overall shoot specimen was not higher than in the control.

Roots of Azolla, grown in the presence of uranium, copper, and cadmium lost much of their magnesium content in all regions, and the most significant loss was observed in the cytoplasm (Fig. 11).

There was no effect of uranium and copper on the iron content in the root (data not shown). Roots grown in the presence of cadmium exhibited an increase of iron content in the cell wall of epidermis from 4 to 7 RCU, and three- to eightfold in the other regions. Sodium content in the copper-treated roots was decreased to half that of the control (from 1.0 to 0.5 RCU) and was not affected by uranium and cadmium.

Fig. 2. Azolla shoot cross section. (A) Microscopic photograph (bar = 30 μm); (B) schematic presentation. OCE = outer cell wall of epidermis, BU = bundle cell, Cyt = cytoplasm throughout the section.
Fig. 3. Distribution of heavy metals in Azolla roots. Values described by the same letter, within the regions for each metal treatment, are not significantly different by Duncan’s multiple range test at P = 0.05. Root regions are: 1, OCE; 2, ICE; 3, TCP; 4, RCP; 5, BU; 6, Cyt (key for abbreviations is in Fig. 1).

DISCUSSION

The studies reported in this communication provide evidence for the pattern of intracellular heavy metal localization of Azolla and for the mobility of heavy metals from the root to the shoot. High quantities of cadmium, copper, and uranium were localized within the cell wall moiety in the shoot and the root of Azolla. These observations agree with previous reports for rice (4) and Zea mays (13) where cadmium was primarily bound to the cell walls of the root. Copper was retained mainly in Agrostis cell wall fraction (26), and as much as 95% of the total content of uranium taken up by Chlorella was associated with the cell walls (10). On the other hand, as much as 70% of cadmium absorbed by beans was stored in the cytoplasmic fraction in roots and leaves (28), and in Becium leaves copper was mainly stored in the cytoplasm (21).

A low mobility rate of copper is suggested in view of its low content in the shoots compared to its content in the root. Similarly, copper was mainly restricted to the roots of Agrostis (30), where its content was 163 times higher than in the shoot. Roots of Triticum aestivum contained 53 times more copper than its shoots (24). In contrast, a major fraction of copper absorbed by Becium (20) was translocated from the roots to the shoots.

The appearance of high concentrations of cadmium in aggregates containing phosphate and calcium, especially in the bundle cell walls of Azolla, is demonstrated. Such deposition of cadmium may provide a possible means for its detoxification. Localization of cadmium in electron-dense deposits in endodermal cells of Zea mays roots was also reported (13). The involvement of calcium and phosphate in the precipitation of cadmium was suggested for tomato plants (27), where cadmium may be deposited alternatively as an extremely insoluble salt of cadmium phosphate, or incorporated to crystals of calcium oxalate, or interacted with deposits of calcium phosphate in the epidermal layers of tomato stem. An insoluble form of stored cadmium in electron-dense granules was demonstrated inside root parenchyma cells of Agrostis giganta and Zea mays (19).

In contrast to low mobility of cadmium reported for rice (4) and twenty other plant species (11), a relatively high mobility of cadmium was observed in Azolla. High mobility of cadmium was also reported for radish, where the tops contained 403 ppm compared to 174 ppm cadmium in the roots (14).

A number of heavy metal binding groups were suggested to play a major role in plant cells: polyuronic acid in mosses (25), pectic acid in Agrostis (18), polyphosphates in Plectonema (12), and phytochelatins in higher plants (9). Our studies have provided evidence for the involvement of phosphate or calcium phosphate granules as a specific means for cadmium binding.

The fixation procedure of the Azolla tissue employed in these studies immobilized the soluble monovalent ions, such as potassium, sodium, and chloride and the less soluble bivalent ions, calcium, magnesium, and iron. We have therefore analyzed the content of these ions, as affected by the heavy metal treatment, to search for possible mechanisms of heavy metal damage and toxicity in Azolla.

The influence of uranium on the content of essential elements was characterized by increasing calcium and decreasing magnesium in Azolla root. Uranyl ions have been shown to compete with magnesium and calcium on their binding sites in the lichen Cladonia (2). The loss of magnesium from Azolla root treated
FIG. 5. Cadmium-containing grains in xylem cells of the shoot bundle at low (A) and high (B) magnification (bar in (A) = 3 μm and in (B) = 0.7 μm).

FIG. 6. EDAX spectrum obtained from (A) xylem cell wall of shoot bundle of control Azolla; (B) dark grains described in Figure 5. Note that the spectrum of K in (B) is overlapping with the second peak of cadmium, and the real content of potassium is very low. Calcium is absent in (A) and appears only in (B); the overlapping peak to calcium in (A) is contributed by potassium.

FIG. 7. Cadmium-containing oval bodies in cell walls of bundle cells of shoot (bar = 0.4 μm).

with uranium could be explained by competition between the two cations. The losses of magnesium from cadmium-treated Azolla roots were similar to those reported for Plectonema treated with cadmium (12).

Copper was found to cause a decrease in potassium content in Azolla roots, similar to earlier reports on the leakage of potassium induced by copper in Agrostis roots (30) and Chlorella (15). The decrease in magnesium content observed in Azolla roots treated...
FIG. 8. Effect of cadmium, copper and uranium on calcium in Azolla roots. (—) = information not available; (*) = significantly different from control sample values, by Duncan’s multiple range test at P = 0.05. Numbers correspond to regions as in Figure 3.

FIG. 9. Effect of cadmium, copper, and uranium on the distribution of potassium in Azolla roots. Legend as in Figure 8.

FIG. 10. Effect of cadmium, copper, and uranium on the distribution of chloride in Azolla roots. Legend as in Figure 8.

FIG. 11. Effect of cadmium, copper, and uranium on the distribution of magnesium in Azolla roots. Legend as in Figure 8.

with copper agrees with that in wheat roots (24) and Plectonema (12).

Acknowledgments—We are most grateful to Dr. J. Dainty for improving the quality of this manuscript and to Mrs. N. Ben-Yeheskel for editorial assistance.

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