Differences in X-Ray Absorption Due to Cadmium Treatment in *Saponaria officinalis* Leaves

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KEY WORDS microradiography; leaf details; cadmium; phytoremediation

ABSTRACT A method for detecting cadmium uptake in leaves of *Saponaria officinalis* doped with a solution of cadmium acetate is described. The technique based on the exposure of dried leaves to X-rays of a wavelength close to that of the metal K-edge could be useful for phytoremediation studies as it could reveal the bioaccumulation in plants due to the treatment either in vivo or in vitro with heavy metals. X-ray microradiography measurements are in agreement with those from peroxidase enzyme assay utilized to follow the oxidative damage induced by heavy metals. At present, as we will see in this report, microradiography has still poorer sensitivity in comparison with enzyme assay, but it has the advantage of being faster, not destructive, and usable even at very high doping levels, where the enzyme assay technique results are fully saturated. Further analysis of the optical density values could lead to a quantitative measurement of the heavy metal in the sample. Thus, the technology developed in this article could be useful for tracing the intake in phytoremediation studies. *Microsc. Res. Tech. 64:21–29, 2004.*

INTRODUCTION

Soft X-rays produced by laser plasma sources have been widely used for imaging of biological samples, and various techniques have been developed for the X-ray image reconstruction. Of particular relevance is soft-X-ray contact microscopy of living cells using X-ray absorption in the water window (λ = 2.2–4.3 nm) and microradiography of leaves or small animals using energies of around hv = 1–1.5 KeV or a wavelength range of 0.8–1.2 nm (Albertano et al., 1997a,b; Bollanti et al., 1995, 1996, 1998; Conti et al., 1997; Cotton et al., 1992, 1995; Fletcher et al., 1992; Ford et al., 1991; Panessa et al., 1981; Panessa-Warren et al., 1989, 1991; Stead et al., 1988, 1992; Schneider et al., 1995).

The production of special zone plate lenses allows the setting up of projection microscopy where X-rays passing through a small size biological sample (typically 20 × 20 μm^2) are projected on a CCD detector to provide an image of magnification around ×1,000 and a spatial resolution as high as 30 nm (Medenwaldt et al., 1998).

Moreover, by the use of spherical bent mica or quartz crystals (Flora et al., 2001; Pikuz et al., 2001), it is also possible to obtain projection micro-radiographies of larger biological samples (typically a few mm) with a resolution limit of some microns and with the possibility of selecting a specific wavelength using the crystal as a monochromator.

In the past ten years, numerous scientific reports on the use of these and other techniques have highlighted the importance of these new methods (Thieme et al., 2003; Wang et al., 2000; Weiss et al., 2000).

X-ray imaging is a useful tool in biology because the penetration depth of X-rays inside the sample can provide more structural information than is possible with conventional techniques like optical microscopy and electron microscopy. In fact, the short wavelength of X-rays can improve the resolution limits relative to optical microscopy to around 30 nm, while still allowing whole living cells to be imaged. This is not possible using electron microscopy.

Moreover, with X-rays it is possible to perform measurements of trace elements (as Ca,Fe,Ni,Cd), and accumulation site mapping in a variety of biological samples by different techniques such as X-ray absorption, phase contrast imaging (Cloetens et al., 2001), X-ray fluorescence microprobes (Sutton et al., 1995), and fluorescence microtomography (Schroer et al., 2002). The detection limits of the element of interest can provide semi-quantitative estimates of concentration down to a few pg/cm^2 (Penner-Hahn and Pearlso, 2000) with submicron spatial resolution in relatively large (mm^2) fields of view.

This property is very useful for sample imaging when it is necessary to localize a particular element either

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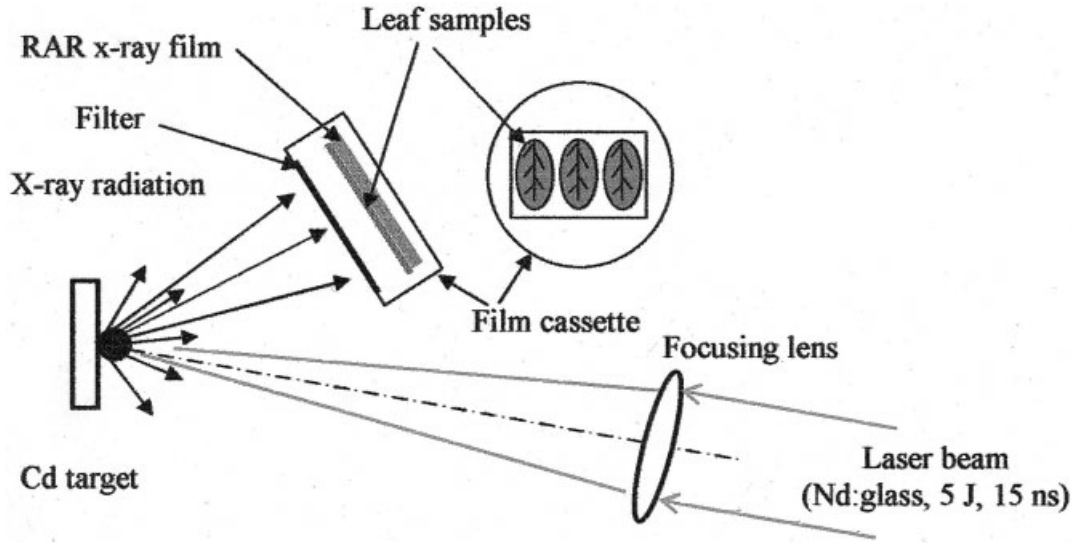

Fig. 1. Experimental apparatus to obtain microradiographies from leaf samples treated with different concentrations of cadmium.

already present in the sample structure or even artificially absorbed by the sample by a procedure of doping. In this way, it is possible to highlight inside the sample the biological structures where a particular chemical element or substance is present.

In the present study, leaves have been doped with cadmium and imaged with 1 KeV soft-X-rays, with the aim of measuring the level of cadmium doping inside the leaves based on X-ray opacity of the leaves in non-monochromatic X-ray microradiography. This method is compared with the peroxidase enzyme activity standard test. In fact, cadmium ions can inhibit and sometimes stimulate the activity of several anti-oxidative enzymes (Sanità di Toppi L and Gabrielli R, 1999). Peroxidase enzyme is normally produced by plants to protect them against the oxidative damage induced by doping with heavy metals. Quantitative measurement of enzyme concentration in the leaves thus gives information on the dopant concentration (Sanità Di Toppi, 2000).

The defence mechanism of plants includes the production of active oxygen species and peroxidase is a key enzyme involved in this metabolic pattern to protect against the free radical oxidative damaging action (Navarri-Izzo and Quartacci, 2001; Salin, 1998; Sanità di Toppi, 1999, 2000; Scebba et al., 2001). In fact, it allows detection of the stress-induced alterations even if genotoxic symptoms are not visible.

As mentioned above, an increase of the peroxidase activity is usually explained as a plant defense against the oxidative processes, as well as the scavenging role of H_2O_2 and organic peroxides. Peroxidase reduces peroxide compounds, thus avoiding their damaging action throughout the cell. In the reaction, catalyzed by this enzyme, an electron donor is also involved, which is oxidized at the end of the process. The reaction phases are as follows: first, the peroxidase oxidation from H_2O_2 produces the enzyme oxidized form, and, second, the electron donor is oxidized releasing the native form of the enzyme.

On the basis of this process, it is possible to dose the peroxidase activity, choosing the appropriate electron donor and pH for the reaction (Pichorner et al., 1993).

MATERIALS AND METHODS

Potted *Saponaria officinalis* plants were grown for two months in the open air. Each plant was treated for 3 or 10 days with different concentrations of cadmium acetate: 0.1, 2, 5, and 15 mM.

Untreated control plants grown under the same conditions were maintained with pure water instead of cadmium acetate. Both treated and control samples were dehydrated by the same procedure and length of time. They were mounted together on a sample holder with the same polypropylene filter, and, to avoid errors due to fluctuations in intensity of the shots, treated and untreated samples were exposed simultaneously. From each plant, untreated and treated sample leaves were cut off for enzyme assay of the protein peroxidase (Scebba et al., 2001) and others were dried for the exposure to X-rays.

Leaf samples of similar morphology were chosen. No matter what the doping was, the leaves had the same mass per unit of surface within a few percent (3.8 mg/cm^2 in our specific case) after being dehydrated as explained in the following section).

Peroxidase Enzyme Assay

Leaves (1 gr) were homogenized and diluted (1:5) with a buffer NaH_2PO_4, 50 mM, at pH 6. The homogenate was centrifuged at 13,000 rpm at 4°C for 15 minutes, and the supernatant was collected and immediately used for the estimation of the peroxidase soluble form activity. The presence of the peroxidase enzyme was detected spectrophotometrically (UV/visible spectrophotometer Varian CARY 50 SCAN) by monitoring the increase in absorbance A_{430} with pyrogallol (20 μl) and hydrogen peroxide (10 μl) as substrates. Then, buffer was added to give a final volume of 1 ml. Specific activity was calculated and expressed

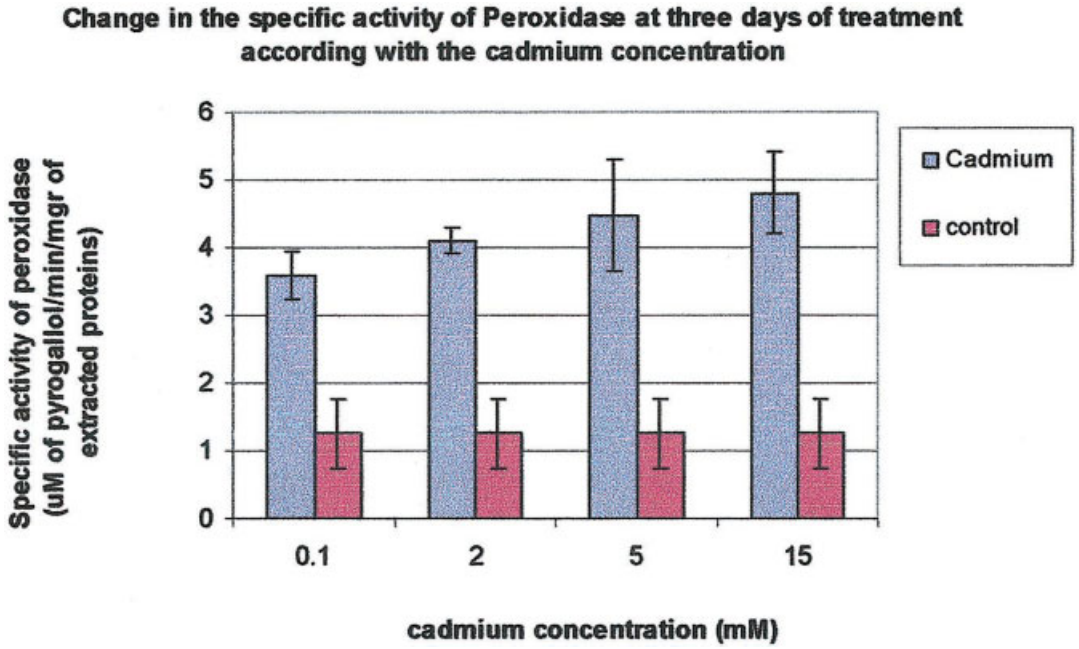

Fig. 2. Change of the peroxidase activity in leaves after 3 days treatment with cadmium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

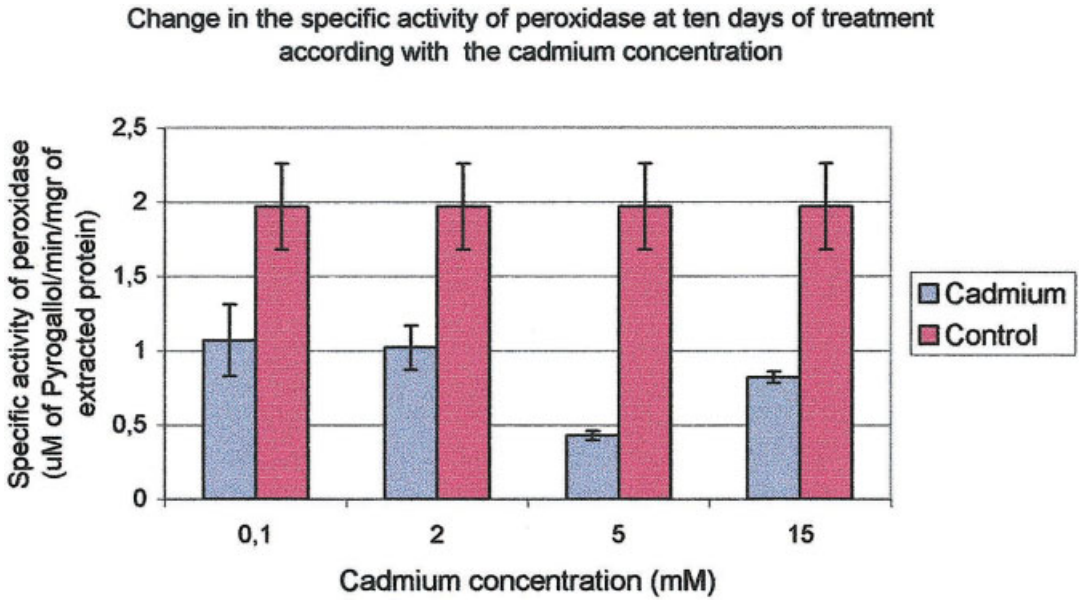

Fig. 3. Change of the peroxidase activity in leaves after ten days treatment with cadmium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

as μmol of pyrogallol/min/mg extracted proteins. Protein concentration was determined using a Protein Assay system (Bio-Rad, Milano, Italy).

X-Ray Microradiography Method

The leaves were exposed to soft X-rays produced by a laser plasma source with a cadmium target in a vacuum chamber (Albertano et al., 1997a,b; Bollanti et al., 1995, 1996, 1998; Cotton et al., 1992, 1995; Conti et al., 1997; Fletcher et al., 1992; Ford et al., 1991).

With this target, a soft X-ray-emission in a range around 1–1.5 KeV is produced. This radiation in the 0.8–1.2-nm range of wavelength includes the K-edge of absorption of cadmium itself and hence it is mainly absorbed by the sites, inside the sample structure, where the intake of cadmium occurred. In order to dry them out, the leaves were put in an oven. In fact, at an energy of around 1 KeV, the water content can absorb the X-ray radiation preventing useful images from being obtained.

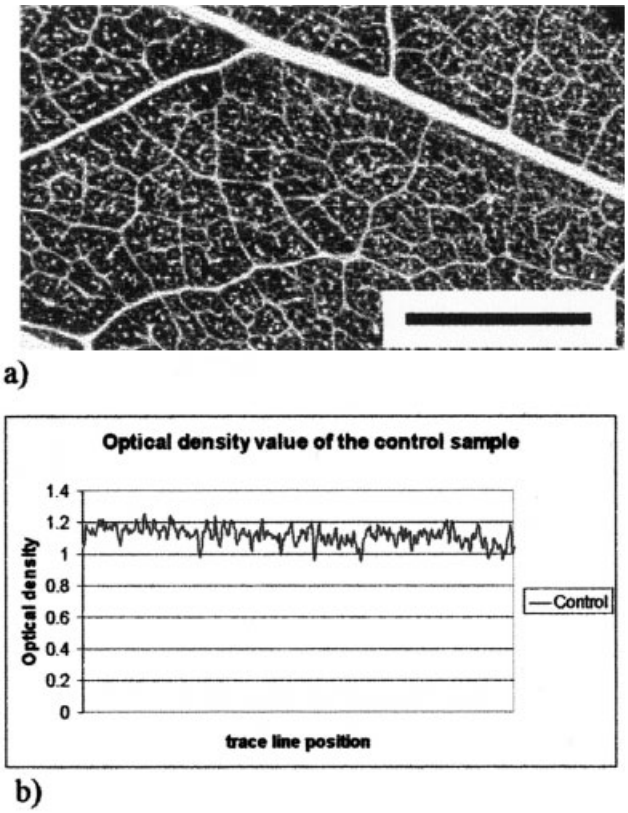

Fig. 4. **a:** Small region of the control sample; 4,000 dpi scanning resolution. **b:** Corresponding optical density profiles (not including the larger vein visible in the figure). Scale bar = 1 mm.

Leaves, treated and untreated, were positioned in the sample holder and exposed to X-rays at the same time for a total of four shots of the laser plasma source at a distance of 15 cm from the source. After the exposure, the X-ray photosensitive Kodak film RAR 2492 was analyzed with a scanner at 4,000 dpi resolution (CanoScan FS4000US) to obtain a high-resolution X-ray image of the leaf. The greyscale levels of the digitized images were then converted into different optical density values by using the scanner's calibration curve (obtained by means of calibrated neutral filters). Thus, the final profile of the image details of each sample was converted to a profile of optical density.

Experimental Apparatus

The experimental apparatus used for the X-ray analyses is shown in detail in Figure1: the laser energy was around 5 Joule/shot, the pulse duration was 15 ns, and the estimated X-ray fluence on the sample, placed at 15 cm from the source, was around 30 μJ/cm/shot on 2 π steradiane in the 1–1.5 KeV spectral range. The experimental system consisted of a laser Nd:YAG, whose beam was focused by a lens onto a cadmium target that emitted the soft X-rays.

This physical process normally occurs in a vacuum chamber allowing the soft X-rays propagation, although in some studies helium is used at a pressure up to one atmosphere (Albertano et al.,1997b; Bollanti et al.,1998).

The diameter Ø of an X-ray source is around 300 μm. This is a rather large value compared with the sizes of typical laser plasma sources, due to the small numerical aperture (NA ~0.03 μm) of the focusing lens. The distance from the plasma source and the holder is d = 15 cm.

The relatively large size of the source could give some problems of penumbral blurring. For this reason, the sample is placed as close as possible to the film (at ~1 mm). The film RAR 2492 has a small grain size and low sensitivity, and the visible light was removed with a 2-μm polypropylene filter coated with 0.4-μm aluminum to give a total thickness of 2.4 μm . This laser equipment has been designed and manufactured by Quantel and it is located at the Rome Tor Vergata University, STFE (Scienze e Tecnologie Fisiche ed Energetiche) Department, INFM-Tor Vergata research unit (Bellucci et al., 2000; Petrocelli et al., 1993a,b).

RESULTS AND DISCUSSION

First, the variation in peroxidase activity in treated leaves was investigated. This enzyme is the main nonspecific marker of the negative effect of environmental pollutants or other stresses (Navarri-Izzo and Quartacci, 2001).

Figure 2 shows the peroxidase activity changes after 3 days of treatment with different cadmium concentrations. Specific activity mean values are significantly different ($P < 0.01$, Student's t-test) compared with the control. On the basis of our observations, *Saponaria officinalis* plants survive up to 3 days of treatment with high doses of contaminant.

The concentrations listed in the Materials and Methods were selected in order to follow current studies on peroxidase assay. These values of contaminant dose appear to be rather high, but in reality the concentration of Cd in the leaves reached after some days is much lower than the value of concentration in the solution at the root level. Usually, concentration values in the order of μM are used (Sanità di Toppi and Gabrielli, 1999). Here a higher dose in the mM range has been used, to specifically test whether these plants could tolerate and hyperaccumulate contaminants up to a dose that could occur in an environmental disaster. In such cases, the plants could be exposed to even higher doses.

For a 10-day treatment (Fig. 3), a decrease in peroxidase activity and a general flattening of the values are observed in comparison with the control. (Specific activity mean values were significantly different, $P < 0.01$, Student's t-test.) This means that the oxidative stress is so large that the negative effects of Cd could not be avoided by the cellular recovery system (i.e., for longer doping treatment, plants die and no longer are of interest for our research).

Next, the micro-radiographs of the leaves were scanned at 4,000-dpi resolving power. Small regions were selected along the length of the leaf image, with the same average size for all samples. The optical density value on the film and also the degree of homogeneity were measured for each region.

The microradiographs were evaluated quantitatively by measuring the optical density (O.D.). To obtain

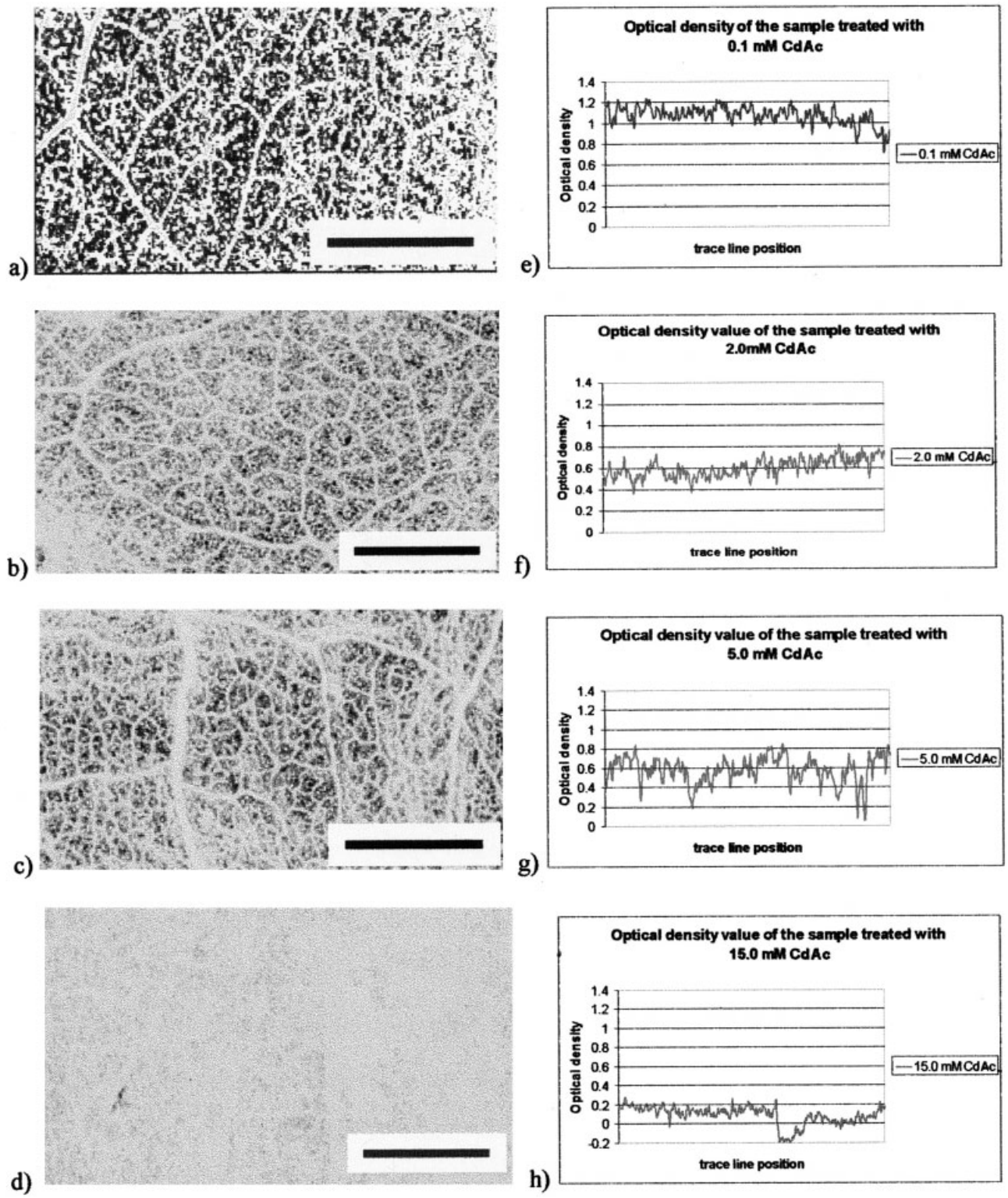

Fig. 5. **a–d:** Details of the 0.10-, 2.0-, 5.0-, 15-mM treated samples; 4,000-dpi scanning resolution. **e–h:** Corresponding optical density profiles. Scale bars = 1 mm.

TABLE 1. Comparison of different optical density average values and degree of homogeneity for treated and control samples

	Control	0.1 mM	2.0 mM	5.0 mM	15 mM
Optical density average value	1.12	1.07	0.60	0.58	0.089
Degree of homogeneity	Low	Low	Average	High	Highest

these data, it is necessary to measure the grey-scale level in the image and then to evaluate the O.D. using the scanner's calibration curve.

In Figure 4, a small region of the untreated leaf is shown, together with the optical density value of the whole area of the region itself (each point of the trace of Fig. 4b is obtained by averaging the OD(x,y) of Fig. 4a along the vertical coordinate on the same horizontal coordinate). From these data, the average optical density of the untreated leaf is 1.12. Differences in absorption between the veins and the background in the micro-radiograph of the leaf are evident. The white area in the veins and the spots among secondary veins distributed on the leaf area are due to increased thickness.

Figure 5a–d shows the details of the leaves treated for 3 days with 0.1, 2.0, 5.0, and 15 mM cadmium acetate solution, respectively. The corresponding optical density profiles of the whole area of each detail (as explained for Fig. 4b) are also shown in Figure 5e–h, and the average optical density values are summarized in Table 1 together with the degree of homogeneity for the different treatment concentrations. Clearly, the higher the treatment concentration, the higher is the leaf opacity and hence the lower is the film optical density.

As already mentioned (Materials and Methods), the differences in average opacity of the samples and in the corresponding optical density of the microradiographic films cannot be caused by differences in leaf mass per area, and so are significantly determined by the metal content.

In the sequence of the X-ray microradiographic images of Figure 5, a significant change in the internal details in relation to the cadmium concentration must be pointed out, showing a dose-effect. For a 0.1-mM treatment (even if a slight increase in absorption still exists), the sample, like the control, does not show relevant absorption (Figs. 4 and 5a). On the other hand, the images corresponding to the higher dose treatments (2.0 and 5.0 mM) show an increase in average and in distribution of absorption due to the internal structure (Fig. 5b,c). In the image corresponding to the highest treatment concentration (Fig. 5d), it is not possible to recognize any leaf structure because of the high density of the absorbing areas.

These changes in leaf image morphology obtained for different treatment concentrations are probably due to the changes of opacity of the leaf structures. Plants use phytochelatin molecules to chelate heavy metals that accumulate in vacuoles (Salt et al., 1998). Other possible bioaccumulating sites are the cell wall (Nishizono et al., 1998; Sanità di Toppi, 1999; Salt et al., 1998), and the apoplast compartment.

As soon as cadmium enters the plant, it reaches the xylem through an apoplastic and/or a symplastic pathway (Salt and Rauser, 1995), and then is complexed by ligands such as organic acids or possibly by phytochelatin (Cataldo et al., 1988; Salt et al., 1995). These mechanisms for cadmium uptake operate mainly at the root level although there can be a smaller uptake into the shoots (Cataldo et al., 1983; Sanità di Toppi and Gabrielli, 1999). Either phytochelatin synthesis and vacuolar compartimentalization or peroxidase production occur as a defence process activated by the plants against the damage induced by cadmium: a fan-shaped-response (Sanità di Toppi and Gabrielli, 1999).

There is a possible overlapping effect of the closely spaced veins in the leaf which produces a uniform background in the absorption signal. This could be due to the higher concentration of cadmium that is present. The effect shows a variation according to the dose of cadmium that permeates the leaves. Previous studies refer to the possibility that contaminant intake occurs mainly in veins, e.g., in *Thlaspi arvense* (Vazquez et al., 1992), which could account for the possible overlapping effect of the closely spaced veins. Different plants could bio-accumulate into different structures in the leaf, as already discussed for *Thlaspi coerulescens* (Vazquez et al., 1992).

Looking at the average value of optical density in Figure 6, one sees a non-linear dependence of the O.D. decrease with respect to cadmium concentration. There might be a threshold concentration above which the absorption signal is different from control. In fact, the difference in O.D. between the smallest treatment concentration, 0.1 mM, and the control seems to be the least significant among all the other values

The results show that it is possible to detect the uptake of cadmium into the leaf of *Saponaria officinalis*. In principle, it would be possible to detect in the X-ray microradiographs the uptake of any heavy metal in the leaf, at least when high levels of doping are reached as in this study.

The changing degree of homogeneity in the leaf microradiographs is a further effect of the doping as shown in Figure 7.

Figure 7 differs from Figures 5 or 6: a single line profile is shown, without integration along the vertical direction, and the grey levels are not converted to optical densities. As is clearly visible, the degree of homogeneity increases with increasing Cd concentration. This means that as much as the absorption in the leaves is high and the optical density is low, the degree of homogeneity increases. In fact, for the control and the lowest concentration (0.1 mM) of Figure 7, the profile shows sharp peaks, whereas for the 2.0- and 5.0-mM treatment, the degree of homogeneity increases, and at the highest point of concentration (15 mM), the signal is really uniform, almost a flat horizontal line.

On the other hand, if the peroxidase values for each treatment concentration are compared with the control (Fig. 2), even at the lowest concentration, 0.1 mM, the peroxidase test shows a significant difference from the control ($P < 0.01$). This effect clearly shown by the peroxidase test could be due to the plant's ability even at the lowest treatment, 0.1 mM, to react against the oxidative damage caused by heavy metals, whereas this is not evident with the microradiography method.

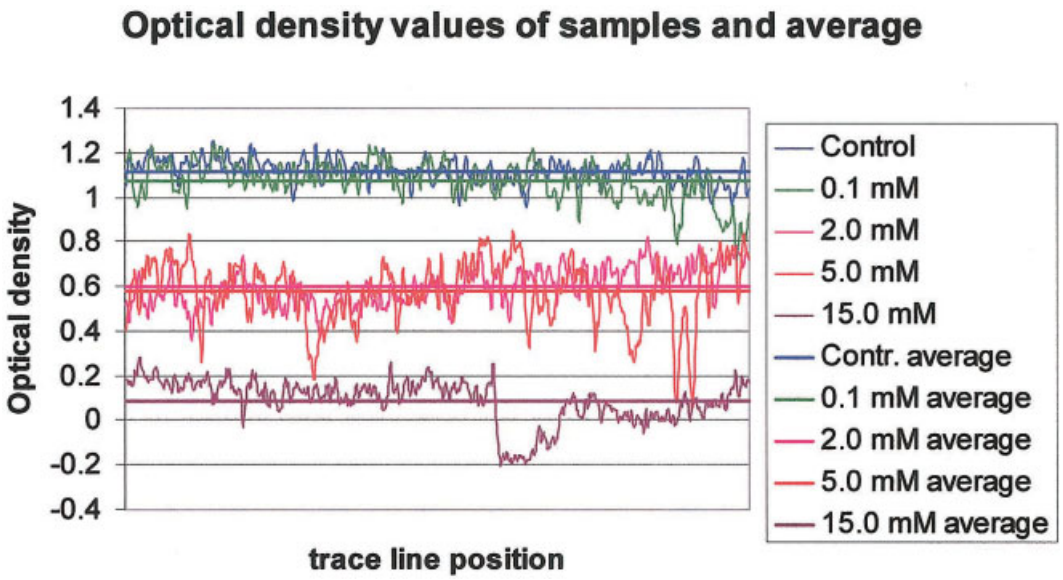

Fig. 6. Profiles of the optical density values of the control and treated samples and average value for each sample; note how optical density increases as the treatment concentration increases. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

This means the above method, still under development, provides a faster detecting procedure for the accumulation of a heavy metal in a sample, but with less sensitivity. However, the radiography method is not destructive and enables the sample to almost remain in its natural state.

In our opinion, this poor sensitivity of the method here presented is due to the wide band width of the radiation used. A much higher sensitivity and a quantitative measure of heavy metal in the sample are expected by using a monochromatic radiation. In this way, it could be possible to directly detect the amount of metal up to pg/cm^2 as already pointed out in the Introduction (Penner-Hahn and Peariso, 2000). An appropriate source of monochromatic radiation is the synchrotron radiation source of X-rays, which allows us to select a wavelength value very close to the edges of a particular element, which could be a possible way to obtain elemental distribution maps of plant leaves. In fact, this non-destructive method can detect and pinpoint the distribution of heavy metals in plant systems (Toaspern et al., 2000). Using monochromatic radiation could possibly perform a dual energy analysis that could improve the reliability of the technique. This method, when based on the radiation having a photon energy higher than 1 KeV and a corresponding penetration length in the atmosphere larger than a few millimeters, could perhaps be used to image live leaves.

On the other hand, as mentioned before, the microradiography with a monochromatic radiation or with a broad band radiation (as used here) allows the determining of the dopant amount even at high concentration values, while peroxidase is a sensitive method only for low concentration and then it saturates. The comparison between the two methods is reported in Figure 8 where the experimental values of the peroxidase and of the optical density already seen in Figures 2, 4, 5 are normalized to the maximum. The empirical best fit functions are shown as well.

Conclusions

We have shown that X-ray microradiography at energy of 1 KeV provides a possible method to detect the uptake of heavy metals by plants. We have compared it with the much utilized test of peroxidase activity. A good correspondence between the microradiographic method and the peroxidase enzyme assay was found, even though microradiography could only be applied to higher concentrations.

In the microradiography analyses, there were decreases in the optical density values with increasing dopant concentrations.The optical density value was measured calibrating the greyscale from the small scanned regions of the sample and then converting the greyscale levels into optical density. Results showed that the uptake of cadmium in the leaves increased with increasing Cd treatment concentration allowing a clear detection of heavy metal uptake. It is reasonable that the technique could be applied for phytoremediation studies.

This heavy metal uptake analysis could be done on plants other than the *Saponaria officinalis* as well as with heavy metals other than cadmium, provided that concentration exceeds the sensitivity threshold and could be tolerated by the specific plant being studied. It should, therefore, be possible to find the best plant for bioaccumulation of specific heavy metals, to identify the accumulation sites, and to evaluate the average concentration of heavy metal uptake.This technique could improve our understanding of the biological mechanisms of hyper-accumulation and may help in the development of superior plants for the phytoremediation of metals. The technique could be important for

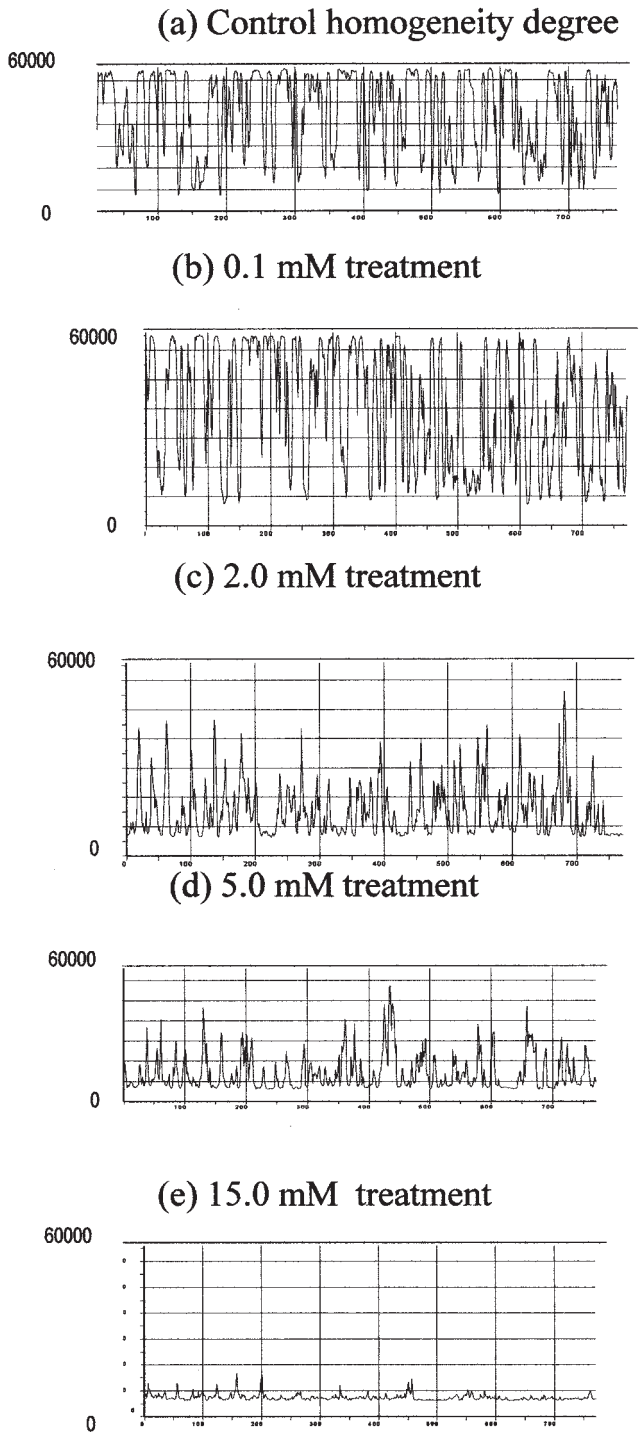

Fig. 7. Signal profile showing the degree of homogeneity of each sample; note how the homogeneity increases as the treatment concentration increases.

detecting heavy metals in a plant for the phytoremediation process, even at an early stage of the process.

Further developments of the technique could allow quantitative measurement of heavy metal in dried or wet leaf samples. Different structures could be analyzed even down to the single cell level by imaging in the water window wavelength region. Microradiography also has the great advantage of being non-destructive. Further development could also give a three-dimensional reconstruction of samples (tomography) by X-ray exposure at different tilt angles.

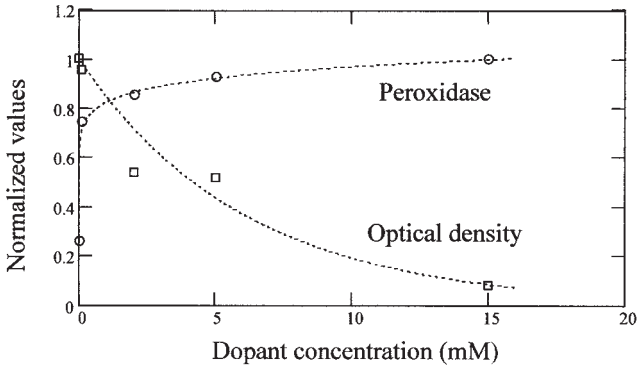

Fig. 8. Peroxidase (circles) and optical density (squares) values (normalized to their maximum) vs. the dopant concentration (C) of cadmium. The corresponding empirical best-fit functions, which are $pero_0 + 0.6 \cdot \sqrt[10]{C/2}$ (where $pero_0$ is the peroxides of control leaves) and $e^{-C/6}$, respectively (with C in mM units), are also reported (dashed lines).

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