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The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the waterlily (Nymphaeaceae)

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Abstract. Co-localization of polyphenols and peroxidase activity was demonstrated in epidermal glands of the waterlily (*Nymphaea*) by histochemistry. Total phenols, tannins and peroxidase activity were determined quantitatively in plant extracts. Polyphenols were partially identified and were found to consist mainly of hydrolyzable tannins, gallic and tannic acid derivatives. *Nymphaea* polyphenols were shown to chelate Cr, Hg, and Pb in vitro, and Cd-binding by polymerized polyphenols was demonstrated in leaves exposed to Cd in vivo. Both polyphenols and peroxidases were found at very high constitutive levels, which were not induced or altered by external conditions, such as light and heavy-metal stress. It is suggested that the polymerization of polyphenols by peroxidases, enhanced after heavy-metal uptake and detoxification, is responsible for the binding of heavy metals in *Nymphaea* epidermal glands.

Key words: Cadmium accumulation – Glandular trichome – Heavy metal – Nymphaea (Cd accumulation) – Peroxidase – Polyphenol

Introduction

Recently we have shown that epidermal glands on the abaxial side of the leaf lamina, and in the petiole and rhizome epidermis of *Nymphaea* plants, accumulate heavy metals such as Cd, Pb, Hg, Mn and Fe. The *Nymphaea* plant can accumulate substantial quantities of heavy metals with no observed toxicity to the plant (Lavid et al. 2000).

As early as 1905, Conard described the high levels of tannins (polyphenols that precipitate proteins) in all parts of waterlilies. These tannins were used for fabric dyeing and leather tanning (Swindells 1983). A few hydrolyzable tannins and anthocyanins from waterlilies have been described (Strack et al. 1992; Saeed and Hamdy 1996; Vergeer et al. 1997; Fossen and Andersen 1997), some of which demonstrate antibacterial activity (Nishizawa et al. 1990; Kurihara et al. 1993).

Polyphenols, especially tannins, are well known for their ability to chelate heavy metals such as Fe, and recently they have received much attention as potential antioxidants (Rice-Evans et al. 1996). These phenomena have been studied in detail in cattle diets, where tannins, present in forage feeding, are suggested to inhibit Fe uptake (Terrill et al. 1994). Plant material rich in tannins has also been used as a filter for water purification (Matsuo et al. 1995; Nakashima et al. 1996; Gloaguen and Morvan 1997). Tea and wines, among other foods rich in tannins, are recommended as natural antioxidants in the human diet (Fuhrman et al. 1995; Cook and Samman 1996; Rice-Evans and Miller 1996). Thus, interactions of polyphenols with heavy metals can involve chelation, antioxidative activity against active oxygen species caused by heavy metals (Halliwell and Gutteridge 1989), or both. However, only a few reports describe the action of tannins in the growing plant on heavy-metal chelation or protection against active oxygen species caused by heavy-metal stress. Aoba (1986) suggested that tannin-rich plants such as tea, which are tolerant to excess Mn, are protected by the direct chelation of Mn by tannins and that the typical brown spots on leaves exposed to excess Mn are actually caused by tannin oxidation.

An important aspect of phenols in plants is their role as lignin precursors, via their polymerization under natural lignification processes (Strack et al. 1989). Phenol polymerization is also involved in plant-pathogen interactions, where extracellular peroxidases in the parasite react with phenolic compounds that are lignin precursors of the host, resulting in host resistance due to the formation of lignin layers (Antonova and

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TerBorg 1996). In other cases, induction of plant peroxidases by pathogens may be responsible for the final steps in the oxidative coupling of phenolic monomers to form cell wall polymers (Alcázar et al. 1995).

Many vascular plants bear trichomes that show a highly specialized morphology, and that also occur as chemically distinct types in that they store a variety of substances at high concentrations (Beckman et al. 1972). These authors have suggested that phenol-containing trichomes might be specialized organs that sense injury and elicit a protective response. Following biosynthesis, the phenols are apparently stored in specialized cellular compartments, where they are kept in a reduced state until some disturbance (injury or infection) occurs. The phenols then diffuse from these storage compartments and become generally distributed throughout the storage cells and adjacent cells, where they are oxidized and polymerized to form coloured products.

A well-studied example is the glandular trichome on the leaf epidermis of many *Lycopersicon* and *Solanum* species. These glandular trichomes have been shown to contain high phenol levels, as well as polyphenol oxidase and peroxidase activities. This combination enables rapid conversion of the clear, viscous trichome exudate into a hard, brown substance following pathogen attack. Polymerization and hardening of this exudate traps small insects (Kowalski et al. 1992; Yu et al. 1992; Thipyapong et al. 1997). The enzymes involved in these polymerization processes are phenol oxidases such as laccase, polyphenol oxidase and peroxidase, known to be involved in the polymerization and de-polymerization of phenols in plants. The process of natural lignification has yet to be elucidated (Bao et al. 1993; Sterjiades et al. 1993; Münzenberger et al. 1997; Richardson et al. 1997). It is not clear to which stage this is an enzymatic or non-enzymatic process, and whether the same enzymes and mechanisms are responsible for natural lignification and phenol polymerization under pathogen attack. Peroxidases have been shown to react with flavonoids in a coupled H_2O_2 detoxification mechanism (Yamasaki et al. 1997). They are involved in lignification (Polle et al. 1994; Schopfer 1996), and in the metabolism of plant phenolics in general (Takahama 1989). High peroxidase activities have been found in aquatic macrophytes, including members of the Nymphaeaceae (Roy et al. 1992), related to their tolerance to water pollution caused chiefly by pulp and paper-mill effluents. In *Arabidopsis thaliana*, the responses to lead toxicity and to bacterial infection are similar and include an increase in peroxidase activity together with the accumulation of polyphenol deposits (Lummerzheim et al. 1995). The latter report is one of a very few dealing with possible interactions of polyphenol polymerization by peroxidase and heavy-metal tolerance in plants.

The objective of the present study was to investigate the possible involvement of polyphenols and peroxidases in the heavy-metal accumulation and detoxification mechanisms in *Nymphaea* epidermal glands.

Materials and methods

Plant material and growth conditions. Plant material and growth conditions were as described previously (Lavid et al. 2000). Three well-developed, mature plants were treated with 100 mg/l Cd for 5 d and their Cd content, phenol content and peroxidase activity were determined. These conditions were chosen after three concentrations of Cd (20 mg/l, 50 mg/l, and 100 mg/l) given over 1–7 d failed to bring about any change in phenol or peroxidase contents. Dark treatments were conducted on plants covered with aluminium foil.

Sampling for quantification experiments. Samples were prepared for quantification experiments as described previously (Lavid et al. 2000). Samples were immediately frozen in liquid nitrogen, ground with a mortar and pestle and lyophilized for 48 h. They were then stored at −70 °C until analysis.

Metal analysis. Metal content in plant samples was determined on freeze-dried material as described by Lavid et al. (2000).

Phenol analysis: phenol extraction. Freeze-dried samples (200–500 mg) were successively extracted by stirring with 20 ml methanol for 5 h four or five times at ambient temperature, until the phenols no longer exhibited a positive reaction in the supernatant (see further on). After filtering through Whatman No. 1 paper, phenols and tannins were quantitated in each extract separately. The determinations of all the extracts from each tissue were summed to calculate the total phenol content. To dissolve condensed tannins, parallel samples were extracted four or five times with methanol containing 1% (v/v) concentrated HCl. Chlorophyll present in the laminae and petioles was removed by several extractions with hexane (2× volume), until no green colour, which can interfere with phenol and tannin determinations, was observed. Extracts of methanol and acid-methanol treatments were used for further analysis of phenols and tannins according to Price and Butler (1977).

Assays for total phenols. The objective here was to quantify total polyphenol content in waterlily, to localize the polyphenols, and to provide an initial characterization. Phenol content of the extracts was determined by a modification of the Prussian blue assay of Price and Butler (1977). Plant extracts (50 µl) were diluted with 3 ml of H_2O. Then 100 µl of 50 mM $FeCl_3$ in 0.1 N HCl plus 100 µl of 8 mM $K_3Fe(CN)_6$ were added to the samples, which were incubated for 20 min at ambient temperature. Absorbance at 720 nm was determined (Uvicon 810 spectrophotometer) against the reagent mixture with methanol instead of plant extract. Gallic acid (Sigma) was used as a standard.

The Folin-Denis, Folin-Ciocaultou, and violet-complex tests (Hagerman and Butler 1989) were also used for total phenol determination. However, results for the total phenol analysis are given for the Prussian blue assay only, because it is less susceptible than the Folin assays to interference by proteins (Hagerman and Butler 1989).

Tannin determination by protein precipitation. The quantitative tannin assay is based on their ability to precipitate protein. Tannin content was determined as described by Hagerman and Butler (1978). Crude methanol extract (1 ml) and 2 ml acetate buffer (0.2 M CH_3COOH, pH 5.0; 0.17 M NaCl) containing 1 mg/ml bovine serum albumin were incubated for 15 min at ambient temperature. After centrifugation at 12,000 *g* for 15 min, the pellet was washed with 0.2 M acetate buffer at pH 5.0, and dissolved in 4 ml of a solution consisting of 1% w/v SDS + 5% (v/v) triethanolamine in water. A 1-ml aliquot of $FeCl_3$ (10 mM in 0.01 N HCl)

was added and after 15 min incubation at ambient temperature the absorbance was determined at 510 nm in a Uvicon 810 spectrophotometer. Tannic acid (Sigma) was used as the standard.

Thin-layer chromatography (TLC). Chromatography was carried out on silica gel 60 plates (Merck), using butan-1-ol: acetic acid: water (3:1:1, by vol.; BAW) as the developing solvent. Acid hydrolysis of methanol and acid-methanol extracts was conducted by adding an equal volume of 40% concentrated HCl, which was then heated at 100 °C for 6 h until hydrolysis was complete. For each tissue type, methanol and acid-methanol extracts were separated by chromatography before and after acid hydrolysis. The dry TLC plates were observed under UV light before and after spraying with $AlCl_3$ salt or after spraying with 0.1% $FeCl_3$ in methanol to visualize spots and characteristic groups of compounds. The resultant spots were identified by comparison with gallic and tannic acid standards (Porter 1989; Harborne 1998; Lewis et al. 1998).

Assay for heavy-metal binding (dialysis). To determine polyphenol chelation, 5 ml of crude extract (prepared from 10 g fresh rhizome in 100 ml methanol and used for all replicates) was placed in dialysis tubing (Medicell International, London, UK; 2–18/32″). The tube was stirred in 100 ml of the metal solution (10–200 ml/l concentrations, 5 °C) with a magnetic stirrer at 60 rpm. The tested metals were aqueous solutions of Cd (as $Cd(NO_3)_2$, pH 5.55), Cr (as CrO_3, pH 2.95), Pb (as $Pb(NO_3)_2$, pH 5.3) or Hg (as $HgCl_2$, pH 4.60). These metals were chosen because they had already been tested in in-vivo experiments (Lavid et al. 2000); results for Cr (a chelating agent used in leather tanning) were, however, not described in the previous report. After 3–48 h, the entire contents of the tube were collected, measured and digested as described for metal determination (Lavid et al. 2000) using inductively coupled plasma (ICP) spectroscopy (Spectro, Kleve, Germany). Due to chelation, brown colloids were formed in tubes containing Cr, Pb and Hg, small parts of which could not be removed; this residue was estimated to be no more than 5% of the colloid mass. Samples were taken from the outer solution for metal determination by ICP spectroscopy.

Enzyme determinations: enzyme extraction. Aliquots (250 mg) of freeze-dried powder were transferred into 10 ml of extraction buffer (0.2 M sodium phosphate buffer containing 1% w/v insoluble polyvinylpolypyrrolidone and 0.1% w/v SDS, pH 7.0) and homogenized for 1 h at 4 °C. This extraction buffer was chosen to prevent protein precipitation by tannins during extraction, a known problem in tannin-rich plants like *Nymphaea*. The homogenates were centrifuged for 30 min at 17,640 g at 4 °C and the supernatants were passed through cheesecloth. These crude extracts were used immediately for spectrophotometric determination of enzymatic activities (see below).

Peroxidase assays. Peroxidase activities were assayed as described by Ryan et al. (1982) except that the 3-ml reaction mixture contained sodium phosphate buffer (0.2 M, pH 6.5), 30 mM H_2O_2, 5.3 mM guaiacol, and 50 µl crude enzyme. Increases in absorbance at 470 nm were monitored in a Uvicon 810 spectrophotometer.

Enzyme activity was determined by measuring the initial rate of the reaction. Guaiacol peroxidase activity was calculated using an extinction coefficient of 26.6 OD for 1 mmol oxidized guaiacol. Blanks were recorded in the absence of either substrates or enzyme extract, or with boiled crude enzyme extracts; in both cases, activity was negligible (Münzenberger et al. 1997).

Histochemistry. Fresh plant material was washed thoroughly with deionized water. Tissue sections were obtained using a sharp razor, then washed again with deionized water and stained. Light microscopy of fresh samples was performed with an Olympus model BH-2 microscope.

Phenol staining with Prussian blue. Ferric chloride (50 mM in 0.1 N HCl) and 8 mM $K_3Fe(CN)_6$ were mixed in equal proportions before dipping. Reactive phenols gave blue spots after a 15-min incubation at ambient temperature (after Swain 1965). Phenol and tannin localization was reconfirmed with $FeCl_3$ and toluidine blue stain, according to Gahan (1984).

Auto-fluorescence. Samples were viewed with a Zeiss Axioscope epifluorescence microscope equipped with a Dapi FilterSet (excitation 365 nm, emission 420 nm).

Peroxidase activity stains. These were conducted by a modification of the method described by Fielding and Hall (1978). Tissues were incubated in 50 mM phosphate buffer (pH 7), containing 5.3 mM guaiacol and 30 mM H_2O_2, for 15 min at ambient temperature. Staining was not observed in the absence of H_2O_2.

Statistics. Each treatment was conducted with three replicates, and each experiment was conducted at least three times. These experiments were performed at different times in a glasshouse under natural conditions and therefore differences among absolute values were quite large. However, the trends were similar in all experiments; therefore, only representative experiments are presented.

Results

Phenol content of Nymphaea

Phenol and tannin contents were high in all of the *Nymphaea* plant parts grown under light conditions (Table 1). Phenol content was not affected in plants kept in the dark or grown in the presence of Cd for 5 d under the conditions of this study (data not shown).

Phenols were partially identified in methanol extracts of leaf laminae and rhizomes, before and after full hydrolysis, separated by TLC on silica gel (Fig. 1). The pattern of extracted lamina and rhizome phenols was very similar to the tannic acid standard, and after hydrolysis of the phenol extract yielded mainly gallic acid. This pattern was also found for petiole, rhizome epidermis and roots with methanol or acid-methanol extracts (data not shown). Condensed tannins were not detected in acid-methanol extracts by typical assays of

Table 1. Phenols, tannins and peroxidase activity in tissues of *Nymphaea* plants grown on gravel (control)

Compound/activity	Root	Rhizome		Leaf	
		Epidermis	Medulla	Petiole	Lamina
Total phenols[a]	108 ± 7	268 ± 3	311 ± 41	39 ± 5	113 ± 5
Tannins[a]	87 ± 0	229 ± 14	270 ± 37	20 ± 0	96 ± 5
Peroxidase activity[b]	91 ± 3	10 ± 1	10 ± 1	173 ± 13	114 ± 10

[a] mg/g lyophilized tissue
[b] mmol oxidized guaiacol min^{-1} g^{-1} lyophilized tissue

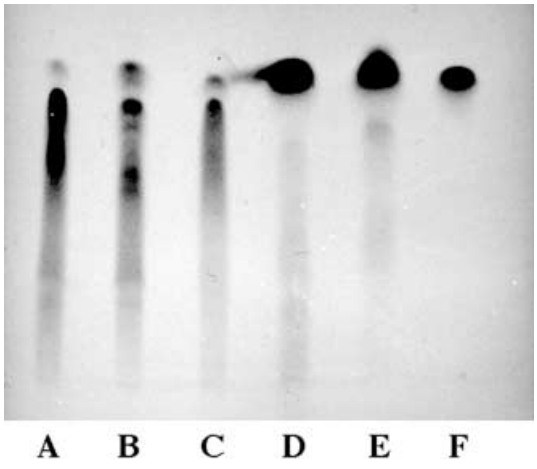

Fig. 1. Thin-layer chromatography of *Nymphaea* polyphenols. *A*, methanol extract of rhizome. *B,* methanol extract of leaf lamina. *C*, tannic acid standard. *D*, rhizome extract hydrolyzate. *E*, leaf lamina extract hydrolyzate. *F*, gallic acid standard

vanillin-HCl and butanol-HCl according to Butler (1982). These results suggest that the phenol moiety in *Nymphaea* consists mainly of polyphenols, hydrolyzable tannins, and gallic and tannic acid derivatives.

Metal chelation by Nymphaea polyphenols in vitro

The chelating abilities of *Nymphaea* polyphenols were examined by dialysis assay. A correlation between polyphenol concentration and Cr uptake from the solution and binding to the polyphenols is shown in Fig. 2A. Chromium chelation exhibited a good correlation with polyphenol concentration, but excess polyphenol content in solution was less effective at binding due to auto-polymerization of polyphenols, a well-known phenomenon in tannin chemistry (Hagerman and Butler 1989). Figure 2B shows the effect of Cr concentration on polyphenol chelating ability; increased Cr concentration in solution resulted in greater Cr-binding by the polyphenols. Figure 2C shows the time-course of the chelation process, which occurred mainly during the first 12 h of interaction between Cr and rhizome polyphenols. Binding of Hg and Pb by rhizome polyphenols was similar, but lower than that of Cr. Cadmium, which accumulated to very high levels in the plant in vivo (Lavid et al. 2000), did not bind to rhizome polyphenols under these conditions (data not shown).

Histochemical localization of phenols in the presence and absence of Cd in Nymphaea

In controls, glands in the unstained abaxial epidermis of the leaf lamina were clearly delineated by surrounding anthocyanin pigments (Fig. 3A). Staining with Fe salts (Prussian blue) showed that these glands contain phenols (Fig. 3B). Phenols were similarly detected in epidermal glands of petioles and rhizome, as well as in whole roots (data not shown). Brown deposits were observed in detached leaf lamina epidermal glands 3 h after exposure to 50 mg/l Cd solution (Fig. 3C). These are apparently oxidized polyphenols.

Auto-fluorescence of polyphenols in control and Cd-treated Nymphaea

Polyphenols are known for their auto-fluorescence under UV light (Harris and Hartley 1976). Figure 4A shows this auto-fluorescence under a UV filter, in the abaxial epidermis of a *Nymphaea* lamina, where epidermal glands are clearly observed. Similar auto-fluorescence was observed in epidermal glands in the petioles and rhizome as well as in whole roots (data not shown). Figure 4B shows auto-fluorescence of a detached leaf lamina exposed to 50 mg/l Cd for 6 h; the spread of fluorescence to adjacent cell walls can be seen.

Determination and localization of peroxidase activity in Nymphaea, in the presence and absence of Cd

Peroxidase activity were localized to the same tissues as those accumulating Cd: leaf lamina, petiole and roots (Lavid et al. 2000). Histochemical localization of peroxidase activity in the abaxial epidermis of leaf laminae (Fig. 5A) demonstrated that, as with polyphenols, it is localized in epidermal glands. Similar observations were made in epidermal glands of the petiole, rhizome and whole root. Figure 5B shows spread of peroxidase activity to adjacent cell walls in detached leaf laminae exposed to 50 mg/l Cd for 6 h. The total level of peroxidase activity found under light (control) conditions (Table 1) was identical to that found for dark-treated and Cd-grown plants. Polyphenol oxidase activities were negligible in all plant tissues, as tested according to Ryan et al. (1982).

Discussion

Cadmium and other heavy metals have been found to accumulate in the epidermal glands of the lamina, as well as in the epidermal glands of the petiole and rhizome of *Nymphaea* (Lavid et al. 2000). Polyphenols and peroxidase activity were also co-localized in the glands.

In the presence of 50 mg/l Cd, colourless polyphenols in the epidermal glands of the leaf lamina turned brown. These deposits gradually appeared in adjacent cell walls together with peroxidase activity, following a pattern similar to that of Cd uptake (Lavid et al. 2000; Figs. 3–5). These observations support our working hypothesis that polyphenols and peroxidases are involved in Cd accumulation by *Nymphaea*. These findings agree with those of Lummerzheim et al. (1995) on the similarities between the response of *A. thaliana* to lead toxicity and bacterial infection, and with the description of Beckman et al. (1972) of plant-pathogen

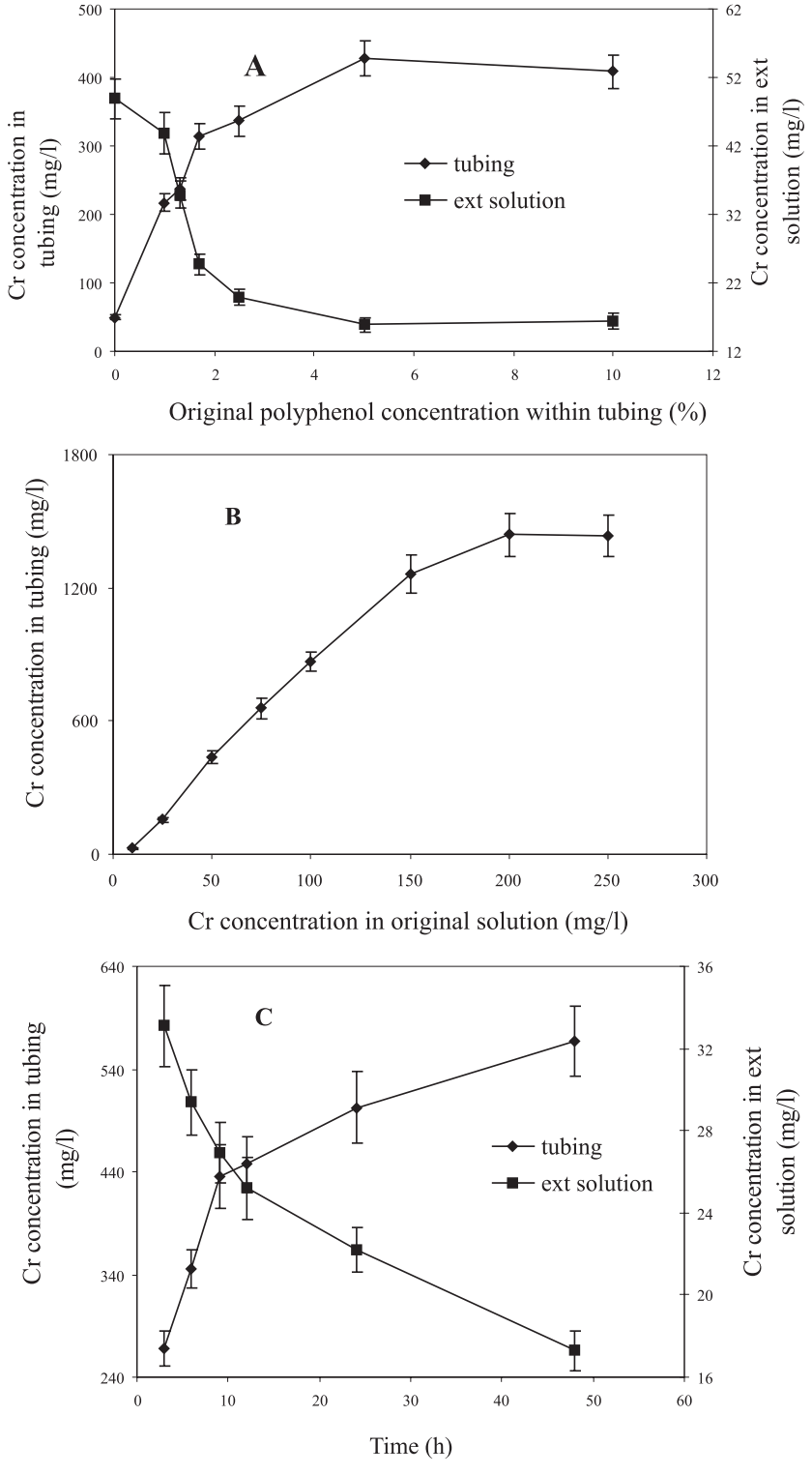

Fig. 2A–C. Chelating abilities of *Nymphaea* rhizome extracts containing polyphenols. The stock polyphenol extract (concentration 10% in **A**) was made by grinding 10 g fresh rhizome in 100 ml methanol. Other concentrations were made by dilution with methanol. A 5-ml aliquot of the respective extract was placed in dialysis tubing. The tubing was placed in the Cr solution where it was stirred (magnetic stirrer at 60 rpm) for the time mentioned. The concentrations of Cr in the tubing and external solution were then determined. "Tubing" refers to Cr that was chelated by polyphenols and consequently trapped within the tubing. "Solution" refers to Cr in the outer solution, i.e. the amount of Cr that was not chelated by the polyphenols. **A** Effect of rhizome polyphenol concentration on Cr-binding (after 24 h). Original Cr concentration in solution was 50 mg/l. Means ± SE, $n = 3$. **B** Effect of Cr concentration on binding to rhizome polyphenols for stock extract (24 h). Means ± SE, $n = 3$. **C** Effect of time on Cr-binding by rhizome polyphenols for stock extract. Original Cr concentration in solution was 50 ml/l. Means ± SE, $n = 3$

interactions. In both studies, these interactions were accompanied by enhanced peroxidase activity and the differential distribution of phenols, both throughout the storage cells and to adjacent cells. These phenols become oxidized and polymerize to form coloured products. However, whether the observed distribution of phenols and peroxidase activity in *Nymphaea* was due to their diffusion out of the glands or just to the increased permeability of those glands to the heavy metal and to the stains is not clear.

Direct chelation, or binding to polyphenols, was observed with methanol extracts of rhizome polyphenols for Cr (Fig. 2), as well as for Pb and Hg. The stoichiometry of this chelation can be computed once the exact structure of *Nymphaea* polyphenols and their degrees of polymerization are determined. Other ele-

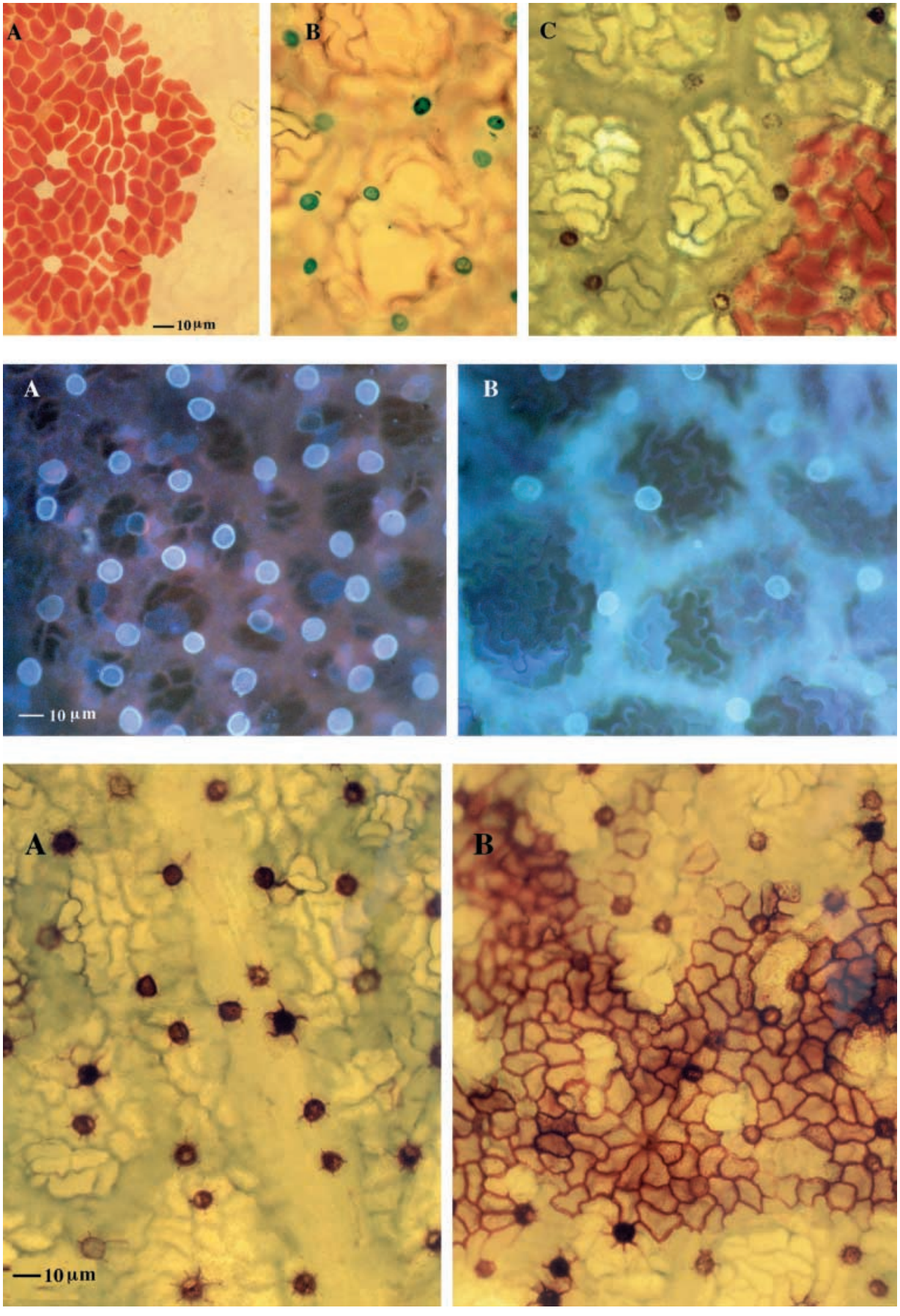
A
B
C
10 µm
A
B
10 µm
A
B
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Fig. 3A–C. Polyphenol localization in epidermal glands on the abaxial lamina of a *Nymphaea* leaf. **A** Control lamina; unstained. **B** Leaf lamina after staining with Prussian blue. **C** Detached leaf lamina after 3 h exposure to 50 mg/l Cd; unstained

Fig. 4A,B. Auto-fluorescence of polyphenols in epidermal glands on the abaxial lamina of a *Nymphaea* leaf. **A** Control lamina. **B** Detached leaf lamina after 6 h exposure to 50 mg/l Cd

Fig. 5A,B. Localization of peroxidase activity in epidermal glands on the abaxial lamina of a *Nymphaea* leaf. **A** Brown stain indicating peroxidase activity in epidermal glands of a control lamina. **B** Peroxidase activity stain in detached leaf lamina after 6 h exposure to 50 mg/l Cd, showing spread to adjacent cell wall

ments (i.e. Fe, Mo, Al and B) that bind to polyphenols are also used in different stains (Harborne 1998). However, Cr accumulation in *Nymphaea* in vivo is very low (≈15 mg/l out of 10 mg/l CrO_3 solution), while Cd, Hg and Pb accumulation in vivo is very high (Lavid et al. 2000). Cadmium was not chelated under our dialysis conditions. Therefore, it seems that direct chelation of heavy metals by *Nymphaea* polyphenols accounts for only a minor part of the heavy-metal accumulation in vivo, particularly Cd.

The possibility that peroxidases participate in heavy-metal accumulation, along with the polymerization of polyphenols, which then trap heavy metals, was investigated. Peroxidases were found to be involved (Fig. 5) and H_2O_2-dependent oxidation apparently enhances Cd accumulation in daylight (Lavid et al. 2000). Peroxidase is one of the major enzymes catalyzing the oxidative metabolism of xenobiotics in plants. In a report on antioxidative enzymes in aquatic plants that survived in polluted lakes in Finland, peroxidase was found to be the main component associated with tolerant waterlilies exposed to paper mill pollution (Roy et al. 1992). Polyphenol oxidase activity, which has been found in glandular trichomes of *Lycopersicon* and *Solanum* spp., was negligible in *Nymphaea*. Sherman et al. (1991) suggested that the acquisition of polyphenol oxidase, widespread in terrestrial plants but not in algae, may have occurred simultaneously with the adaptation to an oxygenated atmosphere where polyphenol oxidase activity is vital in the photosynthetic apparatus exposed to atmospheric oxygen. Interestingly, another difference in antioxidative enzymes was found with Cu-Zn superoxide dismutase (SOD), present in all higher-plant families surveyed except the Nymphaeaceae, the latter having an Fe form of SOD (Salin and Bridges 1982).

Nymphaea contains high constitutive levels of polyphenols and peroxidases. Light or growth in the presence of Cd did not alter polyphenol or peroxidase activity content. Stress-tolerant plants usually contain high constitutive levels of protective metabolites, while the more sensitive ones show their induction under stress (Constabel and Ryan 1998).

Heavy-metal accumulation in *Nymphaea* plants in vivo differs from heavy-metal chelation by *Nymphaea* polyphenols in vitro. These results suggest that heavy-metal accumulation in *Nymphaea* involves different mechanisms: (1) trivalent ions, such as Fe, Cr and Al, may bind specifically to polyphenols; and (2) divalent ions such as Cd, Mn and Hg, may form oxides or other precipitates, which are then trapped and immobilized in polymerized polyphenols. The typical appearance of iron plaques on aquatic plant roots has been described by Taylor et al. (1984), who suggested that oxygen flow from the root is the driving force for cast formation by oxidation and precipitation of Fe on external cell surfaces. This agrees with our finding that *Nymphaea* roots contain high levels of polyphenols as well as peroxidase activity, and accumulate Cd (Lavid et al. 2000).

The importance of the formation of colloidal metal hydroxides induced by photosynthetic organisms in alkaline environments has been recently described (Capolino et al. 1997). Algal tolerance to Hg has been related to the rapid increase in dissolved O_2 in the medium due to active photosynthesis, which caused the formation of insoluble Hg hydroxide. Similarly, Fe and Ca hydroxides, phosphates and carbonates co-precipitate ionic Cu and Cd. Indeed, Cd co-precipitates with Ca in *Nymphaea* epidermal glands (Lavid et al. 2000).

Photosynthesis may play an indirect role in heavy-metal accumulation by *Nymphaea* by supplying H_2O_2 (a by-product of photosynthesis) for the polymerization of polyphenols by peroxidases, as suggested by Yamasaki et al. (1997), and/or by supplying O_2 for generating oxide precipitates (Capolino et al. 1997).

The nature of heavy-metal accumulation by epidermal glands of *Nymphaea* seems to involve at least two mechanisms: (a) direct chelation by polyphenols and (b) binding and trapping of heavy-metal precipitates during the process of polyphenol polymerization by peroxidases. Polyphenols and peroxidases, highly abundant in the waterlily, seem to play a major role, both through their antioxidant activity and through their polymerization-based trapping potential, and may provide the biochemical and physiological basis for heavy-metal accumulation and tolerance in *Nymphaea*.

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