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ORIGINAL ARTICLE

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# Phenols and phenol oxidases are involved in cadmium accumulation in the water plants *Nymphoides peltata* (Menyanthaceae) and *Nymphaeae* (Nymphaeaceae)

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**Abstract** This comparative study investigates the mechanism of cadmium accumulation in the semiaquatic plant *Nymphoides peltata* (Menyanthaceae) and the aquatic plant *Nymphaea* (Nymphaeaceae). It was conducted as part of an ongoing study of the use of water plants for phytoremediation. Epidermal structures, known as hydropotes, are located on the abaxial epidermis of the leaf laminae of *Nymphoides peltata* and are shown to contain phenols, peroxidase and polyphenol oxidase activities. When plants are subjected to 50 mg/l of cadmium in the growth medium, these hydropotes accumulate cadmium. Cadmium-induced increases in phenols, peroxidase and polyphenol oxidase activities were determined in plant extracts. Cadmium binding by polymerized phenols was demonstrated in vivo. In comparison with *Nymphaeae* epidermal glands, *N. peltata* hydropotes are larger, open, and create bigger crystals, the latter principally composed of calcium and, proportionally, less cadmium. Although both plants showed similar levels of cadmium accumulation, *N. peltata* was sensitive while *Nymphaeae* was resistant to this cadmium level. It is suggested that in these water plants the main mechanism for cadmium accumulation is based on the trapping of cadmium crystals by polymerized phenols in specialized epidermal structures and this is due to peroxidase and polyphenol oxidase activities. *Nymphaeae*, with greater peroxidase activity and more polyphenols, is more resistant to this heavy metal than *N. peltata*.

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**Keywords** Cadmium accumulation · *Nymphaea* (Cd accumulation, trichome) · *Nymphoides* (Cd accumulation, hydropote) · Peroxidase · polyphenol oxidase · Phytoremediation

**Abbreviations** EDS: energy-dispersive spectroscopy · SEM: scanning electron microscopy

## Introduction

Soil and water pollution by toxic heavy metals is a major environmental concern. Use of metal-accumulating plants for environmental phytoremediation, the process by which plants remove, detoxify or stabilize pollutants is a promising technology for environmental clean-up (Salt et al. 1995). Of particular concern are the highly toxic non-nutrient metals such as mercury, lead, chromium and cadmium (Cd). The toxicity of these heavy metals is due to their ability to cause oxidative damage to tissues. Damage includes enhanced lipid peroxidation, DNA damage, enzyme inactivation and the oxidation of protein sulfhydryl groups (Taiz and Zeiger 1998). Aquatic plants have been investigated for their potential use in wastewater treatment because they possess the ability to grow in water bodies where heavy metals are normally discharged into the environment (Reimer and Duthie 1993).

Water provides a uniform, dense and weightless habitat. The most striking structural features in the leaves of water plants, in comparison to terrestrial plants, are the reduction of supporting and protective tissues, the decrease in the amount of vascular tissue, especially xylem, and the presence of air chambers. The epidermis of water plants does not have a marked protective function but it plays a role in the uptake of nutrients from the water and in gas exchange (Fahn 1989).

The epidermis of the immersed surface of leaves of some water plants (e.g. *Aponogeton*, Aponogetonaceae; *Nymphoides peltata*, Menyanthaceae; *Potamogeton*, Potamogetonaceae and *Sagittaria macrophylla*,

Alismataceae) possesses small groups of cells which stain deeply with toluidine blue and Prussian blue. These cell groups, termed hydropotes (water drinkers), are thought to be structures that facilitate water and salt transport into and out of the plant. It was found that the cuticle covering the hydropotes is especially permeable to water and nutrient salts (Fahn 1979). These epidermal features differ from the multicellular glands or trichome glands found in the epidermis of *Nuphar lutea* and *Nymphaea* of the Nymphaeaceae (Kristen 1969).

Recently, we have shown that the epidermal glands in *Nymphaea* are capable of accumulating Cd and other heavy metals, and contain high levels of phenols and peroxidase activities. Incorporation of heavy metals and their accumulation in *Nymphaea* were shown to be localized, with immobilization occurring mainly in the epidermal glands. Typical co-crystallization of Cd and calcium (Ca), which accumulated in epidermal glands, was suggested as a mechanism for Cd storage and detoxification (Lavid et al. 2001a).

Co-localization of polyphenols and peroxidases in epidermal glands of *Nymphaea* was also demonstrated by histochemistry. Both polyphenols and peroxidases were found at a very high constitutive level in plant extracts, which was not induced or altered by external conditions such as light and heavy-metal stress (Lavid et al. 2001b).

It was suggested that polymerization of polyphenols by peroxidases, enhanced under stress conditions caused by the presence of toxic heavy metals, is responsible for binding and detoxification of heavy-metals in *Nymphaea* epidermal glands. The mechanism proposed for heavy-metal accumulation in *Nymphaea* was based on the combination of special epidermal structures, phenols and phenol oxidases (Lavid et al. 2001b).

The objective of this study was to investigate the mechanisms of Cd accumulation in hydropotes in other water plants, to establish the applicability of the suggested model for such plants, and thus to contribute to the understanding of factors influencing phytoremediation efficiency. The semi-aquatic submerged plant *Nymphoides peltata* (Osborne et al. 1996) was chosen since it is sensitive to Cd levels that are tolerated by *Nymphaea*.

## Materials and methods

### Plant material and growth conditions

Rhizome explants (*Nymphoides peltata* and *Nymphaea* 'Aurora', obtained from Kibbutz Hazorea, Israel) were grown under the growth conditions described previously (Lavid et al. 2001a). Well-developed, mature plants were treated with 50 mg/l Cd (as $Cd(NO_3)_2$) for 4 days and their Cd content, phenol content, peroxidase and polyphenol oxidase activities were determined (see below). No toxic effects (such as leaf chlorosis or wilt) were observed in *Nymphaea* while *N. peltata* was severely damaged during the 4-day study.

### Analytical procedures

#### *Sampling for quantification experiments*

Samples were prepared for quantification experiments as described previously (Lavid et al. 2001a, b).

#### *Metal analysis*

Metal content in plant samples was determined on freeze-dried material by inductively coupled plasma spectroscopy (Spectro, Kleve, Germany), as described by Lavid et al. (2001a).

#### *Determination of enzyme activities*

Extraction and assay of peroxidase activity were as described in Lavid et al. (2001b). Polyphenol oxidase activity was assayed by a modification of the method described by Ryan et al. (1982). Crude enzyme extracts (0.25 ml) were added to reaction mixtures containing 2.75 ml sodium phosphate buffer (50 mM; pH 6.5), and 50 mM catechol. Increases in absorbency at 420 nm were monitored in a Uvicon 810 spectrophotometer. Enzyme activities were determined by measuring the initial rate of the reaction. In both test systems, blank rates recorded in the absence of either substrates or enzyme extracts, or utilizing heat-inactivated (100 °C, 3 min) crude enzyme extracts, were negligible.

#### *Analysis of phenols*

Assays for total phenols; tannins and condensed tannins were performed as described previously (Lavid et al. 2001b).

### Histochemistry

#### *Analytical microscopy*

Carbon-coated samples were examined in a JSM-6300 scanning electron microscope equipped for Link energy dispersive spectroscopy (EDS) as described by Lavid et al. (2001a).

#### *Light microscopy*

Fresh samples were studied with an Olympus model BH-2 microscope. Sample preparation, phenol staining with Prussian blue and peroxidase activity stains were done as described before by Lavid et al. (2001b). Polyphenol oxidase activity stains were conducted by a modification of the method described by Ryan et al. (1982). Fresh tissue slices were incubated in 50 mM phosphate buffer (pH 6.5) containing 50 mM catechol, for 15 min at ambient temperature. Staining was not observed in the absence of catechol.

### Statistics

Each treatment was done in triplicate, and each experiment was conducted at least three times. These experiments were performed at different seasons in a glasshouse under natural conditions, and therefore differences among absolute values were quite large. However, the trends were similar in all experiments.

## Results

Exposure of *N. peltata* to 50 mg/l Cd for 4 days caused severe leaf damage, starting with typical dotted chlorosis (Fig. 1A), in comparison to non-treated control plants (Fig. 1B). *Nymphaea* was tolerant of the same treatment (Lavid et al. 2001a).

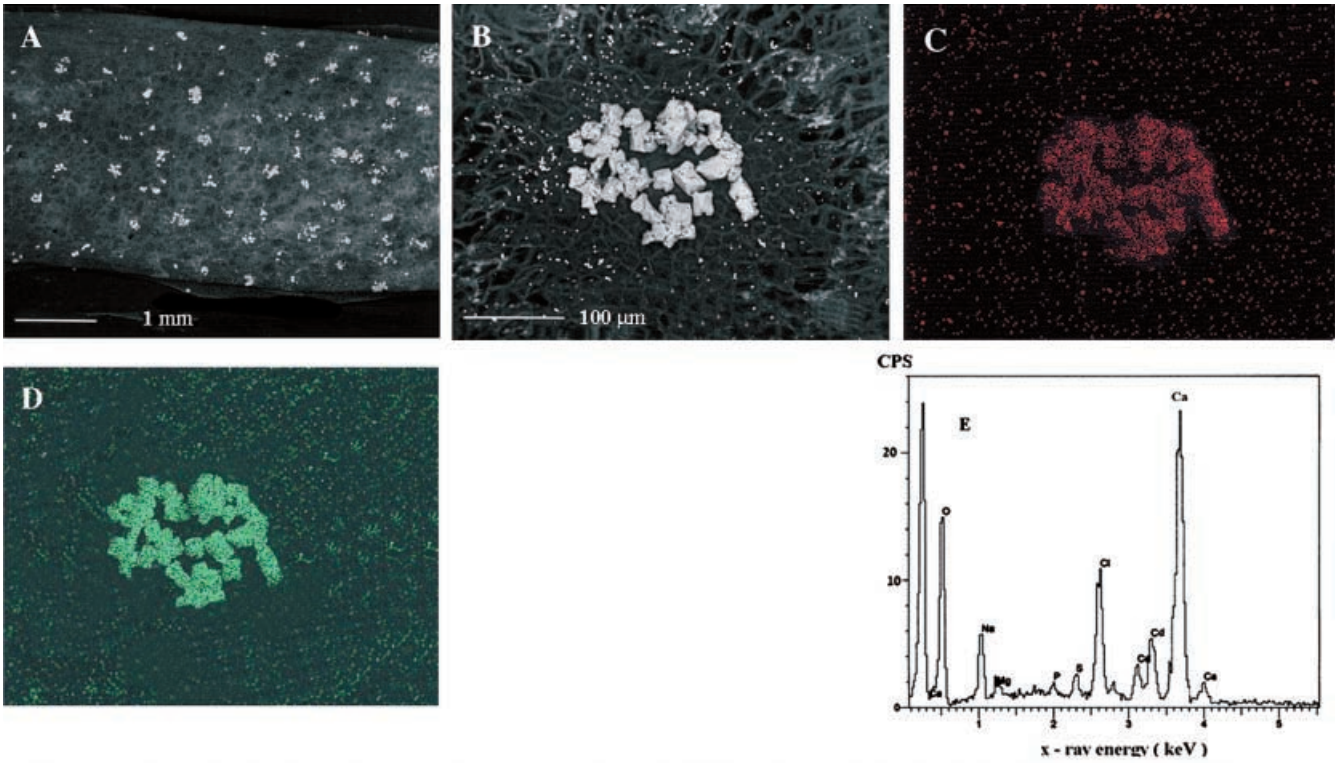

**Fig. 1A, B** Effect of 50 mg/l Cd treatment for 4 days on *Nymphoides peltata*. **A** Treatment; **B** control

Scanning electron microscopy (SEM) with EDS showed selective accumulation of Cd in hydropotes in the abaxial epidermis of the mature laminae of *N. peltata* after 24 h exposures to 50 mg/l Cd. An overall picture of the lamina (Fig. 2A) and an enlargement displaying a single hydropote (Fig. 2B) are shown. The crystals formed in the hydropote were mapped and found to contain both Cd and Ca (Fig. 2, C and D, respectively). A representative X-ray emission spectrum of a single hydropote is shown in Fig. 2E. The spectrum of a single crystal in this hydropote displayed similar peaks of Cd and Ca and is not shown. Untreated plants (Fig. 3A, B) showed no such accumulations. This observation was verified quantitatively by inductively coupled plasma spectroscopy (5.4 mg/g Cd, 40.2 mg/g Ca compared to control values of 0.0 mg/g Cd, 12.9 mg/g Ca).

**Fig. 2** Images of epidermal hydropotes on the laminae of mature *N. peltata* leaves, as yielded by SEM (**A–D**) and EDS spectra (**E**). **A** General view of the abaxial epidermis after 24 h of 50 mg/l Cd treatment. **B** SEM image of a single hydropote in **A**. **C** Cd X-ray mapping of **B**. **D** Ca X-ray mapping of **B**. **E** Spectrum of **B**. *CPS* Counts per second

## Histochemical localization of phenols in the presence and absence of Cd in *N. peltata*

In controls, hydropotes could be seen in the unstained abaxial epidermis of the leaf lamina (Fig. 4A). Staining with Fe salts (Prussian blue) showed that these hydropotes contained phenols (Fig. 4B). Brown deposits



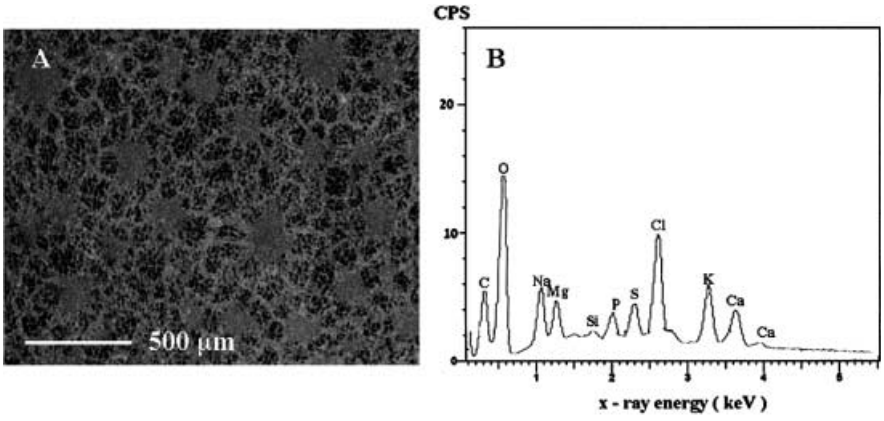



**Fig. 3** Images of epidermal hydropotes on the laminae of mature *N. peltata* leaves, as yielded by SEM (**A**) and EDS spectra (**B**). **A** General view of the abaxial epidermis of an untreated lamina (control). **B** Spectrum of a single hydropote in **A**. *CPS* Counts per second

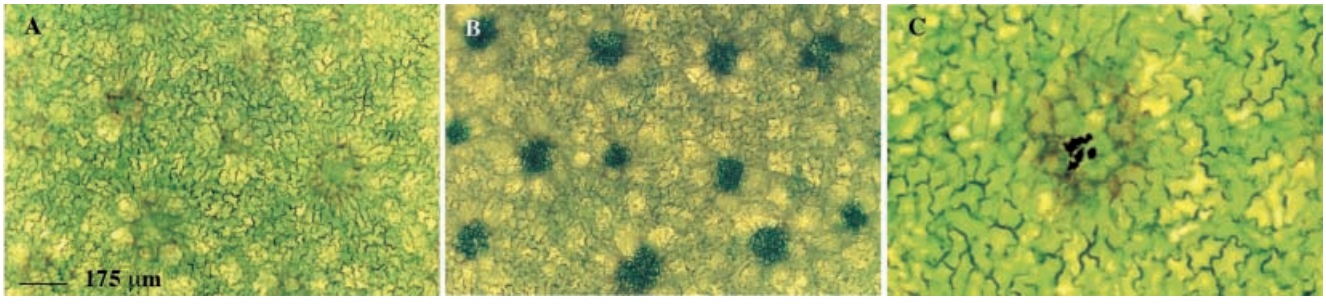



**Fig. 4A–C** Localization of phenols in epidermal hydropotes on the abaxial lamina of an *N. peltata* leaf. **A** Control lamina; unstained. **B** Leaf lamina after staining with Prussian blue. **C** Detached leaf lamina after 3 h exposure to 50 mg/l Cd; unstained

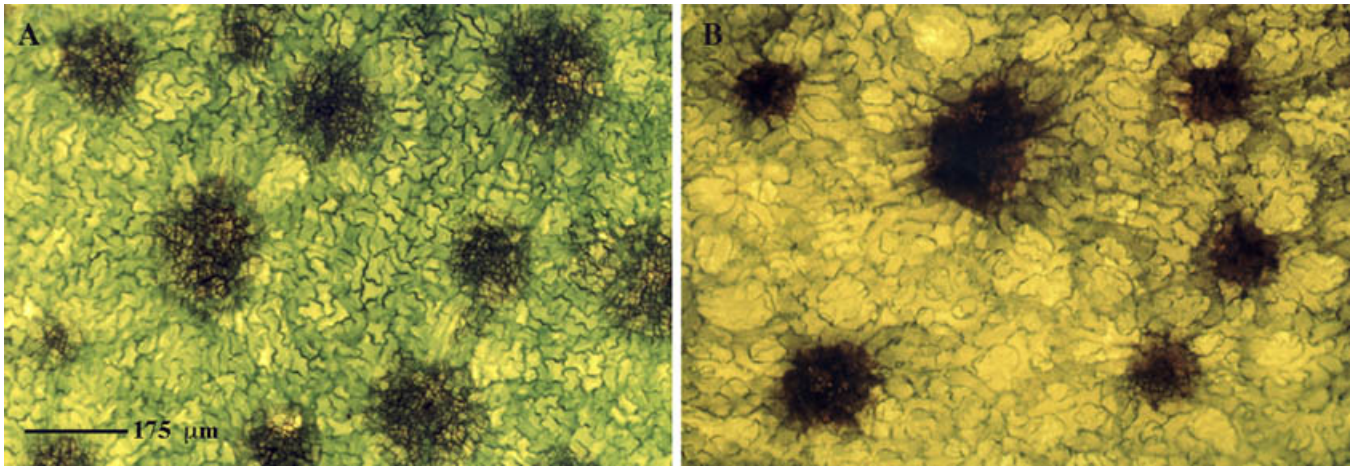
were observed in the hydropotes of detached leaf laminas 3 h after exposure to 50 mg/l Cd solution (Fig. 4C). These deposits are apparently oxidized phenols that seem to envelop cube-shaped structures, possibly Cd-Ca crystals.

**Fig. 5A, B** Localization of peroxidase and polyphenol oxidase activities in epidermal hydropotes on the abaxial lamina of an *N. peltata* leaf. **A** Brown stain indicating peroxidase activity in epidermal hydropotes of a control lamina. **B** Brown stain indicating polyphenol oxidase activity in epidermal hydropotes of a control lamina

## Histochemical localization of peroxidase and polyphenol oxidase activities in *N. peltata*

Histochemical localization of peroxidase activity (Fig. 5A) and polyphenol oxidase activity (Fig. 5B) demonstrated that, like Cd and phenols, these activities are localized in hydropotes.

Total phenols, total tannins, peroxidase and polyphenol oxidase activities in *N. peltata* and *Nymphaea* laminae extracts, as well as Cd and Ca content in laminae samples were determined during 4 days exposure to 50 mg/l Cd (Fig. 6). The metal content of plant samples revealed that after 4 days, specimens of the two plant species had accumulated approximately the same levels of Cd, but *N. peltata* accumulated much more Ca than *Nymphaea* (Fig. 6A). Figure 6B shows that in *N. peltata*,



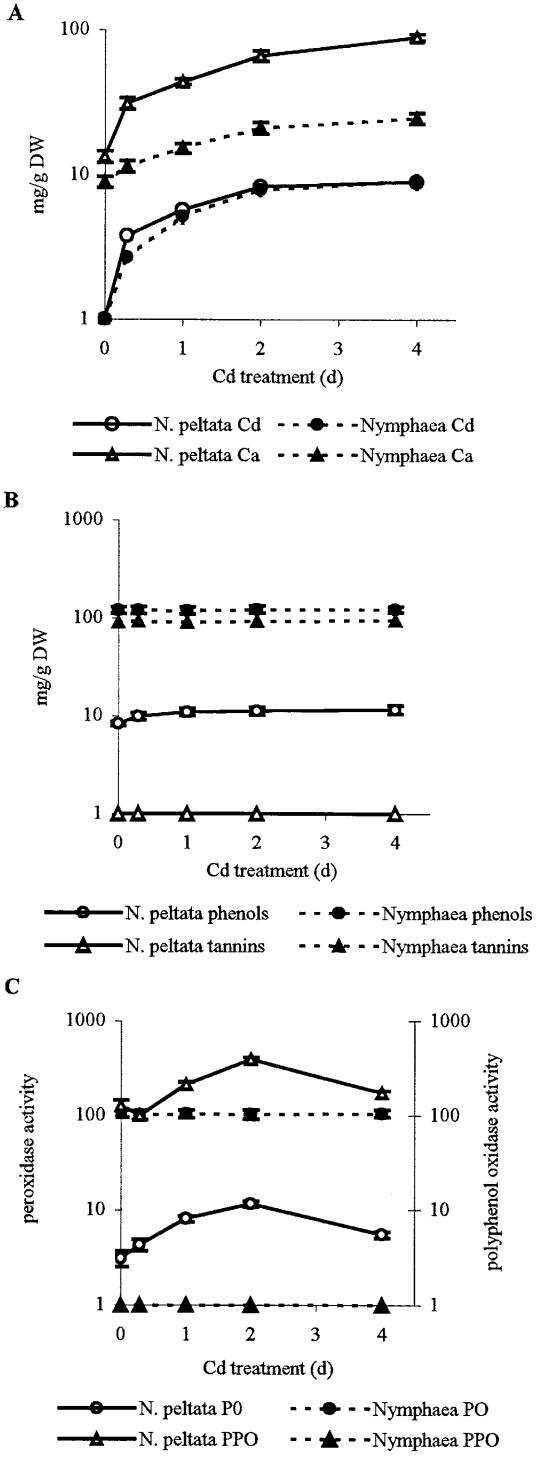



**Fig. 6A–C** Metal content and metabolites in laminae of *N. peltata* and *Nymphaea* as determined during 4 days exposure to 50 mg/l Cd. Means ± SE, $n = 3$. **A** Cd and Ca contents in lamina samples. **B** Phenols and tannins in lamina extracts. **C** Peroxidase [*PO*; mmol oxidized guaiacol (g lyophilized tissue)$^{-1}$ min$^{-1}$] and polyphenol oxidase [*PPO*; $\Delta A_{420}$ (g lyophilized tissue)$^{-1}$ min$^{-1}$] activities in lamina extracts

phenols level slightly increased while both peroxidase and polyphenol oxidase activities increased 3-fold (Fig. 6C) after exposure to Cd. In comparison, *Nymphaea* showed no Cd-induced increase in phenols or peroxidase activity and lacked polyphenol oxidase activity (Fig. 6B, C). Enzymatic activities of *N. peltata* decreased after 4 days (Fig. 6C), probably due to the severe damage observed by that time.

Table 1 summarizes the similarities and differences in Cd accumulation for *N. peltata* and *Nymphaea*. In both plants, Cd accumulates simultaneously with Ca in special epidermal structures, which contain phenols and phenol oxidases, and its accumulation results in browning (oxidation) of these phenols. The differences lie in levels and constitutions of these metabolites: *Nymphaea* lacks polyphenol oxidase activity and shows high constitutive levels of peroxidase and phenols (including tannins), while *N. peltata* shows induction of these enzymes and different phenols (no tannins). For the same Cd level, *Nymphaea* showed resistance while *N. peltata* was severely damaged.

## Discussion

In this study, we describe different aspects of Cd accumulation in *N. peltata*. As was found in *Nymphaea* (Lavid et al. 2001a), Cd is accumulated in special epidermal structures, probably in the form of Cd-Ca crystals. Co-localization of phenols and phenol oxidases was found in these structures as well. In the presence of 50 mg/l Cd, colourless phenols in the hydropotes turned brown, similar to a phenomenon observed in waterlily epidermal glands (Lavid et al. 2001b). These observations support a previous suggestion that phenols and phenol oxidases are involved in Cd accumulation by *Nymphaea* epidermal glands (Lavid et al. 2001b). The mechanism of Cd accumulation in these two plants seems to be similar and to involve the formation and trapping of Cd-Ca crystals during the process of phenol polymerization by phenol oxidases. However, although *Nymphaea* tolerates these Cd levels, *N. peltata* does not.

It appears that the combination of special epidermal structures (i.e. glands or hydropotes) containing phenols and phenol oxidases enables Cd accumulation in these water plants. However, the nature of the crystals formed, amount of Cd accumulated, and ultimately the tolerance of the plant depend on the unique nature of the components involved. Cd-Ca crystals were demonstrated in *Nymphaea* epidermal glands and in *N. peltata* hydropotes. The composition of these crystals and their size differ between these two plants. Compared with the *Nymphaea* crystals (Lavid et al. 2001a), *N. peltata* crystals were larger, and contained less Cd and more Ca. This finding agrees with that of Smits et al. (1992), who demonstrated that leaf development in *N. peltata* depends on the presence of a minimum level of Ca in the water; unlike *Nymphaea*, this plant is not found in acidic low-Ca water. In addition, it appears that the non-glandular, bigger and open hydropote structure enables the growth of Cd-Ca crystals to a size not allowed by the smaller closed-gland structure of *Nymphaea*.

**Table 1** Comparison between *Nymphoides peltata* and *Nymphaea* laminae with respect to the components involved in Cd accumulation and detoxification. Means ± SE, $n = 3$

| Trait | *Nymphaea* | *N. peltata* |
|---|---|---|
| Cd-tolerance (50 mg/l) | Tolerant | Sensitive |
| Cd accumulation[a] (50 mg/l; 4 days) | 9.0 ± 0.6 | 8.9 ± 0.3 |
| Special structures in abaxial lamina epidermis | Glands | Hydropotes |
| Co-localization of Cd/phenols/phenol oxidases in glands/hydropotes | + | + |
| Total phenols[a] (control) | 119.6 ± 11.3 | 8.3 ± 0.4 |
| Total tannins[a] (control) | 90.4 ± 9.3 | – |
| Peroxidase activity[b] (control) | 105.4 ± 4.0 | 3.1 ± 0.6 |
| Polyphenol oxidase activity[c] (control) | – | 126.6 ± 17.6 |
| Cd-induced increase in phenol oxidases | No | 3-fold |
| Cd-induced browning of phenols in epidermal structures | + | + |
| Cd-dependant Ca-accumulation[a] (50 mg $l^{-1}$ Cd; 4 days) | 24.5 ± 3.3 | 88.6 ± 2.9 |

[a] mg (g lyophilized tissue)$^{-1}$
[b] mmol oxidized guaiacol (g lyophilized tissue)$^{-1}$ min$^{-1}$
[c] $\Delta A_{420}$ (g lyophilized tissue)$^{-1}$ min$^{-1}$

The types and levels of phenol oxidases found in each of these species are also different. 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 its activity is vital in the photosynthetic apparatus exposed to atmospheric oxygen. *Nymphoides peltata*, a semi-aquatic plant, shows a low level of peroxidase content compared with *Nymphaea*, but a high level of polyphenol oxidase, in agreement with this hypothesis.

*Nymphaea* contains high constitutive levels of polyphenols and peroxidases. Constabel and Ryan (1998) suggested that stress-tolerant plants usually contain high constitutive levels of protective metabolites, while the more-sensitive ones show their induction under stress. Induction of phenols, peroxidase and polyphenol oxidase has been shown here for the Cd-sensitive *N. peltata* (Table 1), and was found also in the semi-aquatic Cd-sensitive plant *Marsilea minuta* L. (Garg et al. 1994). 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). Increases in phenol concentration in response to heavy-metal stress have also been noted in several plants, such as pine (Giertych et al. 1999), tobacco (Edreva and Apostolova 1989), maize (Baccouch et al. 1998), *Albizia* (Tripathi et al. 1999), *Acer* (Zobel and Clarke 1999), and birch (Loponen et al. 1998). Aoba (1986) and Wang et al. (1997) suggested a similar mechanism in tannin-rich plants such as tea, which are tolerant to excess Mn.

The pattern of the phenols and their contents also differ between the two aquatic plants we tested. *Nymphaea* contained 10 times as much total phenols, including tannins, as compared to *N. peltata* (Fig. 6B, Table 1), which lacks tannins. Our present objective was to quantify the total content of polyphenols in *N. peltata* compared with *Nymphaea* and to localize them anatomically. *Nymphaea*'s polyphenols were partially identified and were found to consist mainly of hydrolyzable tannins, and derivatives of gallic and tannic acids (Lavid et al. 2001b). The phenols of *N. peltata* have not yet been identified, but were shown by TLC and the tannin precipitation test to be different from those found in *Nymphaea*. Interestingly, sclereids are abundant in these two plants, but only *Nymphaea* possesses Ca-oxalate crystals on these sclereids. Other plants such as oak and acacia, which possess Ca-oxalate crystals on their sclereids, are also known for their high tannin levels (Esau 1978). Tannins from these plants are used for adsorption of heavy metals (Gloaguen and Morvan 1997; Nakashima et al. 1996). It might be that some metabolic pathways leading to tannin production are also involved in the creation of sclereids, and their co-existence with phenol oxidases in plants might indicate a high potential for heavy-metal binding by these plants.

*Nymphoides peltata* accumulates Cd by a parallel mechanism to *Nymphaea* and to the same content, but shows sensitivity to this heavy metal (Fig. 1, Table 1). This sensitivity may be due to lack of tannins and overall lower level of phenols. The main phenol oxidase in the *N. peltata* hydropote is polyphenol oxidase (substrates phenol and oxygen), which does not act as an antioxidant, while, in *Nymphaea*, high levels of peroxidase (substrates phenol and hydrogen peroxide), an effective antioxidant, are present in the glands. This suggests less antioxidant activity of both phenols and phenol oxidases in *N. peltata*. It is also possible that the oxidized products formed in *N. peltata* hydropotes, differing from those of *Nymphaea* because the phenols and enzymes are different, were toxic to the plant.

*In conclusion*, in this study we demonstrate the involvement of phenols and phenol oxidases in Cd accumulation by *N. peltata* hydropotes. This plant accumulates Cd by a mechanism similar to that occurring in *Nymphaea* epidermal glands, and to the same levels. However, *N. peltata*, unlike *Nymphaea*, is sensitive to these levels of Cd. Compared with *Nymphaea*, *N. peltata* contains a lower level (as well as different constitution) of phenols, low activities of peroxidase and high activities of polyphenol oxidase, and Cd induces their increase. In these water plants, the main mechanism suggested for Cd accumulation is based on a combination of special epidermal structures, phenols and

phenol oxidases. Based on our evidence, it appears that plants containing high levels of peroxidase activities and polyphenols are likely to be more tolerant to Cd.

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