Zinc Reversal of Cadmium-induced Energy Transfer Changes in Photosystem II of *Ceratophyllum demersum* L. as Observed by Whole-leaf 77K Fluorescence

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ABSTRACT

We have examined the mode of interaction between cadmium (Cd) and zinc (Zn), toxic and micronutrient elements, on the photosynthetic apparatus, with respect to energy transfer in leaves of the free-floating macrophyte *Ceratophyllum demersum* L. Low temperature fluorescence spectroscopy enabled *in situ* detection of changes in energy transfer from pigments energetically coupled to PS I and PS II, induced by Cd and Zn in *C. demersum*. The two-point normalization algorithm permitted a direct comparison of the shape of excitation spectra in the wavelength range including absorption maxima of main photosynthetic pigments (from 438 to 555 nm). The results indicate that Cd and Zn in concentrations up to 1000 μM can induce remarkable changes in energy transfer from carotenoids to reaction centers of PS II and antenna complexes LHC II in *C. demersum* leaves. Cd at 1000 μM, after 48 hr incubation, induced the most pronounced increase of fluorescence from PS II/LHC II, attributable to excitation of the carotenoid pool (482 and 486 nm bands). The presence of Zn (100 μM) together with Cd (1000 μM) suppressed the observed increase of carotenoid contribution to energy transfer to photosynthetic reaction centers in PS II/LHC II. The changes in the contribution of carotenoids to energy transfer, as observed by low-temperature fluorescence spectroscopy show no direct correlation with relative changes in concentrations of these pigments in untreated versus metal-treated *C. demersum*, as measured by HPLC. In conclusion, the presence of Zn in the environment may prevent effects on photosynthetic energy transfer, induced by high concentrations of Cd in PS II/LHC II of aquatic plants.

Keywords: cadmium toxicity, carotenoids, photosynthetic apparatus, zinc amelioration

INTRODUCTION

Heavy metals are reported to have deleterious effects on plants. A large body of literature is available on the effects of exposure to elevated concentrations of heavy metals on plant growth, physiological and biochemical processes. Heavy metals may interact with the photosynthetic apparatus at various levels of organization and architecture (Greger 1999; Prasad and Strzałka 1999; Myśliwa-Kurdziel *et al.* 2002). In particular, they may alter the functions of chloroplast membranes and components of the photosynthetic electron transport chain, mainly photosystem II (PS II) and photosystem I (PS I) thus impairing the light phase of photosynthesis (Myśliwa-Kurdziel *et al.* 2002).

Cadmium (Cd) and zinc (Zn) were found to be inhibitors of PS II activity in different plant species (Prasad and Strzałka 1999; Myśliwa-Kurdziel *et al.* 2002). Also, low doses of Cd have been shown to inhibit PS II electron flow *in vitro*, in isolated chloroplasts (Bazzaz and Govindjee 1974; Chugh and Sawhney 1999). Zn inhibited PS II activity in millimolar concentrations (Rashid *et al.* 1991; Krupa and Baszyński 1995) and induced a pronounced decrease in both the variable (Fv) and the maximal (Fm) chlorophyll fluorescence as measured at room temperature (Szalontai *et al.* 1999). On the contrary, no effect of Cd up to 1000 μM, on these chlorophyll fluorescence parameters was observed (Szalontai *et al.* 1999). Higher doses (above 1 mM) of Cd promoted degradation of several PS II polypeptides (Nedunchezhian and Kulandaivelu 1995). Zinc at 1-5 mM was found to inhibit oxygen evolution in isolated chloroplast membranes, by release of Mn from the oxygen evolving

complex (Miller and Cox 1983). Further, the donor side of PS II was found to be a site of Zn interaction (Rashid *et al.* 1991). In comparison to PS II, the influence of Cd and Zn on PS I activity is far less known. It was found that Cd inhibited electron flow in PS I on its reducing side (Siedlecka and Baszyński 1993). However, other authors reported that in *Vigna*, 3-day treatment with Cd, in concentrations up to 9 mM, did not change significantly PS I activity as compared to PS II (Nedunchezhian and Kulandaivelu 1995). There are only limited data on inhibition of PS I activity by Zn (Myśliwa-Kurdziel *et al.* 2002).

The substitution of the Mg ion in chlorophyll (Chl) molecules by certain heavy metals (Cu, Zn, Cd, and Hg) has been shown to occur after prolonged (1-2 weeks) exposure of several species of submerged water plants like *Elodea*, *Callitriche*, *Lemna*, *Ceratophyllum* and the green alga *Scenedesmus* (Küpper *et al.* 1996). The formation of metal-substituted chlorophylls, mostly unsuitable for photosynthesis, was detected in these plants by *in vivo* absorption spectroscopy and by *in situ* fluorescence microscopy at room temperature (Küpper *et al.* 1996, 1998). Consequently, the chemical modification of photosynthetic pigments was postulated to play a role in the inhibition of photosynthesis under heavy metal stress in water plants (Küpper *et al.* 1996). However, information both on the susceptibility of Chl molecules in different photosynthetic complexes to modification by heavy metals and the effect of metal-modified chlorophylls on energy transfer in both PS I and PS II remains unknown.

Heavy metals are known to inhibit Chl synthesis at several points of its biosynthetic pathway (Myśliwa-Kur-

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dziel and Strzałka 2002; Bhattacharjee and Mukherjee 2003). One of the most common effects induced by heavy metals is the loss of chl from leaf tissues. As metal exposure continues, chlorosis increases progressively. Chlorosis is frequently expressed as the loss of specific forms of Chl, typically Chl *a* and Chl *b*, or as a change in the ratio between the two. Several reports suggest that the Chl *a:b* ratio increases as chlorosis progresses (Bhattacharyya and Choudhuri 1994; Moreno-Caselles *et al.* 2000; Bindhu and Bera 2001). These observations suggest that a specific pattern of Chl loss during metal-induced chlorosis represents either a direct altering of the two Chl pools or indirect influence by the altered metabolic process due to metal toxicity.

Recently it was established that Zn (an essential element) could alleviate stress induced by Cd (a non-essential element) in *C. demersum* L. The observed suppression of Cd uptake in the presence of Zn indicates a strong competition between Cd and Zn in plant cells (Aravind and Prasad 2003, 2004a, 2004b, 2005a, 2005b, 2005c). However, no information is available on the influence of the system: micronutrient/toxic metal on photosynthetic apparatus of water plants. To initiate a study on the interactive influence of toxic and micronutrient elements on the photosynthetic apparatus we have examined the mode of interaction between Cd and Zn with respect to energy transfer in leaves of free-floating macrophyte *Ceratophyllum demersum* L.

The fluorescence spectroscopy at 77 K is a useful tool for studying physical processes in the photosynthetic apparatus, especially excitation energy transfer (Butler and Kitajima 1975; Markwell *et al.* 1985). In particular, the fluorescence excitation spectra measured at 77 K allowed to semi-quantitatively characterize the contribution of particular photosynthetic pigments to energy transfer to PS II and to PS I *in situ* (Więckowski and Waloszek 1993).

In this study, low temperature fluorescence spectroscopy enabled *in situ* detection of changes in energy transfer from pigments energetically coupled to PS I and PS II, induced by Cd and Zn in *C. demersum*.

MATERIALS AND METHODS

Plant material and metal treatment

Ceratophyllum demersum L. (var. *brasiliensis*) from the collection of the Botanical Garden of the Jagiellonian University was cultured in an aquarium, at ambient temperature (22 ± 2°C) under shade in daylight. One week before the experiment began the plant material was transferred to modified Hoagland's medium, as described by Bonner and Galston (1959), diluted 1:10. Fragments of *C. demersum* stems containing apical meristems (fresh weight about 3 g) were treated for 48 h with Cd (cadmium sulphate) in concentrations of 10, 100 and 1000 μM and Zn (zinc sulphate) in concentrations of 100 and 1000 μM, in the culture solution, in 150 ml glass beakers. Zn supplementation was done by adding zinc sulphate (100 μM) to culture solutions containing increasing Cd concentrations as above. All experiments were performed on leaves taken from the apical part of the growing stem.

Whole-leaf fluorescence measurements

C. demersum leaves were loaded into capillary tubes and immediately frozen in liquid nitrogen. Fluorescence spectra were recorded at 77 K using an LS-50B (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK) spectrofluorometer with automatic correction for the wavelength dependence of the detection system. Fluorescence excitation spectra (380-580 nm) with emission at 682 nm (PS II reaction centers and LHC II antenna complexes) and 730 nm (PS I) were recorded (Więckowski and Waloszek 1993). The obtained spectra were normalized by a two-point algorithm according to following formulas:

(1) $F_n(\lambda_i) = F(\lambda_i) - F(\lambda_1)$

(2) $F_{norm}(\lambda_i) = F_n(\lambda_i) / F(\lambda_2)$

where:

$F(\lambda_i)$ is the fluorescence intensity at certain excitation wavelength (λ_i);

$F_{norm}(\lambda_i)$ is the normalized value of the fluorescence intensity;

$F_n(\lambda_i)$ is the fluorescence intensity value after the 1st step of normalization;

$F(\lambda_1)$ is the fluorescence intensity at minimum ($\lambda_1 = 555$ nm);

$F(\lambda_2)$ is the fluorescence intensity at maximum ($\lambda_2 = 438$ nm).

After normalization, the spectra of untreated plant material (control) were subtracted from the spectra of leaves incubated with different concentrations of heavy metals. The resulting differential spectra were presented and compared with respect to their shape and relative fluorescence intensity.

HPLC analysis of photosynthetic pigments

C. demersum leaves, dried by overnight lyophilization, were extracted with acetone: methanol (7:2 v/v) and centrifuged for 3 min at 4°C with a Sigma K-18 centrifuge (20,000 × *g*). The supernatant was evaporated under nitrogen gas and extracted pigments were dissolved in 98% ethanol. Pigments were separated on the Prostar HPLC system (Varian, Palo Alto, USA), equipped with a photodiode-array spectrophotometric detector Tidas I (World Precision Instruments, USA) and a Zorbax SB-C18 reversed phase column, 5 μm particle size (Agilent Technologies, Wilmington, USA), using the gradient method described by Lagarde *et al.* (2000). Samples (100 μl) were filtered by a stainless steel filter (φ = 0.22 μm) and loaded on a column equilibrated with solvent A (acetonitrile: water: triethylamine, 9:1:0.01 v/v/v). The column was eluted with a one-step linear gradient (15 min) of solvent B (ethyl acetate 100%) with a constant flow rate 1.5 ml/min. The absorption spectra of the eluate (380-800 nm) were recorded every 0.2 s. The relative content of each pigment was estimated by comparison of peak areas on chromatograms recorded at 440 nm and normalized per gram of the fresh weight of leaves. All experimental values were the means of at least three individual experiments.

Statistics

The values are means of three individual experiments (three independent batches of the plant material) with triplicates (value averaged to one) for each experiment. The results were subjected to statistical analysis using the Student's *t*-test ($n = 3$). The significance level (α) was set at 0.2 for fluorescence measurements and at 0.1 for pigment analysis.

RESULTS

The two-point normalization algorithm enabled a direct comparison of the shape of excitation spectra in the wavelength range including absorption maxima of main photosynthetic pigments (from 438 to 555 nm). The subsequent subtraction of the spectrum of untreated leaves (control) from the spectra of metal-treated material revealed semi-quantitative changes in the relative impact of particular pigments on the fluorescence from PS II/LHC II (682 nm) and PS I (730 nm). Thus, this approach allowed the detection and semi-quantitative analysis of pigments energetically coupled to reaction centers and antenna complexes in the photosynthetic apparatus *in situ*.

C. demersum leaves treated with Cd and Zn showed a remarkable increase of the fluorescence excitation bands with maxima at 482 and 486 nm, for emission observed at 682 nm. As 482 and 486 nm reflect absorption maxima of carotenoids, this result indicates the relative increase of the carotenoid contribution to the energy transfer both to reaction centers of PS II and to LHC II antenna complexes, which are characterized by emission at 682 nm (**Fig. 1**). In leaves treated with Cd or Zn only, this increase was most pronounced at highest metal concentrations applied (1000 μM), but Cd was *ca.* 30% more effective than Zn (**Fig. 1C**). Interestingly, if Zn at 100 μM was present in the culture medium during Cd treatment, the relative increase of carotenoid contribution to PS II/LHC II energy transfer was significantly lower at Cd concentration of 1000 μM (**Fig. 1C**). As can be seen in **Fig. 2**, Zn supplementation (100 μM) abolished the increase of carotenoid contribution (482 and 486 nm bands) to PS II/LHC II energy transfer induced by
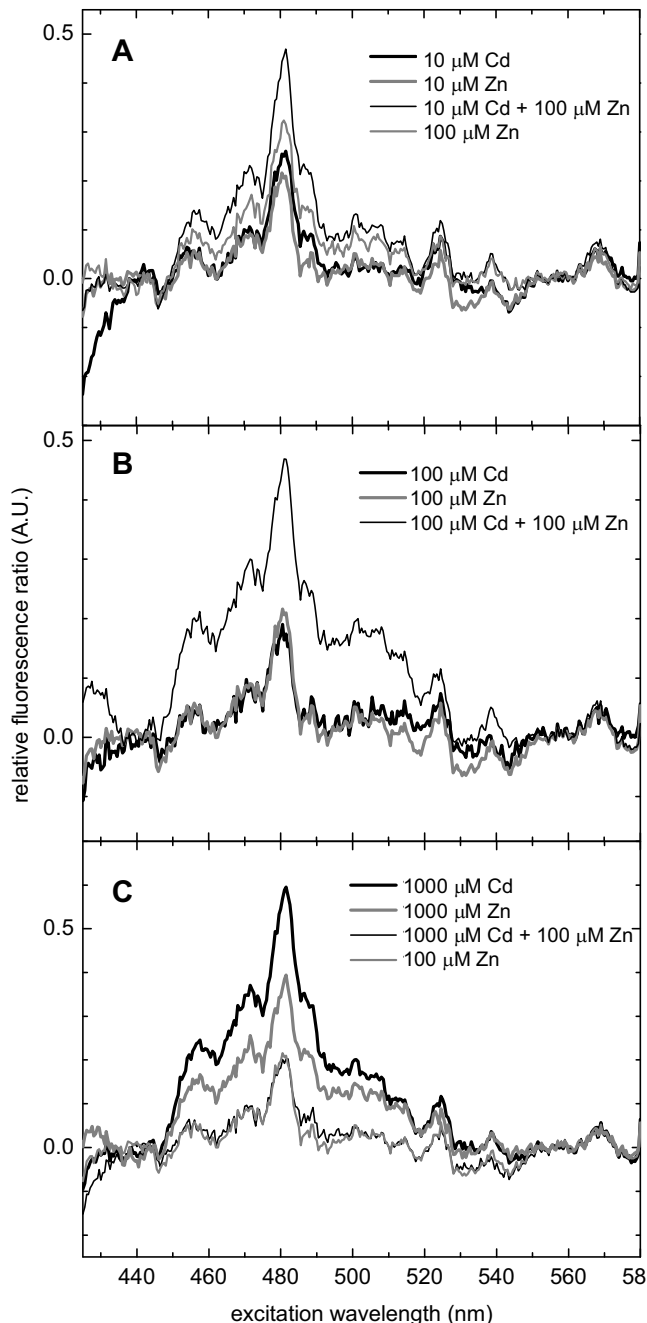
Fig. 1 Low temperature differential fluorescence excitation spectra for emission observed at 682 nm (PS II) of *C. demersum* leaves treated with increasing concentrations (10 μM - **A**; 100 μM - **B**; 1000 μM - **C**) of Cd (bold black lines), Zn (bold grey lines) and Cd in the presence of 100 μM Zn (light black lines). The spectrum of leaves treated with 100 μM of Zn is shown on panels **A** and **C** for comparison (light gray lines). The apparent changes in the relative intensity of bands with maxima at $\lambda_{max} = 482$ nm and $\lambda_{max} = 486$ nm are observed.

Cd at 1000 μM. In contrast, if Zn (100 μM) was applied simultaneously with lower Cd concentrations (10 and 100 μM) the elevation of both 482 and 486 nm bands was observed, indicating a relative increase of the carotenoid contribution to energy transfer (**Fig. 1A, 1B**).

The differential excitation spectra recorded for emission observed at 730 nm, a wavelength characteristic for PS I reaction centers, showed changes in emission intensity at 482, 486 and 526 nm (**Fig. 3**). However, the total level of these changes was *ca.* 60% lower in comparison to emission at 682 nm (**Fig. 3**) whereas excitation maxima at 482 and 486 nm could be correlated with carotenoids, the maximum 526 nm reflects energy transfer from pheophytin. As seen in **Fig. 4**, under our experimental conditions, the observed changes in carotenoid contribution to PS I fluores-
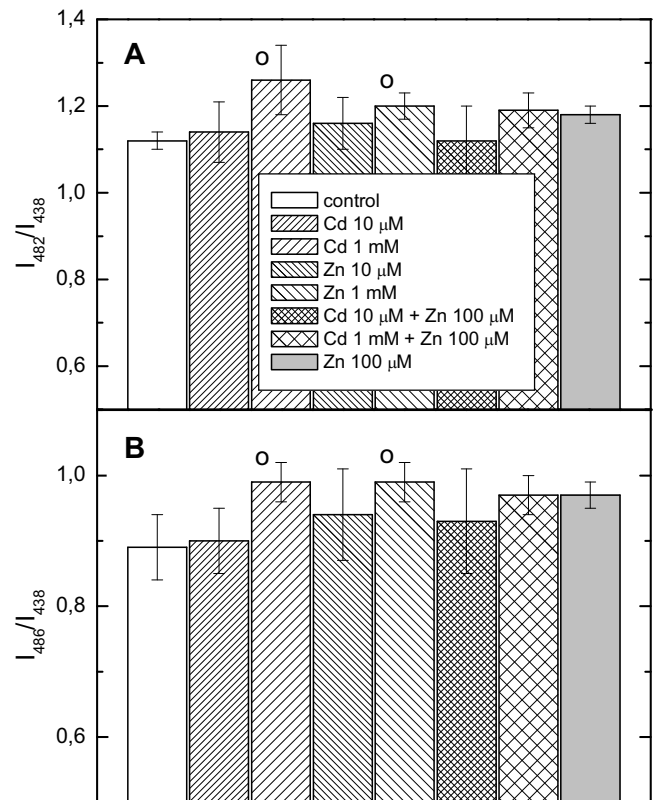

Fig. 2 The effect of different metal concentrations on the relative intensity of excitation band at $\lambda_{max} = 482$ nm for emission observed at 682 nm (PS II), normalized to the excitation intensity at $\lambda = 438$ nm. Open circles indicate differences statistically significant from the control material at $\alpha = 0.2$.

Table 1 The relative content of chlorophyll a in *Ceratophyllum* leaves as measured by HPLC. The values represent the area under Chl a peak on the chromatograms, recorded at 440 nm and normalized per gram of the fresh weight [mAbs*s/g FW] ±SE [%]. For details, see text.

Control	Cd (1000 μM)	Zn (1000 μM)	Cd (1000 μM) + Zn (100 μM)
5118 (±14.6%)	9430 (±15.9%)	9682 (±19.8%)	6289 (±14.9%)

cence were below the level of statistical significance.

The fluorescence excited at 526 nm, indicating an increase in the content of pheophytin energetically coupled to PS I, was slightly enhanced if Cd at low concentration (10 μM) was present in the culture medium (**Fig. 3A**).

To find out if the changes observed in low temperature fluorescence excitation spectra of *C. demersum* leaves treated with Cd and Zn correlate with putative changes in photosynthetic pigment composition, the photosynthetic pigments from *C. demersum* leaves were extracted and separated by reversed-phase HPLC. The relative amounts of main photosynthetic pigments (neoxanthin, violaxanthin, lutein, zeaxanthin, Chl *a* and *b*, β-carotene) were calculated as areas under respective peaks on chromatograms and normalized per gram of fresh weight of the leaf tissue. As it is shown in **Table 1**, the content of Chl *a* was significantly higher in *C. demersum* leaves treated with Cd (1000 μM) and Zn (1000 μM) than in leaves treated with Cd (1000 μM) in the presence of Zn (100 μM), where the increase of Chl *a* content was relatively lower in comparison to leaves treated with Cd or Zn (1000 μM). There was no statistical significance between control material and leaves treated with Cd in the presence of Zn. Also, all other observed differences in the pigment composition were below the level of statistical significance (data not shown).
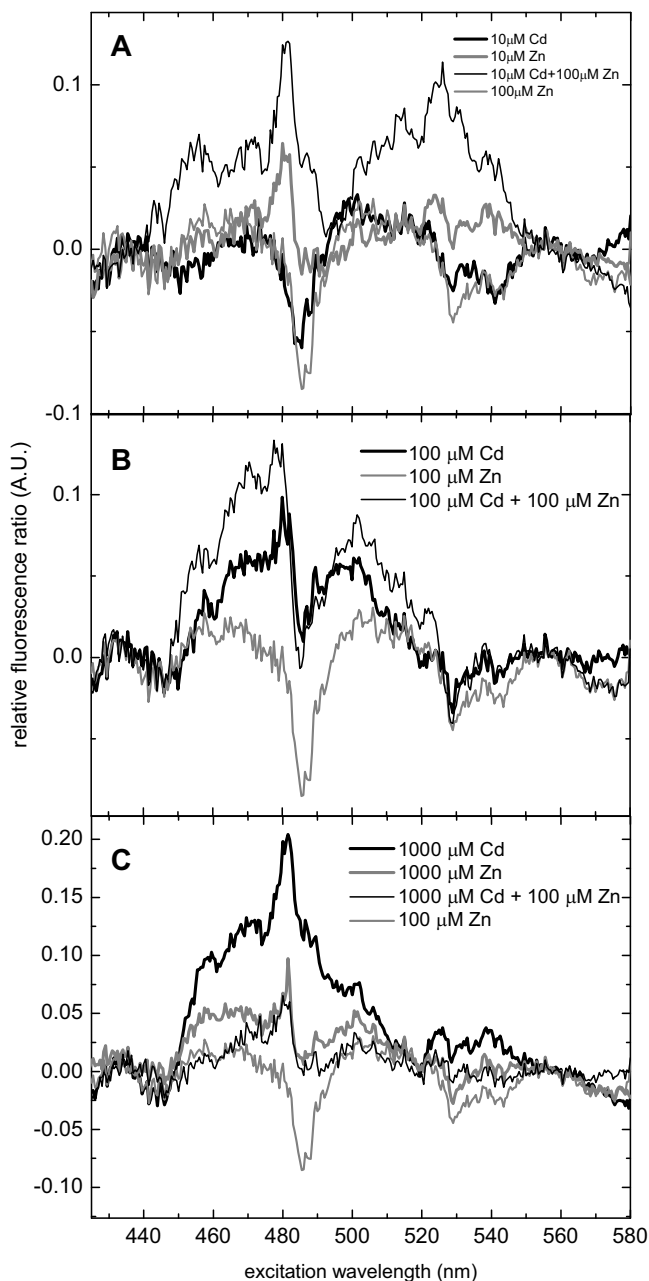
Fig. 3 Low temperature differential fluorescence excitation spectra for emission observed at 730 nm (PS I) of *C. demersum* leaves treated with increasing concentrations (10 μM - **A**; 100 μM - **B**; 1000 μM - **C**) of Cd (bold black lines), Zn (bold gray lines) and Cd in the presence of 100 μM Zn (light black lines). The spectrum of leaves treated with 100 μM of Zn is shown on panels **A** and **C** for comparison (light gray lines). The apparent changes in the relative intensity of bands with maxima at λ_{max} = 482 nm and λ_{max} = 486 nm are observed.

Fig. 4 The effect of different metal concentrations on the relative intensity of excitation bands at λ_{max} = 482 nm (**A**) and at λ_{max} = 486 nm (**B**) for emission observed at 730 nm (PS I), normalized to the excitation intensity at λ = 438 nm.

DISCUSSION

The results presented in this work indicate that Cd and Zn in concentrations up to 1000 μM can induce remarkable changes in energy transfer from carotenoids to reaction centers of PS II/LHC II complexes in *C. demersum* leaves. Cd at 1000 μM induced the most pronounced increase of the fluorescence attributable to energy transfer from the carotenoid pool (482 and 486 nm bands) from PS II/LHC II, after 48 hr incubation. A similar effect was observed at lower Cd concentrations (10 and 100 μM) if Zn (100 μM) was applied simultaneously with Cd. This result suggests that probably two carotenoid pools with different spectral characteristics may become energetically coupled to PS II/ LHC II in Cd- and Cd+Zn-treated *C. demersum* leaves.

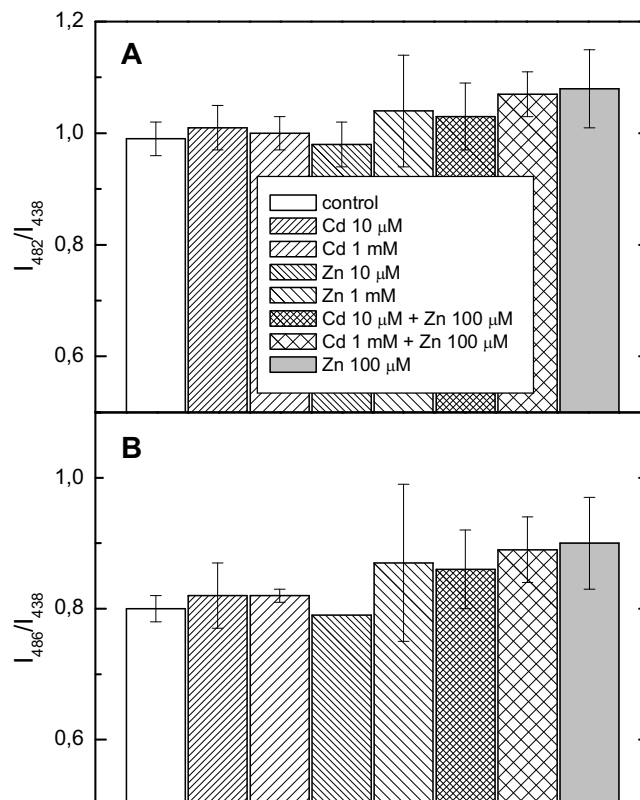Thus, our findings suggest that some carotenoids ex-posed and/or formed after Cd and Zn treatment become energetically coupled to photosynthetic reaction centers and continue to work as light-harvesting pigments in PS II/LHC II complexes under abiotic stress conditions. Alterations in the Chl:carotenoid ratio could appear in photosynthetic complexes as an indirect effect of heavy metal toxicity, e.g. after Cd-induced degradation of some complex subunits (Nedunchezhian and Kulandaivelu 1995), inhibition of Chl synthesis or accelerated senescence induction (Matile *et al.* 1999; Prasad and Strzałka 1999; Lu *et al.* 2001). Also, en-hanced carotenoid biosynthesis under mild stress conditions (Mazurek *et al.* 1990; Rai *et al.* 1998) may promote incor-poration of these compounds into photosynthetic complexes and consequently increase their impact on energy transfer. On the other hand, other authors reported that moderate doses of some heavy metals (Cu, Zn, Cd, Pb) induced a relative decrease in the content of total carotenoids in plant tissues (McFarlane and Burchett 2001; Fargasova 2004). Additionally, other, not-yet-defined structural and/or meta-bolic modifications may promote relative increase of caro-tenoid contribution to energy transfer in photosynthetic complexes after Cd treatment.

The observed intensity changes in fluorescence associ-ated with bands with maxima at 482 and 486 nm as induced by Cd were statistically significant only for PS II reaction centers. This suggests that in comparison to PS II/LHC II, PS I is structurally more resistant for the modification in-duced by heavy metals. This is in agreement with earlier observations in which PS II activity was generally more sensitive for heavy metal toxicity than PS I (Prasad 1995). However, the slight increase of pheophytin contribution to PS I energy transfer observed after 10 μM Cd treatment in the presence of Zn indicates that the action of these metals on PS I is complex and may involve several independent processes.

The presence of Zn (100 μM) together with Cd treat-ment suppressed the observed increase of carotenoid contri-bution to energy transfer to photosynthetic reaction centers in PS II/LHC II, induced by Cd at 1000 μM. Zn has been

found to inhibit the toxic influence of Cd on Chl content, lipid peroxidation, conductivity and total protein content in *C. demersum* (Aravind and Prasad 2003, 2004a, 2004b). Also, it has been reported that Zn decreased the toxic effects of Cd in hydroponic cultures of aquatic plants and *in vitro* cell cultures, when applied at equimolar concentration (Wajda *et al.* 1989; Chakravarthy and Srivastava 1997). Additionally, the role of Zn in protecting plant cells from damage by reactive oxygen species has been also postulated (Cakmak 2000). On the contrary, Zn (100 μM) applied together with Cd at 10 and 100 μM significantly enhanced carotenoid contribution to energy transfer in PS II/LHC II. This paradoxal effect may suggest that the Cd:Zn ratio could be critical for the final response of photosynthetic apparatus to stress induced by these metals.

Changes in the contribution of carotenoids to energy transfer, as observed by low-temperature fluorescence spectroscopy show no direct correlation with relative changes in concentrations of these pigments in untreated versus metal-treated *C. demersum*, as measured by HPLC. The remarkable increase of the Chl *a* content in leaves treated with Cd and Zn (1000 μM) is in agreement with our previous findings in *Lemna*, where Cd, in concentrations up to 2 mM stimulated the accumulation of chlorophylls (Prasad *et al.* 2001). The observed decrease of this Cd-induced stimulation of the accumulation of Chl *a* in the presence of Zn (100 μM) suggests that Zn may prevent effects of Cd on Chl biosynthesis. Alternatively, an increase in the ratio of Chl *a* to Chl *b* can be due to an increase in the ratio of PS I to PS II because PS I and its associated light-harvesting antenna have a higher fraction of their Chl as Chl *a*, compared to PS II/LHC II complexes, which are more sensitive to environmental stress (Prasad 1995; Nishiyama *et al.* 2005).

The fact that observed differences in the pigment composition (with the exception of Chl *a*) as measured by HPLC were below the level of statistical significance suggests that the observed metal-induced changes in energy transfer to PS II reaction centers and LHC II are not a direct effect of changes in concentrations of carotenoids in leaves. In several plant species Cd ions were found to inhibit activity of zeaxanthin epoxidase, and to affect the violaxanthin:zeaxanthin ratio (Latowski *et al.* 2005). In *C. demersum*, low doses of Cd (up to 10 μM) induced slight dose-dependent changes in the Chl *a*:carotenoid ratio (Kumar and Prasad 2004). We hypothesize that even very subtle changes in pigments energetically coupled to PS II and LHC II including e.g. the formation of metal-substituted chlorophylls (Kupper *et al.* 1996, 1998) or modifications of equilibria within the carotenoid pool, may result in significant changes of energy dissipation in pigment-protein complexes and, in consequence, alter an efficiency of energy transfer to reaction centers of PS II. This effect might be the cause of the impairment of PS II activity, even under low doses of Cd, observed by others both *in vitro* and *in vivo* (Li and Miles 1975; Chugh and Sawhney 1999).

Aquatic macrophytes, particularly *C. demersum*, play a significant role in biogeochemical cycling of toxic trace elements (Prasad 2004; Prasad *et al.* 2006). *C. demersum* has been employed for years in water quality assessment and as an *in-situ* bioremediator. It has been widely used to test the phytotoxicity of a variety of heavy metals in the microcosm, storm water streams, experimental ponds and field enclosures (Lewis 1995). The results presented in this study suggest that some toxic effects induced by Cd in aquatic plants might be, at least partially, a consequence of alterations in the dissipation of energy within pigment-protein complexes of the photosynthetic apparatus.

In conclusion, our results suggest that Cd induces changes of energy transfer in PS II/LHC II, even after a relatively short (48 h) exposure of plants to this ion. We hypothesize that qualitative and quantitative alterations in the pool of photosynthetic pigments energetically coupled to PS II reaction centers and LHC II complexes as induced by abiotic stress may be responsible for the changes in energy transfer efficiency observed in this study. Moreover, the presence of Zn in the environment may prevent effects on photosynthetic energy transfer induced by high concentrations of Cd in aquatic plants. The detailed molecular mechanisms of both the observed energy transfer changes and the protective Zn action on Cd-induced modifications of energy transfer in photosynthetic complexes need to be investigated further.

ACKNOWLEDGEMENTS

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