Introduction

The high toxicity of heavy metals for plant metabolism is well known (van Assche and Clijsters, 1990). Inhibition of growth (Titov et al., 1995), nitrate assimilation (Hernández et al., 1997), photosynthesis (Krupa and Baszyriski, 1995), and disturbances of ion- (Wallace et al., 1992) and water balance (Barceló and Poschenrieder, 1990) of plants have been described. The effect of heavy metals was shown to be considerably modified by the composition of the culture solution (Wallace et al., 1992) and the developmental stage of the plants (Skórzyńska-Polit and Baszyński, 1997).

Abbreviations: Chl, chlorophyll; CP, chlorophyll-protein; LHC, light-harvesting complex; PAGE, polyacrylamide gel electrophoresis; PS, photosystem.

Photosynthesis is extremely sensitive to heavy metals (Krupa and Baszyński, 1995). Multiple inhibitory effects of Cd and Pb have been observed at the level of Chl synthesis (Stobart et al., 1985; Padmaja et al., 1990; Böddi et al., 1995), photosynthetic electron transport (Bazzaz and Govindjee, 1974; Hampp et al., 1974; Siedlecka and Baszyński, 1993), activity of Calvin cycle enzymes (Weigel and Jäger, 1980; Malik et al., 1992; Siedlecka et al., 1997), and chloroplast ultrastructure (Barceló et al., 1988). Though the effects of heavy metals on photosynthesis have been intensively studied, their basic impact is still debated. Moreover, very few data are available about their influence on the chlorophyll containing thylakoid complexes (Krupa and Baszyński, 1995) which play important role in photosynthetic energy collection and transduction (Jansson, 1994; Green and Durnford, 1996), and the accumulation of which must be...
strongly influenced in the heavy metal poisoned, chlorotic leaves. Isolation and separation of chlorophyll-protein complexes from greening seedlings of radish with relatively harsh SDS solubilisation and SDS PAGE method revealed that only LHCII was influenced by Cd treatment, the oligomerisation process of which was found to be disturbed (Krupa et al., 1987; Krupa, 1987). In vitro studies showed that Cd at mM concentration caused major conformational changes in LHCII apoprotein by binding to its carbonyl groups or N atoms (Ahmed and Tajmir-Riahi, 1993).

We investigated the effects of Cd and Pb treatment on the pattern of chlorophyll-protein complexes obtained with a gentle isolation procedure in relation to ion content of leaves in order to find specific factors influencing the development or stability of the complexes. Plants were treated in young and more developed stage to understand the differences in the toxic effects of heavy metals on leaves of developing and mature plants.

**Materials and Methods**

The experiments were conducted on cucumber (*Cucumis sativus* L. cv. budai korai) grown hydroponically under standardised laboratory conditions in modified Hoagland solution of 1/4 strength (Fodor et al., 1998). Iron was supplied as Fe-EDTA or Fe-citrate in 4 μM concentration. Heavy metals, Pb(NO₃)₂ and Cd(NO₃)₂, were added to the culture solution in 10 μM concentration starting from one- or four-leaf stage of untreated plants. In some experiments Cd was supplied in lower (1 μM) and Pb in higher (50 μM) concentration. Five-week-old plants were harvested. They developed four (Cd treatment from the beginning) or seven leaves (untreated and all otherwise treated plants) during the five-week growing period. Numbering of leaves started from the oldest (lowest) one. The 7th leaves were of relatively small size, so they were not considered here. Chl content was determined in 80% acetone (Porra et al., 1989).

Chloroplasts were isolated according to Sárvári and Nyitrai (1994). Fluorescence emission spectra (excited at 440 nm) of chloroplasts suspended in isolation buffer and glycerine 1:1 (v/v) were measured at 77K with a Perkin-Elmer MPF-44B spectrofluorimeter. The Chl concentration of samples was 10 μg ml⁻¹.

Chlorophyll-proteins were separated by Deriphat PAGE using mainly glucosidic detergents (dodecyl sucrose : nonyl glucoside : lithium dodecyl sulfate = 4.5:4.5:1) for solubilisation (Sárvári and Nyitrai, 1994).

<table>
<thead>
<tr>
<th>Leaf storeys</th>
<th>Control plants</th>
<th>Plants treated from one-leaf stage</th>
<th>Plant treated from four-leaf stage</th>
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<tbody>
<tr>
<td></td>
<td>Chl a+b [μg cm⁻²]</td>
<td>Chl a/b</td>
<td>Chl a+b [μg cm⁻²]</td>
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<tr>
<td>Fe-EDTA</td>
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<td>28.2</td>
<td>3.31</td>
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<td>3.64</td>
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<td>4</td>
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<td>5</td>
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<td>Fe-citrate</td>
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<td>3.34</td>
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<td>2</td>
<td>33.9</td>
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<td>6</td>
<td>31.8</td>
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Polypeptide patterns were obtained on 10–18% gradient gels according to Laemmli (1970).

Metal contents were determined after a wet digestion with HNO\textsubscript{3}:H\textsubscript{2}O = 1:1 (v/v) by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Záray et al., 1995).

**Results and Discussion**

Leaf growth in the presence of heavy metals was strongly inhibited by Cd and only slightly influenced by Pb (Läng et al., 1999). The growth retardation was hardly seen on leaves of later treated plants emerged before the treatment in spite of the fact that they were still expanding (twofold in surface) during the treatment.

Pb treatment hardly influenced the accumulation of Chl. It was only moderately inhibited in plants grown with Fe-citrate and treated from one-leaf stage, but not affected or even stimulated by other types of Pb treatment (Table I). Chl \(a/b\) ratios were a little lowered only in plants treated with Pb from a younger age. The variations in whole-leaf Chl amounts (Fig. 1) were due to changes both in Chl contents (Table I) and growth (Läng et al., 1999).

The leaves developed under Cd treatment were not only smaller but also pale green. The decrease in the Chl content and Chl \(a/b\) ratio was the most pronounced in plants treated from a young age, particularly in the ones growing with Fe-citrate (Table I, Fig. 1). In later treated plants, the accumulation of Chl was inhibited and the Chl \(a/b\) ratio lowered only in the vigorously growing and newly emerging leaves (3\(^{rd}\) to 5\(^{th}\) leaves). These effects were moderate compared to the ones observed on the most imposed leaves (2\(^{nd}\) and 3\(^{rd}\)) of plants treated for a longer time. However, Chl synthesis proceeded normally in the older (but still expanding) leaves of later treated plants which were exposed to Cd for longer time and contained Cd in more or less the same (or higher) concentration as the chlorotic younger leaves (Figs. 1, 5). This is in agreement with the results obtained by Horváth et al. (1996) showing that neither the synthesis nor the photoreduction of protochlorophyllide was influenced by the Cd treatment, and points out that chlorosis must be the consequence of later steps connected with the stabilisation of the synthesised Chl. Therefore, the longer treatment and higher Cd concentration cannot be the only cause of the stronger effect. This seems to be more closely connected with the developmental stage of the leaves in the time of heavy metal exposition.

Thylakoids isolated from differently treated plants were solubilised and chlorophyll-protein complexes were separated on green gels to discover whether changes in the Chl accumulation were accompanied with distinctive alterations in the amounts of different chlorophyll containing complexes or not. In the chlorophyll-protein pattern of thylakoids (Fig. 2A) green bands were identified on the basis of their Chl \(a/b\) ratio and polypeptide composition (Sárvári and Nyitrai, 1994). Bands 1, 2 and 4 contained PSI with (1,2) and without (4) LHCI, the components of PSII core were found in bands 3, 5 and 6 in complex form (core complex with or without CP43).
Fig. 2. Chlorophyll-protein patterns of thylakoids (A) isolated from plants grown on Fe-EDTA and treated from their four-leaf stage (Co – control, numbers next to the treatment types refer to the examined leaf storeys). The green bands are numbered above the control densitogram. FP – free pigment. (B) Polypeptide patterns of thylakoids isolated from the second and third leaves of control (3) and Cd treated (from their one-leaf stage) plants (1,2) grown on Fe-EDTA (2,3) or Fe-citrate (1). Standard (St) proteins (in kDa): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), α-lactalbumin (14.4). Apo refers to apoprotein.

band 9 (monomeric CP43), the connecting antenna components of PSII were present in band 7 (oligomeric CP29) and bands 10, 11 (monomeric CP29, CP26, CP24 together with a very low amount of solubilised monomeric LHCII), and band 8 contained LHCII in oligomeric form. Densitograms of all more or less mature control leaf storeys (2nd to 5th) showed very similar band pattern (see the Chl a/b ratios of control leaves in Table I and the amounts of complexes in the different leaf storeys of control leaves in Fig. 4). The greatest reduction was observed in the PSI region of the leaf storeys of Cd treated plants that were highly chlorotic, while Pb treatment caused only slight change in the relative ratios of the chlorophyll-protein complexes (Fig. 2A). A decrease in PSI components (P700apo and LHClapo) could be observed also in plants treated in young age (Fig. 2B). In accordance, changes of the long wavelength fluorescence emission band related to the intact PSI particles (Rijgersberg et al., 1979) were observed in Cd stressed plants, but hardly seen in Pb treated ones: there was a decline (in young Cd treated plants) or a blue shift (in young leaves of plants treated later with Cd, and in plants treated with Pb at higher concentration) of the 737 nm band of chloroplasts (Fig. 3). The blue shift referred to disturbances in LHCII antenna accumulation or assembly. Using this mild method for the isolation and separation of chlorophyll-proteins, the oligomeric form of LHCII was not found to be unstable: all the remained LHCII was present in an oligomeric form in treated thylakoids (Fig 2A). The high monomer/oligomer ratio found by Krupa et al. (1987) may have been the consequence of the harsher solubilisation of the supramolecular LHCII complex, which was less stable due to the lower amount of specific phosphatidylglycerol necessary for oligomerisation (Trémolieres et al., 1981). Another possibility is that the rate of greening, including the oligomerisation process of LHCII (Dreyfuss and Thornber, 1994), is slower under Cd treatment, and later on the oligomeric state of LHCII in treated leaves becomes indistinguishable from that of the control.

In parallel with the lowering of Chl content, the absolute amount of all complexes decreased strongly in thylakoids developed under Cd treat-
ment, while Pb treatment was effective only at 50 μM concentration (Fig 4). Comparing the results of the same treatment on differently developed leaves having different Chl content, we found that Cd reduced the amount of chlorophyll containing complexes in the order of PSI > LHCII > PSII-core. Pb treatment influenced LHCII a little stronger than PSI, PSII being the most resistant as under Cd treatment. The higher resistance of upper leaves of plants treated in a more developed stage relative to leaves of young treated plants was supposedly due to the presence of healthy lower leaves, which might help the stabilisation of Chl in chlorophyll-protein complexes and their further growth serving as an energy, inorganic and organic matter source, and which bind or store the majority of heavy metals in a non-toxic manner. The reality of this explanation is presently tested by investigating the development of the photosynthetic apparatus under heavy metal treatment in a short time scale to determine the first appearance of differences. Changes of similar tendency were observed with both Fe-chelators, but the effects of heavy metals were more pronounced in Fe-citrate than Fe-EDTA. As the Cd content of leaves was hardly different depending on the Fe-chelator (Figs. 1, 5), the differences in the development of chlorophyll-protein complexes must be due to some other effect of chelators.

The extraordinary sensitivity of PSI and the relative stability of PSII in Cd treated plants were
similar to the pattern found in iron deficient (Abadia et al., 1989; Fodor et al., 1995) or senescent dicotyledonous (Jenkins et al., 1981; Roberts et al., 1987) plants. Though disturbed Fe metabolism is frequently mentioned as an effect of Cd treatment (Siedlecka and Baszyński, 1993; Alcántara et al., 1994), the examined parameters were rarely correlated with the total Fe content of leaves (Fig. 5, Láng et al., 1999). However, the total Fe content may not reflect the active Fe pool (Mengel, 1995). Therefore, the possibility of physiological iron deficiency needs further investigations. Moreover, the changes were not related to the heavy metal content of leaves. It was the highest in the first of the leaves which were intensively expanding after the treatment: first leaf in the plants treated from the beginning, and the third one in later treated plants. The closest relationship was found between the changing Mn content of the leaves and the measured parameters (Fig. 5). In plants treated from a young age a still better correlation could be seen if the absolute Mn content of treated plants was considered (not shown) because the leaf-storey pattern of Mn distribution was different in control plants (continuously decreased in higher leaf storeys) and Cd treated ones (lowest in the 2nd leaf). Hernández et al. (1998) also found that Cd strongly inhibited the uptake of Mn, and that iron uptake and concentration was less closely correlated with the Cd treatment. Interestingly, the water content of leaf blades seemed to be inversely affected by the Cd treatment.

With respect to premature senescence, Fig. 1 did not refer to Cd induced Chl catabolism rather Cd effect somehow seemed to be exerted at the level of formation or stabilisation of chlorophyll containing complexes. Horváth et al. (1996) also came to the same conclusion on the basis of their greening experiments. Cd induced nitrate deficiency and inhibition of nitrate reductase activity (Hernández et al., 1997), as well as Fe or Mn deficiency (Fig. 5, Hernández et al., 1998) may be the cause of chlorosis. Nitrogen deficiency disturbs (apo)protein synthesis. Fe and Mn are important components of numerous enzymes including the complexes of the photosynthetic electron transport chain. Thus their deficiency may interfere with many biochemical processes in the cell, including the development of the photosynthetic apparatus.

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**Fig. 5.** The relationships between the changes in Chl content, water content (both expressed in µg cm⁻²), the Chl a/b ratio and the metal contents (nmol metal cm⁻²) of leaf blades. Plants were grown in the presence of Fe-EDTA (A,B) or Fe-citrate (C,D) and treated from their one-leaf (A,C) or four-leaf stage (B,D). Lines were drawn just to show the tendency of changes along the successive leaf storeys.
work was financially supported by research grants from EEC (CT930202 and IC-15-CT98-0126) and OTKA (F-021004).

Acknowledgements

The kind supply of Deriphat by Henkel Corp. (Hoboken, USA) is gratefully acknowledged. This


