

Relationship between Changes in Ion Content of Leaves and Chlorophyll-Protein Composition in Cucumber under Cd and Pb Stress

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Hydroponically cultured cucumber plants supplied with 4 μM Fe chelated either with EDTA or citrate and treated with Cd (10 μM) and Pb (10, 50 μM) from their one- or four-leaf stage were grown up to five-week-old age. The decrease in the chlorophyll content was the most pronounced in plants treated with Cd from a younger age, and in the case of Fe-citrate. The chlorophyll *a/b* ratio of Cd stressed plants was also significantly lowered. In later treated plants the accumulation of chlorophyll was inhibited and the chlorophyll *a/b* ratio decreased only in the vigorously growing young leaves. Pb treatment had only a slight effect on both parameters. The changes in the chlorophyll-protein pattern of thylakoids were strongly related to their chlorophyll content but the response of each complex was different. Cd reduced the amount of chlorophyll containing complexes in the order of photosystem I > light-harvesting complex II > photosystem II-core, while light-harvesting complex II appeared somewhat more sensitive than photosystem I in Pb treated plants. In accordance, a decline or blue shift of the long wavelength fluorescence emission band of chloroplasts was observed referring to disturbances also in photosystem I antenna assembly. The accumulation of chlorophyll and chlorophyll-proteins did not show close relationship to the heavy metal content of leaves which was the highest in the first of the intensively expanding leaves in the time of the treatment. The extraordinary sensitivity of photosystem I, and the relative stability of photosystem II under Cd treatment were similar to the case found in iron deficient plants. However, the pattern of chlorophyll content of leaf storeys of Cd treated plants rather followed the changes in their Mn content.

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 Baszyński, 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).

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 Durnfold, 1996), and the accumulation of which must be

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

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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, $\text{Pb}(\text{NO}_3)_2$ and $\text{Cd}(\text{NO}_3)_2$, 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 $\mu\text{g ml}^{-1}$.

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 I. Chl content and Chl *a/b* ratios of the relatively well developed leaves of control, Cd (10 μM), and Pb (10 μM) treated cucumber plants. They were grown either with Fe-EDTA or Fe-citrate, and were treated from a young (one-leaf) or more developed (four-leaf) stage up to their harvesting. The standard deviation was 10–15% and 2–5% of the given Chl content and Chl *a/b* ratio values, respectively.

Leaf storeys	Control plants		Plants treated from one-leaf stage				Plant treated from four-leaf stage			
	Chl <i>a+b</i> [$\mu\text{g cm}^{-2}$]	Chl <i>a/b</i>	Chl <i>a+b</i> [$\mu\text{g cm}^{-2}$]		Chl <i>a/b</i>		Chl <i>a+b</i> [$\mu\text{g cm}^{-2}$]		Chl <i>a/b</i>	
			Cd	Pb	Cd	Pb	Cd	Pb	Cd	Pb
Fe-EDTA										
1	28.2	3.31	10.4	27.6	1.79	3.16	29.6	31.7	3.26	3.47
2	31.2	3.55	5.9	33.9	2.06	3.25	26.7	32.0	3.45	3.56
3	33.1	3.64	8.3	33.3	2.47	3.15	22.2	35.1	3.31	3.55
4	31.4	3.57	9.9	34.8	2.75	3.15	18.7	36.5	3.14	3.52
5	29.1	3.51	–	30.0	–	3.35	12.7	36.1	3.28	3.56
6	32.1	3.38	–	26.4	–	3.41	22.5	32.4	3.03	3.57
Fe-citrate										
1	33.7	3.34	11.2	34.0	1.93	3.03	31.0	32.0	3.28	3.10
2	33.9	3.45	5.3	30.7	2.18	3.22	29.8	37.0	3.40	3.28
3	35.9	3.46	6.5	31.1	2.49	3.22	23.9	36.8	3.03	3.43
4	37.7	3.43	9.3	29.6	2.84	3.27	19.8	40.1	2.95	3.37
5	31.3	3.37	–	25.7	–	3.26	14.2	33.5	2.81	3.41
6	31.8	3.37	–	25.0	–	3.37	12.7	32.8	2.92	3.47

Polypeptide patterns were obtained on 10–18% gradient gels according to Laemmli (1970).

Metal contents were determined after a wet digestion with $\text{HNO}_3\text{:H}_2\text{O} = 1\text{:}1$ (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 (Láng *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 (Láng *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 (3rd to 5th leaves). These effects were moderate compared to the ones observed on the most imposed leaves (2nd and 3rd) 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), and in

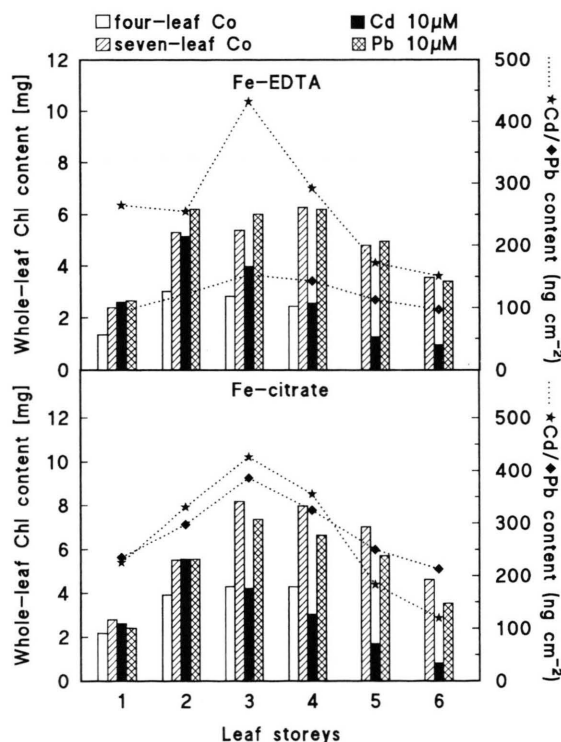


Fig. 1. Accumulation of Chl in leaves of untreated, Cd and Pb treated plants during their growth from their four-leaf to seven-leaf stage. Treatment with heavy metals started when plants were in four-leaf stage. Chl content of whole leaves was calculated before the treatment (four-leaf Co) and in the fully developed untreated (seven-leaf Co), Cd and Pb treated plants. Cd and Pb contents were determined in leaves of fully developed plants. The data of the young 7th leaf were omitted.

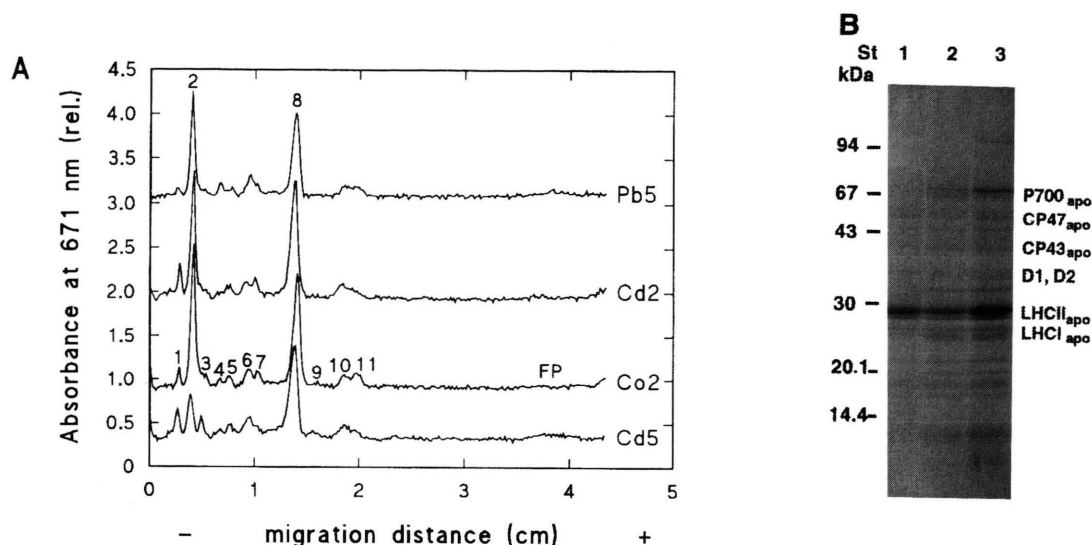


Fig. 2. Chlorophyll-protein patterns of thylakoids (A) isolated from plants grown on Fe-EDTA and treated from their four-leaf stage (Co – control, numbers next to the treatment types refer to the examined leaf storeys). The green bands are numbered above the control densitogram. FP – free pigment. (B) 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 LHCI), 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 (P700_{apo} and LHCI_{apo}) 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 LHCI 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 supra-molecular LHCII complex, which was less stable due to the lower amount of specific phosphatidyl-glycerol 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-

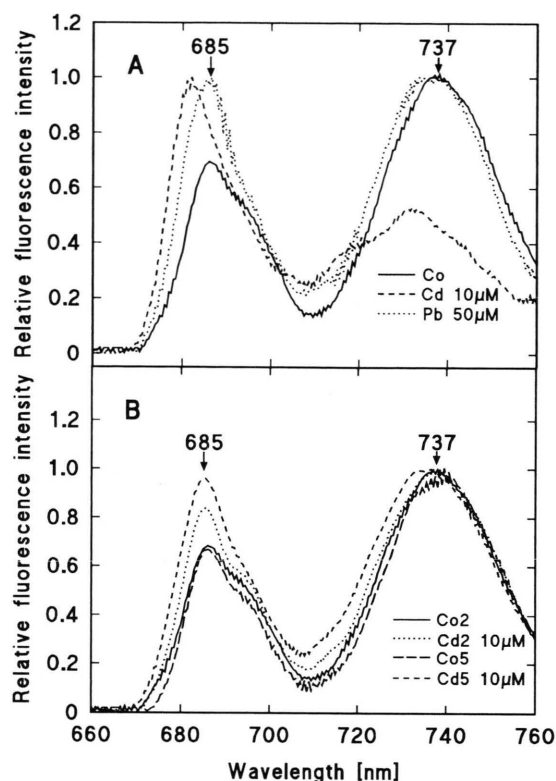


Fig. 3. 77K fluorescence emission spectra (excited at 440 nm) of chloroplasts isolated from the second and third leaves of plants treated from one-leaf stage (A), and from the given leaf storeys (numbers following the type of treatment) of plants treated from their four-leaf stage (B), and grown with Fe-citrate.

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 $\text{PSI} > \text{LHCII} > \text{PSII}$ -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

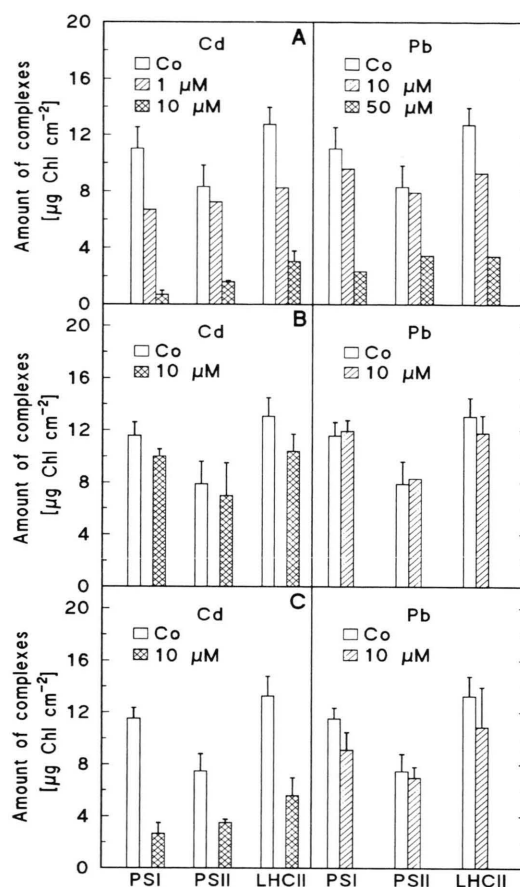


Fig. 4. The absolute amounts of chlorophyll containing complexes of differently treated cucumber plants: second and third leaves of plants treated from one- (A) or four-leaf stage (B), and fifth and sixth leaves of plants treated from four-leaf stage (C). The amount of a given complex shown here was calculated by summing up the densitogram bands corresponding to this complex. Then the Chl content of leaves was shared among the complexes according to their relative proportions.

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.

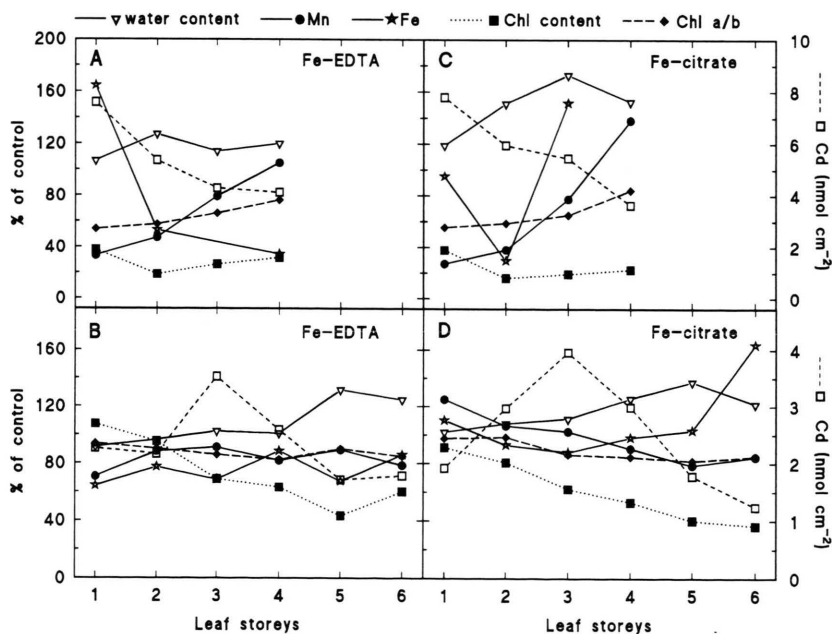


Fig. 5. The relationships between the changes in Chl content, water content (both expressed in $\mu\text{g cm}^{-2}$), the Chl *a/b* ratio and the metal contents ($\text{nmol metal cm}^{-2}$) of leaf blades. Plants were grown in the presence of Fe-EDTA (A,B) or Fe-citrate (C,D) and treated from their 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.

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