

# The effect of Cd and Zn contents in plants on Fe binding into organic substances of spinach biomass

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## ABSTRACT

Partial characterization of iron binding compounds in spinach above ground biomass and differences between binding of Cd, Zn and Fe in spinach biomass were investigated on two soils treated by sewage sludge in model pot experiment. After application of sludge to both soils, Cd and Zn accumulation in spinach biomass was increased in sludge treatments. Total Fe content in biomass showed opposite trend with these treatments and was decreased 2.5–2.7 fold. Results of sequential analysis of spinach biomass showed lower portions of iron in all individual fractions of both sewage sludge treatments compared to control treatments. The largest differences in iron content between control and sludge treatments were determined in ethyl acetate fraction. While majorities of Cd and Zn were found in fractions methanol + H<sub>2</sub>O and H<sub>2</sub>O and methanol + H<sub>2</sub>O + HCl, iron content determined in these two fractions was only 7–8% in control treatments and 15–20% in sludge treatments of total iron. In fraction, Fe portion was 87.7–90.7% in treatments without sludge and 76.1–83.5% in sludge treatments in spinach biomass. Results showed tighter iron binding to plant cell membranes in contrast to cadmium and zinc.

**Keywords:** iron; Fe-binding compounds; sequential extraction; spinach; cadmium; zinc

The use of sewage sludge in agriculture as organic manure is now widespread practice. Sewage sludge is a potential source of organic matter and nutrients for plants and soil microflora, on the other side it contains potentially hazardous trace elements. These elements can affect plant mineral composition. Cadmium and zinc can easily interact with iron, one of the most important elements for plant growth and metabolism. Iron is the essential micro nutrient playing important role in many redox reactions. Iron is present in heme proteins (a, b, c types of cytochromes and siroheme proteins), in bonds of non-heme iron and acid-labile sulphur proteins (for example ferredoxin) and in other iron-sulphur proteins (ferritin). It is a constituent of catalytic enzymes (catalase, peroxidase, nitrogenase etc.) (Marschner 1995). Free ions of iron can be extremely toxic. Hence, plants have developed extra cellular binders – siderophore storage systems and ferritins (Prasad and Hagemeyer 1999). Ferritin plays a critical role in plants not only by acting in buffering Fe but also by protecting the cell from heavy metal toxicity.

Heavy metals, in particular Cu and Zn are known to replace Fe from chelate complexes forming corresponding heavy metal chelates. This may be important in limiting Fe uptake and utilization, either by reduction of Fe chelate translocation to roots or within the plant itself by the effect of the heavy metal on centers of physiological activity for Fe. An excess of Zn leads to a significant reduction in Fe concentration in plants. According to Cakmak (2000) Zn deficiency in plants increased iron concentration. This effect of Zn concentration on Fe absorption by plant *Mentha arvensis* L. was studied by

Misra and Ramani (1991). They found that Zn toxicity induced a Fe deficiency in this plant.

Siedlecka and Krupa (1996) have reported generally much more pronounced toxic effect of cadmium on plant growth parameters under iron deficiency conditions than under normal iron supply. According to Siedlecka and Krupa (1999) strong Fe dependency to Cd mobility within the plant and adaptation of photosynthetic dark phase to Cd stress were found. Sandalio et al. (2001) investigated the activity changes of chloroplastic and cytosolic superoxiddismutases (SOD) caused by Cd. Fe-SOD was affected in lesser extent in contrast to strong reduction of Cu and Zn-SOD. The inhibition of root Fe (III) reductase induced by Cd led to Fe (II) deficiency affecting photosynthesis (Alcantara et al. 1994).

Main objective of our study was focused on partial characterization of Fe binding compounds and differences between bounds of Cd, Zn and Fe in spinach biomass.

## MATERIAL AND METHODS

The experimental plant spinach (*Spinacia oleracea* L.) var. Monores was grown up to full leaves development on Fluvisols and Chernozems treated by sewage sludge in model pot experiment. Fresh homogeneous sewage sludge with 26–28% of dry matter was used in this experiment (Tables 1 and 2). Pot experiment was described by Tlustoš et al. (2001). After harvest, an above ground spinach biomass was gently washed with deionised water, dried, ground and analyzed for total cadmium, zinc

Table 1. Soil type, amount of organic matter and sorption capacity of soils used in the experiment

Treatment	Soil type	pH <sub>KCl</sub>	C <sub>ox</sub> (%)	CEC (mval.kg <sup>-1</sup> )
1	Chernozems	7.2 ± 0.2	1.83 ± 0.41	258 ± 4
2	Fluvisols	4.8 ± 0.4	0.82 ± 0.15	77 ± 13

and iron content. Plant material was decomposed by dry ashing procedure (Mader et al. 1989). The determinations of element concentration were performed by atomic absorption spectrometry (VARIAN SpectraAA-300) with flame and flameless atomization. Quality of plant analyses was verified by reference material RM 12-02-03 Lucerne with certified contents of Cd = 0.136 ± 0.003 mg.kg<sup>-1</sup>, Zn = 33.2 ± 0.5 mg.kg<sup>-1</sup>, Fe = 354 ± 18 mg.kg<sup>-1</sup> for which we obtained Cd = 0.143 ± 0.021 mg.kg<sup>-1</sup>, Zn = 33.2 ± 1.4 mg.kg<sup>-1</sup>, Fe = 323 ± 31 mg.kg<sup>-1</sup>.

For sequential analysis, 0.5 g of grounded spinach dry matter was weighed into column with fritted disc. Extraction solvent was added and stirred with sample. Time of each of first three extractions was 24 hours and time of following extractions was 48 hours. Extraction was performed at laboratory temperature (22–24°C). Fractions of each solvent were collected and evaporated to dryness (40°C). Extraction by solvent was finished at constant weight of each individual fraction.

Sequential analysis of spinach dry matter was conducted according to extraction scheme designed by Pavlíková et al. (2002) (Table 3). Infrared (IR) spectrum of isolated fractions was measured by IR spectrometer (Brucker IFS 88). The determination of element concentration in isolated fractions was performed by atomic absorption spectrometry (VARIAN SpectraAA-300).

## RESULTS AND DISCUSSION

After application of sludge to both soils, Cd and Zn accumulation in spinach biomass was increased by sludge treatments. The increase of Cd accumulation in plant biomass after application of sewage sludge to the soil was also confirmed by Balík et al. (2000). Total Fe content in biomass in these treatments decreased 2.5–2.7 fold (Table 4). Results of sequential analysis of spinach biomass also showed lower portions of iron in all individual fractions of both sewage sludge treatments compared to control treatments (Table 5).

Iron in petroleum ether fraction (A) formed the lowest part of total Fe content (0.08–0.9%). Iron portion was significantly affected by soil type and availability of the element and was one order higher at Fluvisols than Chernozems. Content of this element determined in sludge treatments was by 10–15% lower compared to control treatments. Higher content of iron was obtained in both treatments of Fluvisols containing higher portion of plant-available cadmium and zinc, compared to Chernozems. Iron was bound to long chain hydrocarbons in membranes by chelate bonds to this fraction.

The largest differences in iron content between control and sludge treatments were determined in ethyl acetate fraction (B). Iron content in sludge treatments represented only 25–28% of the iron in control treatments and Cd and Zn contents decreased in sludge treatments as well (Pavlíková et al. 2001, 2002). Cd and Zn contents in sludge treatments equaled 23–29% of cadmium and 12% of zinc in the control treatments. Decreased content of all measured elements showed non-specific binding of these elements to isolated compounds in this fraction. According to our previously published results, elements were bound to saturated and unsaturated fatty acids in the ethyl acetate fraction. The lower weight of isolated ethyl acetate fraction was simultaneously observed and it may be showed that metabolism of isolated compounds was inhibited by toxins from sludge in these treatments.

Methanol fraction (C) mainly contained carboxylic acids, probably acids of the citric acid cycle, amino acids, aromatic, and pyrrole substances (Table 3). Dry mass of this fraction was dominant for all treatments. However, Fe associated with fraction C represented only 0.4–1.5% of total iron content. Fe amounts in treatments without sludge were two fold higher in contrast to sludge treatments. Cd amounts in samples treated and untreated by sludge also represented a low portion of the total cadmium content 1.4–3.9%. Different portion of Zn 11.6–32.8% was determined in fraction C (Pavlíková et al. 2001). We can suggest formation of iron-citrate and iron-malate complexes in plants. Citrate is able to bind other metals

Table 2. Cd, Zn and Fe total content (mg.kg<sup>-1</sup>) in soils and sewage sludge

Treatment	Cd <sub>T</sub>	Zn <sub>T</sub>	Fe <sub>T</sub>
Chernozems	0.321 ± 0.065	145 ± 38	25 713 ± 1 372
Fluvisols	0.147 ± 0.033	70.9 ± 7.5	5 138 ± 730
Sewage sludge	3.68 ± 1.03	1 438 ± 10	17 408 ± 1 184

Table 3. Sequential extraction scheme of spinach biomass and substances in isolated fractions

Fractions	Extraction solvents	Substances in isolated fraction
A	petroleum ether (water free)	saturated and unsaturated long chain hydrocarbons with or without keto group
B	ethyl acetate (water free)	saturated and unsaturated fatty acids
C	methanol (water free)	different carboxylic acids, probably not fatty acids, but acids for example from citric cycle or free amino acids aromatic substances and pyrrole substances
D	methanol + H <sub>2</sub> O (1 + 1; v/v) and H <sub>2</sub> O	substances with amidic bond, for example oligopeptides or extractable polypeptides and proteins, extractable soluble oligosaccharides
E	methanol + H <sub>2</sub> O + HCl (36%) (49.3 + 49.3 + 1.4; v/v/v)	substances with amidic bond, extractable hydrolysate substances from cell walls or cytoskeleton (oligosaccharides, polysaccharides, proteins, glycoproteins)
F	non-extractable residues	

(mainly Zn-citrate complexes), too, but these are not held as tightly as Fe (Brooks 1998).

While majorities of Cd and Zn were found in fraction methanol + H<sub>2</sub>O and H<sub>2</sub>O (D) and in the fraction methanol + H<sub>2</sub>O + HCl (E) (83–96% of total Cd amount and 59.9–86.3% of total Zn amount in sum of these fractions), iron content determined in these two fractions represented only 7–8% in control treatments and 15–20% in sludge treatments of total iron. For this reason, fraction D is one of the most important fractions for metals metabolism. Phytochelatin, metallothioneins, non-heme proteins, enzymes, phytoferritin and various chelating agents – mugineic acid (Takemoto et al. 1978), deoxymugineic acid, avenic acid (Fushiya et al. 1981), amino acid nicotianamine (Buděšínský et al. 1980, Rudolph et al. 1985) can be present in fraction D. Nicotianamine has an optimal molecular structure for chelating iron ions and is considered to be a phytosiderophore with an essential function in cellular iron transport and/or metabolism. Walker and Welch (1987) reported these compounds to form stable anionic complexes with several trace elements. Fraction E (methanol + H<sub>2</sub>O + HCl) contained compounds isolated from cell walls and cytoskeleton after hydrolysis.

In fraction F non-extractable residues were determined. Fe portion of 87.7–90.7% in treatments without sludge and 76.1–83.5% in sludge treatments was found in spinach biomass (Table 5). The amount of Fe determined in this fraction was the highest (90.7%) in treat-

ment with the lowest total Cd and Zn contents. Compounds contained in residues were non-extractable by the used procedure. We expect the presence of cytochromes in this fraction. Cytochromes are associated with the particulate fraction of cell organelles (mitochondria, microsomes and chloroplasts) and they are all bound tightly to the membranes, making extraction difficult. Only cytochrome C is isolated relatively easily from plants. It is located on outside surface of cell membranes (Voet and Voetová 1995). It is probably extracted in the methanol fraction.

Results showed tighter iron binding to plant cell membranes in contrast to cadmium and zinc. We can suggest tight iron bonds as a protection mechanism of the cell against replacement of Fe from chelate complexes by forming corresponding heavy metal chelates in metabolically important processes. For more precise characterization of Fe binding compounds in non-extractable residues, there is a necessity to change the conditions for destruction of cellular biomass (Pavlíková et al. 2002). For extraction by methanol + H<sub>2</sub>O + HCl prolongation of extraction time and/or change of composition of the extraction solvent will be tested in this step. Higher portion of HCl probably will increase hydrolysis of iron bonds to plant cell membranes in this case.

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Table 4. Cd, Zn and Fe content (mg.kg<sup>-1</sup>) in spinach biomass

Treatment	Cd	Zn	Fe
1	2.11 ± 0.07	149.4 ± 2.6	1 610 ± 13
1 + sewage sludge	2.76 ± 0.18	156.9 ± 3.8	604 ± 9
2	3.12 ± 0.23	210.5 ± 1.4	856 ± 8
2 + sewage sludge	4.24 ± 0.25	523.4 ± 5.1	335 ± 4

Table 5. Content of iron in fractions isolated from spinach biomass (mg.kg<sup>-1</sup> dry biomass)

Fractions	Extraction solvents	Treatment			
		1	1 + sewage sludge	2	2 + sewage sludge
A	petroleum ether	1.18	1.02	2.64	2.40
B	ethyl acetate	6.29	1.63	6.42	1.80
C	methanol	13.22	5.89	13.08	7.88
D	methanol + H <sub>2</sub> O and H <sub>2</sub> O	77.09	61.46	45.31	39.92
E	methanol + H <sub>2</sub> O + HCl	51.86	30.33	37.76	27.94
F	non-extractable residues	1 461	504	751	255

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## ABSTRAKT

### Vliv obsahu Cd a Zn v rostlinách na vazby Fe s organickými látkami v biomase špenátu

V nádobovém pokusu byl na dvou zeminách (černozem a fluvizem) s přidavkem čistírenského kalu pěstován špenát. Po aplikaci čistírenského kalu došlo k nárůstu celkového obsahu Cd a Zn v nadzemní biomase, obsah Fe se naopak snížil 2,5–2,7krát. Také výsledky sekvenční analýzy rostlinné biomasy ukázaly pokles obsahu Fe ve všech frakcích špenátu pěstovaného na

variantách s kalem v porovnání s kontrolními variantami. Největší rozdíl v obsahu Fe mezi variantami hnojenými kalem a kontrolními byl stanoven v etylacetátové frakci. Obsah Fe ve variantách hnojených kalem tvořil pouze 26–28% obsahu Fe kontrolních variant. Fe stanovené ve frakci extrahované metanolem (citráty a maláty Fe) představovalo pouze 0,4–1,5% celkového obsahu Fe. Největší množství Cd a Zn bylo stanoveno v frakcích metanol + H<sub>2</sub>O a H<sub>2</sub>O (fytochelatin, metallothioneiny, nehemové proteiny, enzymy, fytoferritin apod.) a metanol + H<sub>2</sub>O + HCl, zatímco obsah Fe v těchto frakcích tvořil na kontrolních variantách pouze 7–8 % a na variantách s kalem 15–20 % celkového Fe. Podíl Fe v neextrahovatelném zbytku (obtížně extrahovatelné sloučeniny) tvořil ve variantách bez kalu 87,7–90,7 % a ve variantách s kalem 76,1–83,5 %. Nejvyšší množství Fe v této frakci bylo stanoveno ve variantě 1, kde byla současně analyzována nejnižší koncentrace Cd a Zn. Výsledky ukazují na rozdíl od Cd a Zn pevnou vazbu Fe v rostlinných buněčných membránách. Lze předpokládat, že pevná vazba v chelátových komplexech Fe chrání proti nahrazení rizikovými prvky.

**Klíčová slova:** železo; sloučeniny poutající Fe; sekvenční extrakce; špenát; kadmium; zinek

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