Chapter 19

Effect of Cadmium and Humic Acids on Metal Accumulation in Plants

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Abstract

The natural ability of plants to accumulate, exclude or stabilize elements could be exploited to remediate soils contaminated with metals. To implement this alternative technology termed phytoremediation, it is crucial to better understand the various processes controlling metal mobilization or immobilization, uptake, and sequestration by the plants. Metal chelation is recognized as a vital biological process that regulates metal solubility, bioavailability, and internal storage in plants. Natural ligands, e.g. soil humates, root exudates components, or synthetic chelators, i.e. ethylenediaminetetraacetic acid or EDTA, can interact in a yet-to-be-defined way to influence metal uptake and sequestration by plants. Here, we investigated the interactive effect of Cd and soil humates on metal acquisition and translocation in wheat plants. Metal contents in tissues and root exudates composition were determined, using X-ray fluorescence for metals and gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) for organic exudates. Cd inhibited biomass production, from –55% to –65% on tissue dry weight basis, and greatly reduced root exudation, of about –84% by dry weight. Cd treatment also resulted in a substantial co-accumulation of transition metals (Fe, Ni, Cu, Zn) and Cd in wheat roots. Moreover, co-treatment with humates alleviated some of the Cd effect showing biomass inhibition reduced by about 10% for the tissues and 17% for the exudates, while accumulation of some metals (Zn, Cu, Ni, Cd) in the root was enhanced. Thus, under Cd treatment, with or without humate, the enhanced accumulation of metals was not mediated via root exudation. This is contrary to the exudate-mediated Fe acquisition under Fe deficiency. The mechanism for this phenomenon is being sought.

Key words: humic acids, cadmium, root exudates, metal ion ligands, phytoremediation

19.1
Introduction

Toxic metals are released into the biosphere through various anthropogenic activities including mining operations, industrial and domestic waste discharges, combustion of fuels, and agricultural operations. Upon accumulation in organisms, many of these metals induce toxic damage, thus threatening both human and ecosystem health (Alloway 1990). To address metal contamination issues, both engineering and in-situ bioremediation are used, with the latter approach generally expected to be more economical for very large-scale remediation efforts. This is particularly the case for plant-based remediation, termed phytoremediation. Furthermore, basic understanding of the mechanisms of phytoremediation is indispensable for assessing biological risk of contamination in already-vegetated sites, or in situations where the goal of remediation is incompatible with physical-chemical treatments, e.g. where the goal is to protect the habitat or the agricultural viability of the soil.

There are three main approaches that can be utilized in phytoremediation of metals: phytoextraction, phytovolatilization, and phytostabilization (Cunningham and Ow 1996). Plants have evolved an extensive ability to mobilize or absorb elements from their environment. In particular, plant roots can acquire micronutrients which are present at very low available concentrations in soils (Marschner et al. 1986). This ability is being exploited for the phytoextraction approach. Plants can also transform metals and metalloids, e.g. Se, into volatile forms for dissipation, as in the case of the phytovolatilization approach (Terry and Banuelos 2000). Moreover, metal availability in soil can be altered by exudation of organic ligands by plants (e.g. Basu et al. 1997). Metal availability can also be modified by the transformation products of plant litters, e.g. humates, which is a major sink for metals in soils. These processes can be utilized in both phytostabilization and phytoextraction approaches. Regardless of the approach, metal ion ligands either directly produced by plants or resulting from transformation of plant matter is expected to play significant roles in phytoremediation. Before any commercial use of this alternative clean-up technology can be realized, it is crucial to acquire a better understanding of these ligands, from their chemical nature to their function in metal bioavailability in soil or sediment matrices. As mentioned earlier, this basic understanding of phytoextraction, phytovolatilization, and phyto-stabilization is also needed for an accurate, pre-treatment assessment of biological risk from contamination at vegetated sites.

It is now generally recognized that plants have evolved several strategies to mobilize metals and enhance their uptake. Along with microorganisms, they can manipulate the chemistry of the rhizosphere, through acidification, electron transfer, and release of ligands via root exudation (Marschner 1986; Laurie and Manthey 1994; Barona and Romero 1996). In particular, metal chelation with various ligands is recognized to be an important factor in regulating metal solubility, bioavailability, and internal storage in plants (Laurie and Manthey 1994; Salt et al. 1998). Biogenic chelating agents present in the soil solution are expected to increase metal solubility by forming highly soluble metal complexes which prevents metal ions from precipitation and sorption (Salt et al. 1995a). The metal chelates can also enhance metal mobility and bioavailability. The ligands that are expected to be present in soil solutions include: *(1)* natural ligands such as microbial chelating agents (e.g. bacteriosiderophores), ligands released in plant root exudates (e.g. phytosiderophores), and humic substances; and *(2)* synthetic organic ligands (most notably EDTA) released from agriculture, industrial and urban activities. These agents are deployed to modulate metal ion solubility and availability.

Both soil humic substances and root exudate ligands are among the most abundant organic chelators in soils, yet least understood, apparently due to the complex nature of the constituents which vary widely in sizes, polarity, and functional groups. The best known ligands in root exudates are the phytosiderophores released by gramineous plants in response to micronutrient deficiency. For instance, an increase in mugineic and avenic acid exudation by gramineous roots have been reported to facilitate Fe and Zn acquisition under Fe and Zn deficiency (Crowley and Gries 1994). There is also evidence that these siderophores might be involved in Cu and Mn uptake (Romheld 1991). Many other ligands have also been found in root exudates (Fan et al. 1997, 2000a), but their involvement in metal acquisition remains unclear. Moreover, phytosiderophores have been assumed to mediate the acquisition of contaminant metals, but whether this is the case remains to be seen. A broad understanding of the exudate chemistry in conjunction with its relation to metal acquisition by plants should help clarify these uncertainties.

Humic substances (HS) are a complex mixture of organometallic macromolecules, arising from the biotic and abiotic degradation of plant and animal residues in the environment. They are generally classified into humic acids, fulvic acids, and humin according to their solubility in acid and basic solutions (Hayes et al. 1989). It has long been recognized that humic substances have many beneficial effects on plant growth and crop productivity (MacCarthy et al. 1990; Chen and Aviad 1990; Chen et al. 1994). For example, they influence nutrient uptake, nitrogen metabolism, enzymes activities, and membrane permeability in plants (MacCarthy et al. 1990; Chen and Aviad 1990; Chen et al. 1994). In addition, HS have been shown to protect plants from toxic effects of excess metals and to alleviate toxic effect of Cd, Zn and Cu (Strickland et al. 1979; Kinnersley 1993). Despite the abundance of descriptive effects, little is known about the chemical mechanism(s) by which HS influence these biological activities.

In this study, we investigated the interactive effect of Cd (a priority metal pollutant) and soil humic acids on plant metal uptake. We also examined the impact of Cd and/or HS on the composition of root exudates, so that ligands that mediate the metal uptake may be revealed.

19.2
Experimental

19.2.1
Plant Growth

Seeds of Chinese Spring wheat were surface sterilized in 10% NaOCl (v/v in water) for 30 min, rinsed extensively with doubly deionized water and germinated on filter paper moistened with $CaSO_4$ at 5×10^{-4} M for 3 d at room temperature (first 24 h in complete darkness). The seedlings were transplanted onto dark polyethylene cups (4 seedlings per cup). Three cups each were positioned on the top of 4 black 9-l polyethylene containers containing one-half strength Hoagland solution as modified from Epstein (Epstein 1972) (composition in mg l^{-1}: K: 118; N: 113; Ca: 80; Na: 23; S: 16; Mg: 12; Si: 14; P: 1.55; Fe: 1.396; Cl: 0.888; B: 0.137; Zn: 0.065; Mn: 0.055; Cu: 0.016; Ni: 0.029; Mo: 0.024). The plants were aerated and maintained in a growth chamber (Sanyo) with 16/8 h light/dark periods, day/night temperature of 25/19 °C and constant humidity at 70%.

On day 10, soil humate and/or Cd was added to the nutrient solution in 3 of the 4 containers. Cd was added as $CdSO_4$ to the medium to reach a final concentration of 5 mg l^{-1} of Cd, in the 'Cd' treatment and 'HS + Cd' treatment. The soil humic acids used were isolated from a freeze-dried soil obtained from Chikugo prefecture, Japan as previously described (Higashi et al. 1998). The extraction and Tiron-treatment (to remove exchangeable metals ions) were performed according to the procedures previously described (Fan et al. 2000b). HS was added to get a final concentration of 1 mg l^{-1} in the 'HS' treatment and 'HS + Cd' treatment. Throughout the experiment, the solutions were maintained at pH 6.0 by daily adjustment with 1 M KOH or HCl. On day 20, roots exudates were collected as described previously (Fan et al. 2001). Immediately following exudate collection, the plants were harvested, rinsed thoroughly with doubly deionized water and separated into roots and shoots. The tissues were freeze-dried, the dry weight determined and the final material pulverized to <5 µm particles before storage at –70 °C.

19.2.2
Determination of Metals Contents

Total shoot and root metals contents were determined by energy dispersive X-ray fluorescence spectrometry (ED-XRF, JVAR Inc. EX3600 spectrometer). The analytical conditions were as follows: for Mn, Fe, Ni, Cu and Zn: high voltage 15 kV, Mo 0.05 mm filter, live time 1 000 s and for Cd: high voltage 45 kV, Mo 0.1 mm filter, live time 500 s. The dry ground tissues (approximately 20 mg per sample) along with a set of tomato leaf standards were pressed into 7 mm diameter pellets for X-ray fluorescence (XRF) measurements using an analytical press (Wilmad Inc.). The quantitative analysis was performed by interpolation from standard curves established with the tomato leaf standards. These standards were prepared by spiking tomato leaf tissues with appropriate multielemental standard solutions. Selected standards and wheat tissues were also analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) after $HClO_4/HNO_3$ digestion. The ICP-AES results, as well as analysis of reference material (#1573a from the National Institute of Standards and Technology) by the XRF method confirmed the accuracy of the XRF method for roots and shoots analysis.

19.2.3
Root Exudates Composition

The lyophilized exudates were redissolved in 1 ml of doubly deionized water and centrifuged to remove particulates. An aliquot was lyophilized and then silylated with 1/1 (v/v) acetonitrile:N-methyl-N-[*tert*-butyldimethylsilyl]trifluoroacetamide (MTBSTFA) under sonication at 60 °C for 2–3 h and left overnight at room temperature. The silyl derivates were directly analyzed by gas chromatography-mass spectrometry (GC-MS) as previously described (Fan et al. 1997). The remaining solution was passed through a Chelex 100 cation exchange resin column to remove paramagnetic ions prior to 1-D and 2-D nuclear magnetic resonance (NMR), as described previously (Fan et al. 1997). Various organic and amino acids were identified and quantified from their GC-MS response calibrated against those of known standards. In addition, acetate, glycinebetaine (GB) and 2'-deoxymugineic acid (2'-DMA) were analyzed by NMR, as described previously (Fan et al. 1997; Fan et al. 2001).

19.3
Results and Discussion

We grew wheat on nutrient solutions containing Cd and/or humic substances (HS), in order to study Cd and HS influence on metal uptake. HS effect was then determined by comparing 'HS' treatment to '*Control*' (nutrient solution without Cd nor HS) and 'HS + Cd' to 'Cd' treatment. Cd influence was evaluated by comparison of 'Cd' treatment to '*Control*' and 'HS + Cd' to 'HS' treatment.

Table 19.1.
Dry weight (g) of roots and shoots as a function of Cd and humic substances (HS) treatments (triplicate samples). Standard deviation in *italic* (3 replicates). %-Values represent dry weight changes relative to: a) 'Control'; b) 'HS'; c) 'Cd' values

	Control	Cd	HS	HS + Cd
Roots	0.61 *(0.33)*	0.21 *(0.02)* −66%^{a}	0.71 *(0.02)* +16%^{a}	0.25 *(0.03)* +18%^{c} −65%^{b}
Shoots	1.92 *(0.20)*	0.85 *(0.14)* −56%^{a}	2.21 *(0.34)* +15%^{a}	0.97 *(0.07)* +14%^{c} −56%^{b}

19.3.1
Effect of Cd and Humic Substances on Biomass Production

Toxic symptoms were visible in wheat plants exposed to Cd, including reduction of the root growth and leaf expansion as well as chlorosis. Both root and shoot dry weight decreased (Table 19.1), regardless of the presence of HS. This reduction was more pronounced for roots (−65%) than shoots (−55%). HS treatment ('HS') resulted in a small enhancement in root and shoot growth, relative to the 'Control', i.e. an increase of about +16% for the roots and +15% for the shoots dry weight (Table 19.1). A similar growth enhancement by HS was observed when Cd was also present ('HS + Cd' vs. 'Cd'), i.e. about +18 and +14%, respectively for roots and shoots.

Cd is known to be highly toxic to humans, animals, and plants. In plants, Cd causes a number of toxic symptoms, including growth reduction, inhibition of photosynthesis, perturbation of enzymatic activities (Reddy and Prasad 1992; Salt et al. 1995b). The observed large reduction in root and shoot growth was in agreement with our previous studies (Fan et al. 2000a; Shenker et al. 2001) and known toxic effects of Cd. The enhancement of biomass production by HS is consistent with the reported effects of HS (e.g. Chen and Aviad 1990). The small increase observed could be due to the low HS concentration ($1\ \mathrm{mg\ l^{-1}}$) employed for the study. More interestingly, this relative enhancement was not eliminated by the presence of Cd (Table 19.1). This positive influence suggests that HS alleviates the toxic action of Cd, as has been reported for plants grown in soils (Strickland et al. 1979).

19.3.2
Effect of Cd and Humic Substances on Root Exudation

Cd treatments ('Cd' and 'HS + Cd') caused a large decrease (about 84%) in the total root exudation, relative to 'Control' and 'HS', while HS plus treatments led to a small increase (21 to 27%) in the dry weight of the exudates relative to HS minus treatments ('HS', 'HS + Cd' vs. 'Control', 'Cd'). Some of this effect was attributable to the influence on root biomass. To correct for this, the exudate dry weight was normalized against root biomass (relative exudation), as shown in Fig. 19.1. A substantial effect of Cd was still observed, as relative root exudation was reduced by about 50%. In contrast, the HS effect on relative exudation was attenuated to only 4 to 8%, which was insignificant.

The large inhibitory effect of Cd on root exudation was also evident in our previous study (Fan et al. 2000a). This effect may be related to the ability of wheat plants to modulate metal uptake under toxic conditions.

Fig. 19.1.
Effect of Cd and humic substances (HS) on relative root exudation (relative exudation = exudate dry weigth / root dry weight)

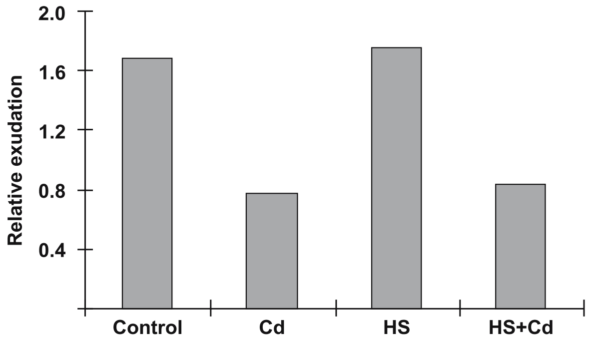

Table 19.2. Effect of Cd and humic substances (HS) on root metal content (in mg kg^{-1}) and % changes. Standard deviation in *italic* (3 replicates). %-Values represent concentration changes relative to: (a) 'Control'; (b) 'HS'; (c) 'Cd' values. *n.d.:* not detected

	Mn	Fe	Ni	Cu	Zn	Cd
Control	35.9 *(14.2)*	425.2 *(141.9)*	66.8 *(9.7)*	40.3 *(2.8)*	90.7 *(4.8)*	n.d.
Cd	44.2 *(10.5)* +23%^{a}	978.7 *(152.1)* +130%^{a}	118.0 *(15.4)* +77%^{a}	160.2 *(20.1)* +298%^{a}	294.6 *(25.2)* +225%^{a}	4 567.1 *(569.9)*
HS	35.8 *(8.4)* 0%^{a}	322.0 *(36.9)* −24%^{a}	78.3 *(5.8)* +17%^{a}	41.7 *(5.3)* +4%^{a}	80.0 *(8.3)* −12%^{a}	n.d.
HS + Cd	48.8 *(11.9)* +36%^{b} +10%^{c}	978.9 *(83.5)* +204%^{b} 0%^{c}	134.2 *(9.2)* +71%^{b} +14%^{c}	142.6 *(3.7)* +242^{b} −11%^{c}	338.7 *(10.6)* +323%^{b} +15%^{c}	4 942.2 *(591.2)* +8%^{c}

19.3.3
Effect of Cd and Humic Substances on Tissue Metal Profiles

Cd accumulated in the root to very high levels, reaching 4 567 ±570 µg g^{-1} for 'Cd' treatment, and 4 942 ±591 µg g^{-1} for 'HS + Cd' treatment (Table 19.2). Large increases of other metal concentrations were also observed in the Cd-treated roots. The most significant variations (% change in Table 19.2) were for Zn (+225% for 'Cd' and +323% for 'Cd + HS'), Cu (+298% and +242%) and Fe (+130% and +204%). Note that the presence of HS slightly enhanced the Cd-induced accumulation of Mn, Ni, Zn, and Cd in the root. In contrast, for Cd-treated shoots a decreasing trend was observed for most of the transition metals, except for Ni and Zn (Table 19.3). 'HS + Cd' treatment resulted in a small increase (+30%) of Ni while the Zn concentration increased by 56–79% when Cd was present. It is also interesting to note that for both roots and shoots, HS alone treatment ('HS') tended to attenuate the accumulation of transition metals, except for Ni in the root (Table 19.2).

Thus, Cd exposure resulted in enhanced uptake for most transition metals into the root. However, except for Zn, the excess metals in the root did not appear to be translocated into the shoot (see section below). There also appeared to be an interaction of HS with Cd in enhancing the uptake of transition metals into the root.

Table 19.3. Shoot metal content: concentrations (in mg kg^{-1} or ppmw) and variation (%) Standard deviation in *italic* (3 replicates).%-Values represent concentration changes relative to: (a) 'Control'; (b) 'HS'; (c) 'Cd' values. *n.d.:* not detected. *HS:* humic substances

	Mn	Fe	Ni	Cu	Zn	Cd
Control	71.9 *(5.4)*	189.6 *(19)*	16.2 *(5.4)*	9.4 *(3.0)*	70.6 *(15.3)*	n.d.
Cd	51.8 *(10.2)* −28%[a]	140.7 *(23.4)* −26%[a]	14.7 *(5.2)* −9%[a]	6.8 *(3.8)* −28%[a]	110.4 *(8.3)* +56%[a]	113.4 *(43.6)*
HS	52.0 *(9.1)* −28%[a]	164.2 *(33.8)* −13%[a]	12.5 *(8.1)* −23%[a]	7.5 *(2.7)* −20%[a]	48.6 *(14.8)* −31%[a]	n.d.
HS + Cd	44.5 *(9.6)* −14%[b] −14%[c]	127.4 *(41.5)* −22%[b] −10%[c]	16.2 *(5.7)* +30%[b] +10%[c]	5.9 *(7.0)* −21[b] −13%[c]	86.9 *(17.8)* +79%[b] −21%[c]	83.6 *(17.8)* −26%[c]

Table 19.4. Root/shoot concentration ratio ($C_{R/S}$) for the various metals. *n.d.:* not detected. *HS:* humic substances

	Mn	Fe	Ni	Cu	Zn	Cd
Control	0.50	2.24	4.12	4.30	1.29	n.d.
Cd	0.85	6.95	8.01	23.66	2.67	40.29
HS	0.69	1.96	6.25	5.58	1.65	n.d.
HS + Cd	1.10	7.68	8.27	24.08	3.90	59.11

19.3.4
Effect of Cd and Humic Substances on Root/Shoot Concentration Ratio ($C_{R/S}$)

To better visualize the effect on metal translocation from roots to shoots, $C_{R/S}$ values were calculated, as shown in Table 19.4. Increasing values should indicate metal accumulation in the roots and restrained movement to the shoot.

Except for Mn, the $C_{R/S}$ values for all other metals were much greater than 1. Mn had the lowest values of $C_{R/S}$ for all treatments while Cd showed the highest values, i.e. about 2 to 100 times greater than those for the other metals. In addition, the $C_{R/S}$ value of 'HS + Cd' was the highest among the four treatments for all metals.

The large $C_{R/S}$ values of all metals but Mn is consistent with the knowledge that plants retain heavy metals in their roots (Reddy and Prasad 1992; Sauerbeck 1991). The $C_{R/S}$ values of Mn and Cd also suggest that Mn was the most "translocatable" while Cd was the most retained metal by the root. Moreover, the higher $C_{R/S}$ values of transition metals with 'HS + Cd' cotreatment than with either treatment alone suggest a synergistic effect of Cd and HS on metal retention in the root. This retention could be mediated through interaction with metal ion ligands such as phytochelatins. This is consistent with a much higher accumulation of phytochelatins in wheat roots than shoots observed in our previous study (Fan et al. 2000a) and with the ability of phytochelatins to sequester Cd in the vacuole of root cells (Dushenkov et al. 1995; Hart et al. 1998).

19.3.5
Effect of Cd and Humic Substances on Root Exudate Profile

To investigate whether root exudation could underlie the effect of HS or Cd on metal uptake into the root, a broad screen of root exudate for metal ion ligands was performed using nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). The most abundant components detected were lactate, alanine, GAB (γ-aminobutyrate), malate, acetate, glycinebetaine (GB) and 2'-deoxy-mugineic acid (2'-DMA). The treatment-dependent profiles of these exudate components are shown in Fig. 19.2. Qualitatively, the exudation profiles for the four treatments were similar, e.g. lactate, acetate, and 2'-DMA remained most abundant. Quantitatively, the exudation of most components for 'Cd' and 'HS + Cd' treatments were distinctly lower than those for 'Control' and 'HS' treatments. In particular, exudation of these components dropped to near detectable limits under 'Cd' treatment. However, HS cotreatment alleviated somewhat this reduction for acetate and 2'-DMA.

These results are consistent with the notion that Cd inhibits root exudation and that Cd-induced metal sequestration into the root is not mediated via these small molecular weight ligands including the phytosiderophore 2'-DMA. This is in contrast to the important role of 2'-DMA in Fe acquisition under Fe deficiency (Marschner et al. 1986). However, the deinhibition of acetate and 2'-DMA exudation under 'HS + Cd' treatment may play a role in the small enhancement of metal sequestration into roots observed for this treatment as compared to the Cd treatment alone (cf. Table 19.2). This lack of dependence of metal accumulation on 2'-DMA appears to extend to several transition metal ions, as summarized in Fig. 19.3. This result indicates that, when Cd is present, the mechanism of accumulation of these transition metal ions – including Fe – is different from that under Fe deficiency and not dependent on low molecular weight organic exudates.

19.4
Conclusion

In conclusion, Cd inhibited the growth of wheat plants and root exudation while enhancing the accumulation of most transition metals in the root, in addition to its own accumulation to 4–5 g kg^{-1} levels. The majority of the excess metals remained in the root except for Zn which was in part translocated to the shoot. The presence of soil HS,

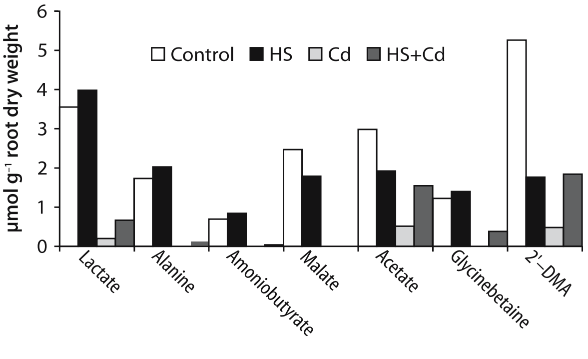

Fig. 19.2.
Effect of Cd and humic substances (HS) on root exudate profiles

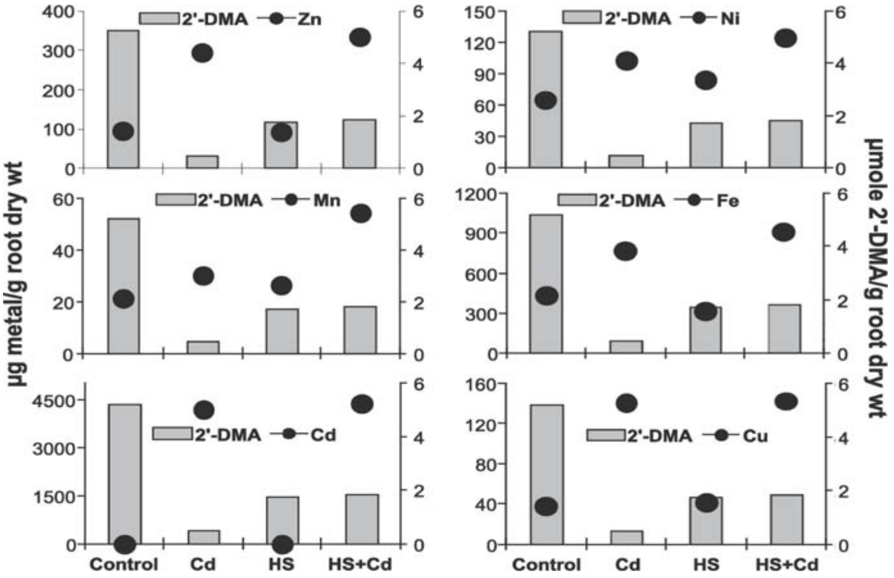

Fig. 19.3. 2'-DMA exudation vs. metals accumulated in wheat roots. *Gray bar:* 2'-DMA concentration in exudates (µmol g^{-1} of root dry weight, *right ordinate*); *Black circles:* metal concentration in the root tissue (µg g^{-1} root dry weight, *left ordinate*)

to a small extent, alleviated the inhibitory effect of Cd on growth and exudation while enhancing Cd accumulation and attendant accumulation of transition metal in the root. HS and Cd also had a synergistic effect on retaining transition metals and Cd in the root. Moreover, the Cd-induced accumulation of metals in the root was not correlated with the exudation of any small metal ion ligand including the phytosiderophore 2'-DMA. This indicates that the excess metal sequestration induced by Cd was not directly mediated by the exudation of the low molecular weight ligands.

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