Plant Rhizosphere Effects on Metal Mobilization and Transport

Teresa W-M. Fan, Dept. of Land, Air and Water Resources, Univ. California, Davis, CA, USA
Richard M. Higashi, Crocker Nuclear Laboratory, Univ. California, Davis, CA, USA
David E. Crowley, Dept. of Environmental Sciences, Univ. California, Riverside, CA, USA
Andrew N. Lane, National Institute for Medical Research, Mill Hill, London, UK

INTRODUCTION & OBJECTIVES

There is only limited information on the mechanism of mobilization, uptake, and metabolism of metal ions by plants. Particularly deficient is the understanding of interactions among pollutant metal ions, key rhizospheric processes such as root exudation of organic ligands that are critical to metal ion uptake, and plant metabolism/adaptation that is linked to metal ion mobilization and sequestration.

A mechanistic insight into these areas will advance knowledge in microbe-plant host interactions, which in turn is essential to applications such as phytobioremediation and microbioremediation. However, the nature of the metal ion-ligand interaction is such that all of the major organic ligands must be determined, regardless of their chemical class.

Our present approach to this problem is:

(1) Determine the major organic composition in root exudates and plants as a function of metal ions;

(2) Examine plant metabolic response(s) to metal ion treatment with emphasis on those of ligand components.

To define major organic ligand composition in plant root exudates and tissues, we employed multi-nuclear/multi-dimensional NMR, pyrolysis/thermal-desorption GC-MS, and peptide gel electrophoresis. The comprehensive ligand information from NMR can be used to "calibrate" the more sensitive GC-MS for quantifying biogenic ligands directly in small quantities of rhizospheric soils.

We have utilized these techniques to define changes in root exudates and tissues from Fe/Cd treated barley, wheat, and rice plants. We demonstrated that the mugineic acid phytosiderophores did not appear to influence the uptake of Cd(II). Instead, a polyhydroxylated component, amino acids, glycolytic metabolites, and SH-rich peptides may be related to Cd(II) sequestration by graminaceous plants.

MATERIALS AND METHODS

(*Please flip up this panel to see details*)

- Plant Growth - Barley and wheat plants were grown for 22 d in nutrient solutions buffered by an excess of EDTA and HEDTA to yield four Fe (pFe = 16.5-18.0) and four combined Fe/Cd treatments

- Root Exudate Collection - Exudates were collected in DDH2O during peak diurnal release of phytosiderophores.

- NMR Analysis - Screening for total organic ligands by NMR included: 1-D ^{1}H and ^{13}C one-pulse, 2-D ^{1}H total correlation (TOCSY), double-quantum filtered correlation (DQF-COSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC), ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) experiments, and watergate TOCSY measurement at 5° C which optimized the detection of exchangeable protons.

- GC-MS Analysis - Broad screening for organic acids were achieved by silylation for GCMS analysis.

- Gel Electrophoresis - Plants were analyzed for peptide chelators.

- Plant Growth - Barley (cv. CM-72) and wheat plants (cv. AgCs) were grown for 22 d in nutrient solutions buffered by an excess of EDTA and HEDTA to yield four Fe (pFe = 16.5-18.0) and four combined Fe/Cd treatments: (i) low Fe/ high Cd; (ii) high Fe/ high Cd; (iii) low Fe/ low Cd; and (iv) high Fe/low Cd. The free metal activities as in pM were: low Fe = 18.2; high Fe = 17.0; low Cd = 10.4; high Cd = 9.1; Zn = 10.1; Cu = 13.5; and Mn = 7.3.
- Root Exudate Collection - Exudates were collected in DDH2O during peak diurnal release of phytosiderophores (2-4 h after light onset) of the last 3 growth days.
- NMR Analysis - Exudates were adjusted to pH2, lyophilized, and redissolved in D2O for NMR measurements at 25° C including 1-D 1H and 13C one-pulse, 2-D 1H total correlation (TOCSY), double-quantum filtered correlation (DQF-COSY), 1H-13C heteronuclear single quantum correlation (HSQC), and 1H-13C heteronuclear multiple bond correlation (HMBC) experiments. Selected samples were dissolved in DDH2O containing 10% D2O+0.9M HCl for watergate TOCSY measurement at 5° C which optimized the detection of exchangeable protons.
- GC-MS Analysis - An aliquot of the exudate at pH2 was lyophilized and derivataized with MTBSTFA before analysis on a Varian 3300 gas chromatograph interfaced to a Finnegan ITD 806 mass spectrometer (Fan et al., 1997).
- Exudate Quantification - all exudate components were quantified by their GC-MS response as compared to that of the respective standard except for the mugineic acids (MAs), glycinebetaine (GB), and Compound X (see Figs 1&2.). The latters were quantified by their 1H NMR responses by comparison with that of alanine (either internal or added), the concentration of which was obtained from GC-MS analysis.

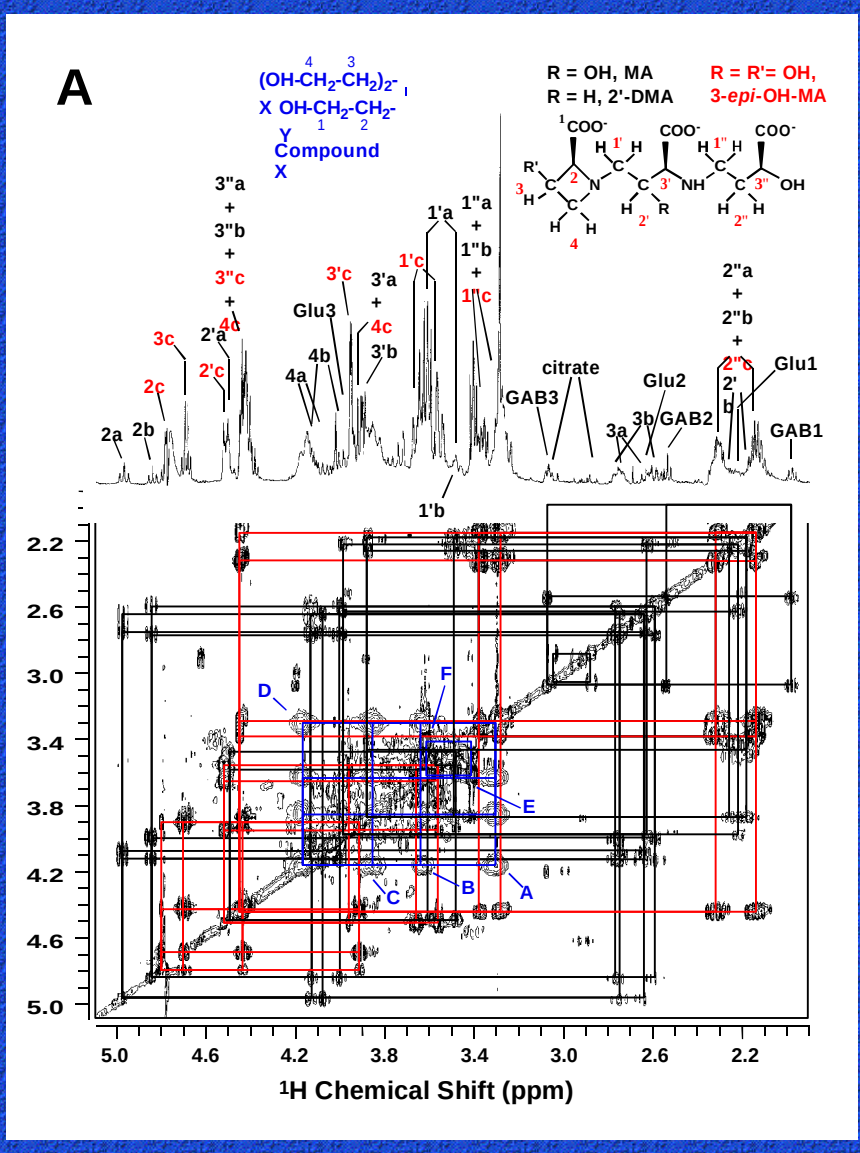
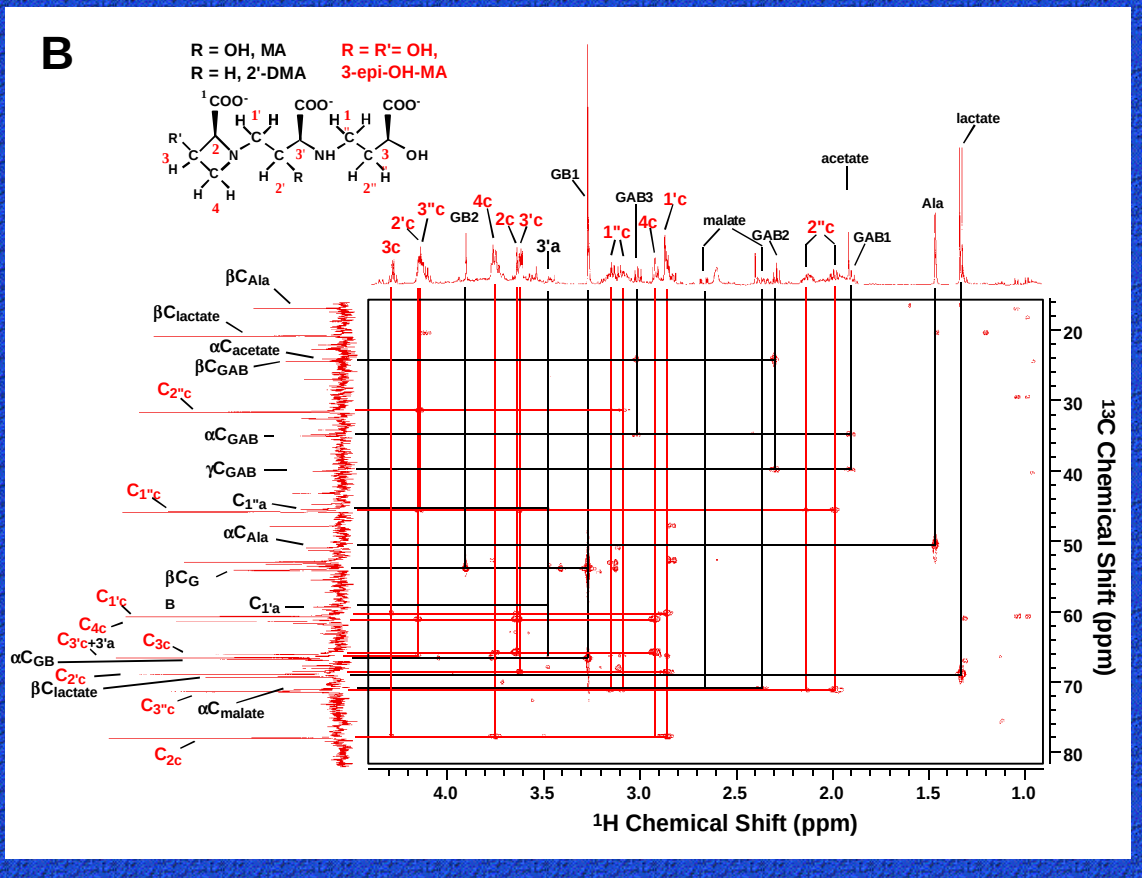


Fig. 1 2-D NMR identifies ligands in whole barley root exudate. 2-D ^{1}H total correlation (TOCSY) (A) and ^{1}H-^{13}C multiple bond correlation (HMBC) (B) spectra were acquired from barley root exudates collected under Fe deficiency. The 2-D spectra are displayed as contour plots while the corresponding 1-D ^{1}H and ^{13}C spectra are presented along the 2-D axes. Also shown are the structures of the phytosiderophores in the mugineic acid (MA) family which are critical to Fe acquisition by grasses.

The boxes in (A) and lines and (B) trace the ^{1}H-^{1}H and ^{1}H-^{13}C connectivity, respectively, of a given molecule. Those in red trace the pattern for 3-*epi*hydroxy MA [c]. These and other 2-D spectra identified the structures of three MA's (2'-deoxy MA [a], MA [b], and 3-*epi*hydroxy MA) directly from whole exudates without fractionation. Also determined are ligands such as Glu: glutamate; GAB: γ-aminobutyrate; A-F: Compound X.



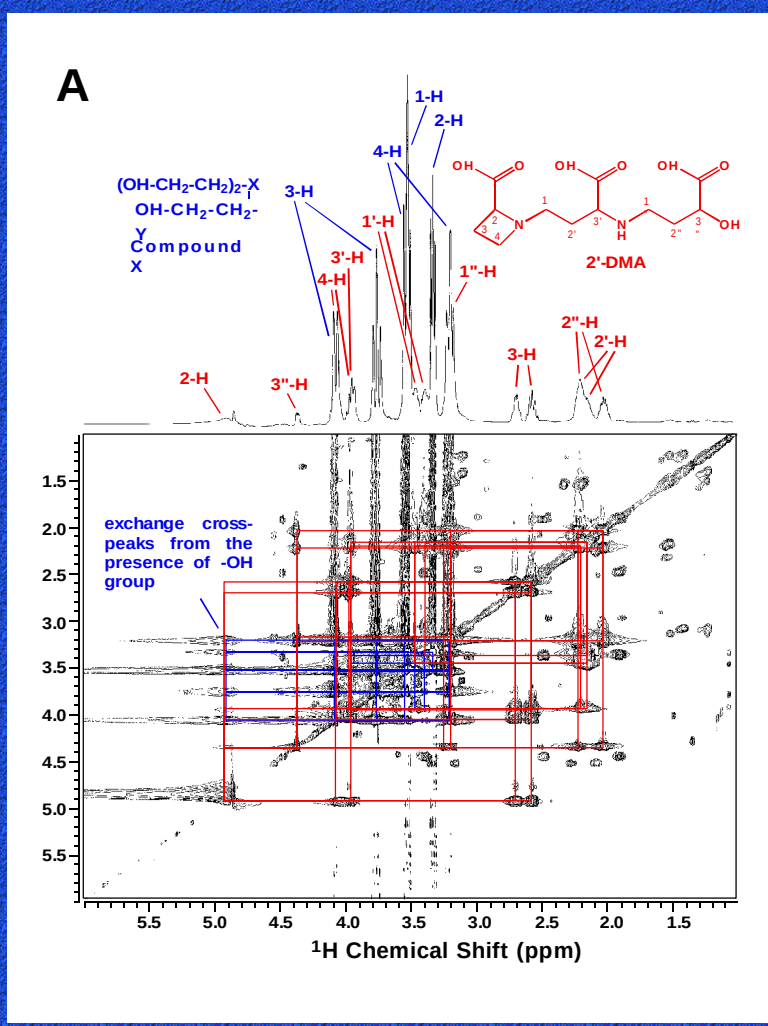
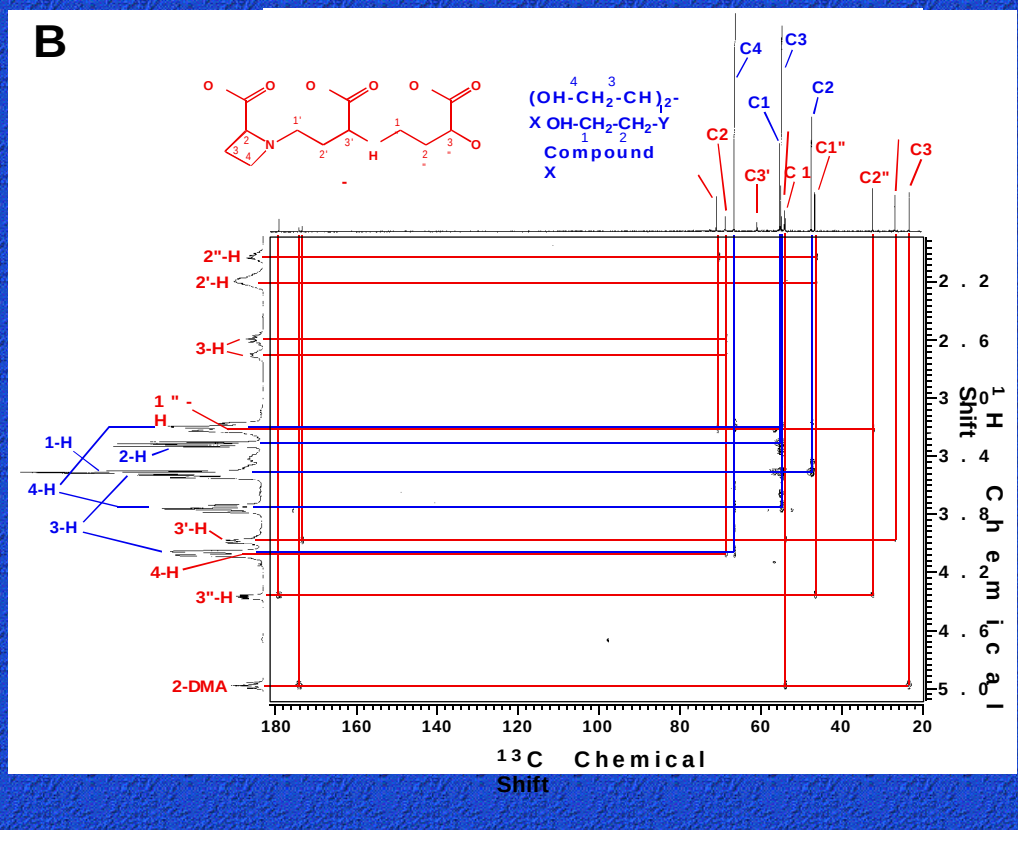


Fig. 2 Use of 2-D NMR spectroscopy for analysis of whole wheat root exudate. The 2-D ^{1}H watergate total correlation (WG-TOCSY) (A), ^{1}H-^{13}C HMBC (B), and the 1-D ^{1}H and ^{13}C spectra were acquired from a wheat root exudate collected under Fe deficiency. The spectral appearances were distinct from that of the barley root exudate (Fig. 1). The red and blue boxes in (A) trace the ^{1}H -^{1}H connectivity of two predominant componants, 2'-deoxy MA (2'-DMA) and an unknown component (X), respectively. Amino and organic acids were generally below observable limits. In addition to revealing covalent linkages, the WG-TOCSY experiment detected protons that exchange with water (e.g. OH group). As in Fig. 1, the HMBC (B) experiment observed ^{1}H-^{13}C linkages of 2 or more bonds apart in 2'-DMA and Compound X (as traced by red and blue lines, respectively).

Based on these and other 2-D spectra, Compound X should have the structure of $(OH\text{-}CH_2\text{-}CH_2)_2\text{-}X\text{-}Y\text{-}CH_2\text{-}CH_2\text{-}OH$. The same component also occurred but less prominently in the barley root exudate (peaks A-F in Fig. 1). Figs. 1 & 2 illustrate the general utility of NMR in comprehensive analysis of organic composition of whole root exudates, with little or no fractionation and “cleanup”, which can lose or alter components.



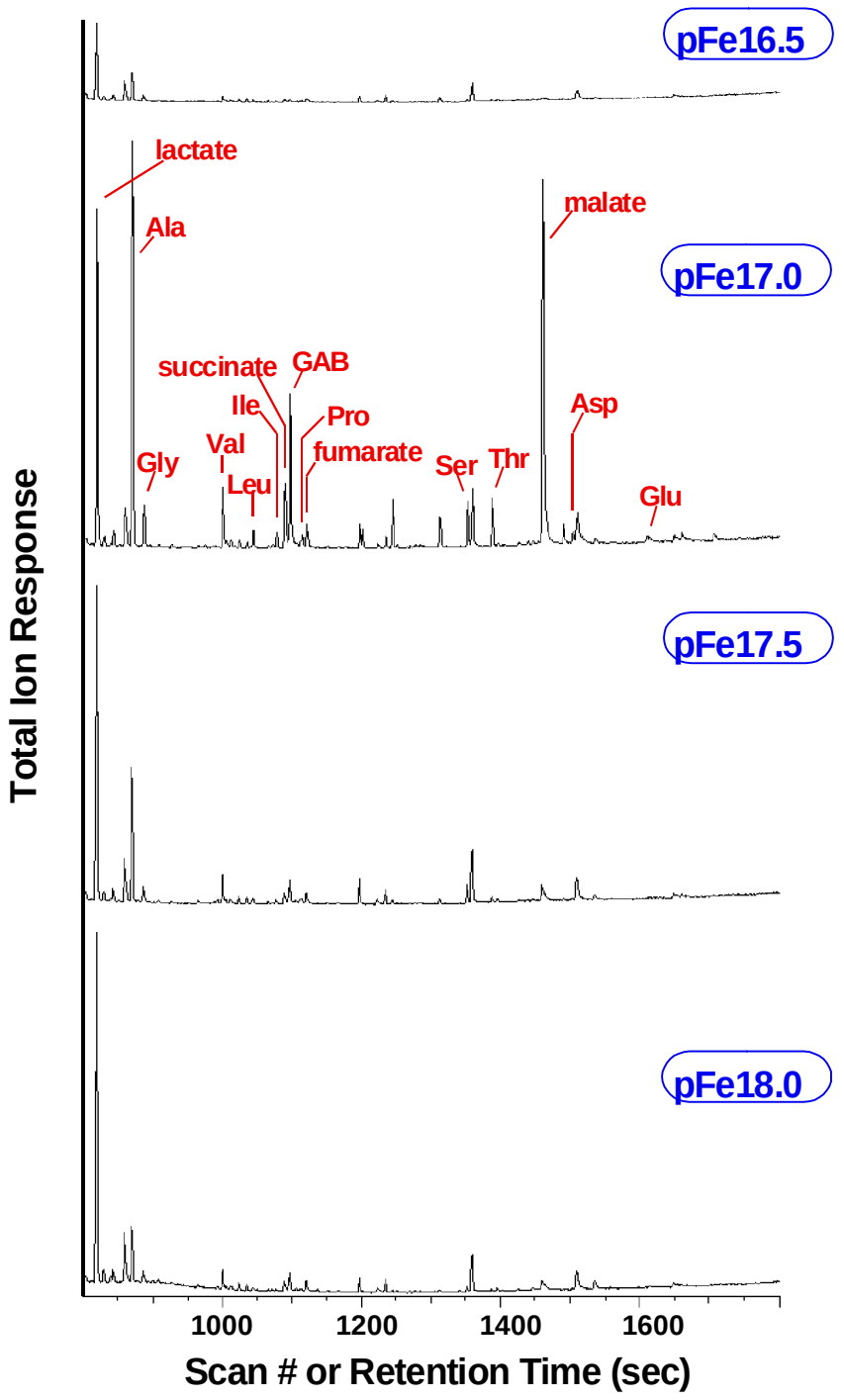


Fig. 3 GC-MS complements NMR analysis of biogenic ligands. The same exudate sample from Fig. 1 was silylated with MTBSTFA, which enabled quantification of a wide range of polar biochemical compounds by GC-MS, with structure confirmation. Shown here is the compositional changes (normalized to barley root mass) of exudates under Fe sufficient (pFe 16.5) and three progressively Fe deficient conditions (pFe 17.0 to 18.0), as revealed by GC-MS analysis.

The combined GC-MS and NMR analyses provided a comprehensive means for determining biogenic ligands in root exudates, without *a priori* knowledge of the chemical composition. NMR identification of ligands, particularly unknown or unexpected ones, can facilitate the development of their quantification by GC-MS. In turn, pyrolysis and thermal-desorption GC-MS has shown promise in the direct analysis of biogenic ligands in rhizospheric soil samples, which is difficult to achieve by NMR or other means.

Table 1. Quantification of the major ligands in root exudates from barley plants grown under four levels of iron availability[a].

	Fe Treatment			
Ligands	pFe 16.5	pFe 17.0	pFe 17.5	pFe 18.0
lactate*	1.11	4.09	5.00	4.82
Ala*	0.31	4.37	1.54	0.66
Gly*	0.07	0.43	0.19	0.16
Val*	0.04	0.58	0.34	0.19
Leu	0.02	0.19	0.07	0.04
Ile	0.00	0.13	0.03	0.00
succinate*	0.04	0.98	0.16	0.14
GAB*	0.02	2.50	0.40	0.27
Pro	0.01	0.17	0.12	0.00
fumarate	0.05	0.29	0.17	0.18
Ser*	0.01	0.39	0.14	0.03
Thr*	0.02	0.74	0.11	0.04
malate*	0.00	2.79	0.23	0.10
Asp*	0.12	0.41	0.36	0.26
Glu	0.02	0.16	0.05	0.00
GB[b]*	0.53	5.91	1.66	2.74
acetate[b]*	1.58	4.68	1.70	2.25
3-epi-OH MA[b]**	1.12	8.09	16.14	17.37
MA+2'-DMA[b]**	–	0.16	1.39	3.59
MAs:FeMS ratio[b]	0.75	1.78	2.93	2.88

[a] Values are in µmoles/g of root, quantified by GC-MS unless specified otherwise; [b] Determined from 1-D ^{1}H NMR analysis. 3-epi-OH-MA: 3-epihydroxymugineic acid; MA: mugineic acid; 2'-DMA: 2-deoxymugineic acid; GB: glycinebetaine; GAB: γ-aminobutyrate; FeMS: iron mobilizing substances determined from the ferrozine assay.

With increasing severity of Fe deficiency, the exudation of the three MAs responded the most. This is consistent with their role in Fe mobilization. Other ligands* (most notably GB, acetate, malate, lactate, GAB, and Ala) also increased in exudation but their response was maximal under mild Fe deficiency (pFe 17.0). These amino & organic acids may contribute to Fe mobilization under mild deficiency, consistent with the decreasing ratio of MAs:FeMS with increasing Fe availability.**

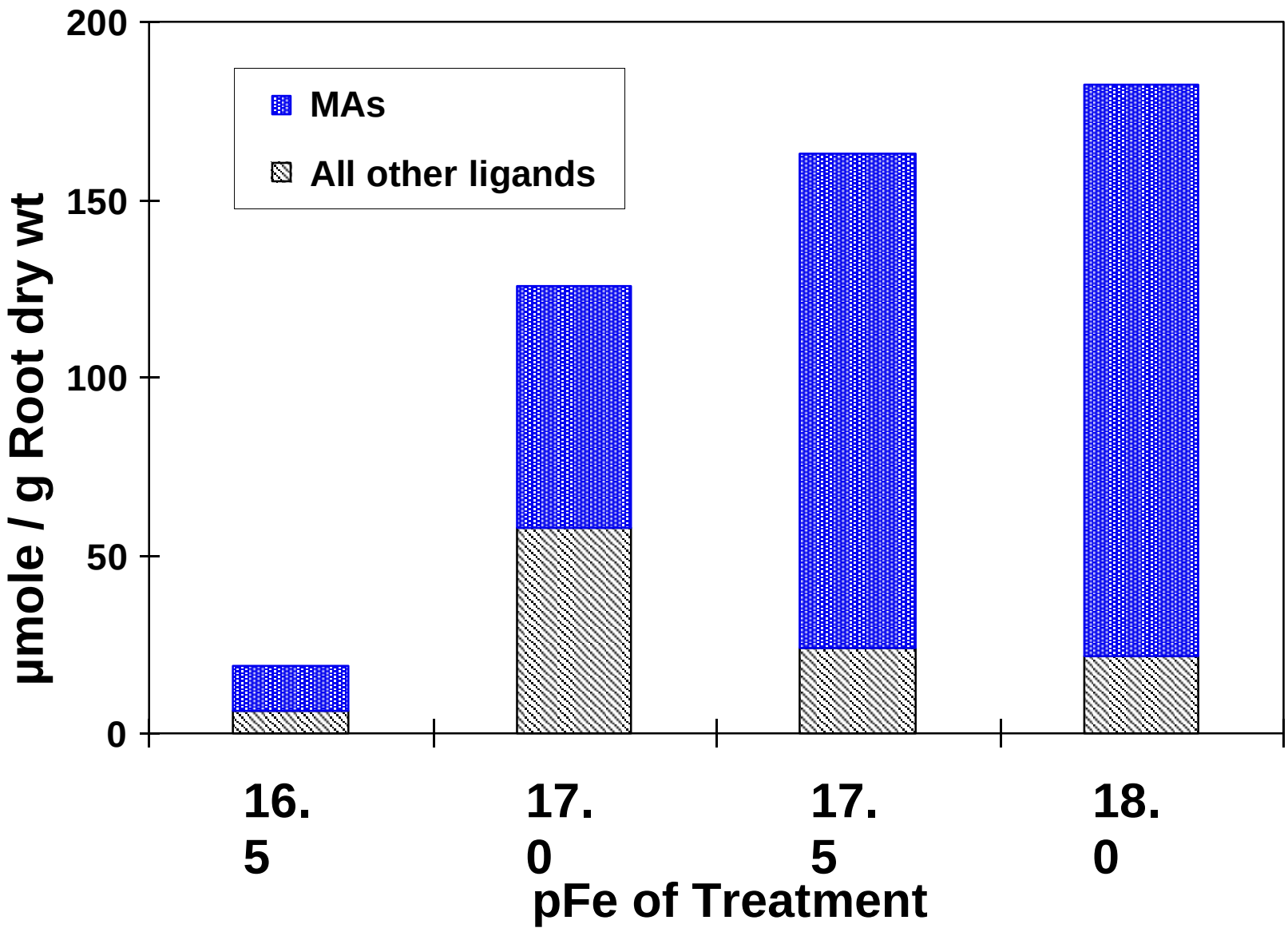


Fig. 4 Information in Table 1, expressed as ionic equivalents. An interesting exercise is to compute the total ionic equivalents represented by the MAs and all other organic/amino acids in Table 1. The total bar height shows that Fe deficiency resulted in the increase of total equivalents exuded by roots. Under mild deficiency (pFe 17.0), the amino and organic acids constituted a significant portion of the total equivalents, while under the more severe deficiency (pFe 17.5 and 18.0), the MAs became the dominant species of organic ligands. Of course, this figure does not reflect the very complex interactions between metal ions and these 19 ligands; we hope to unravel some of those interactions in the future.

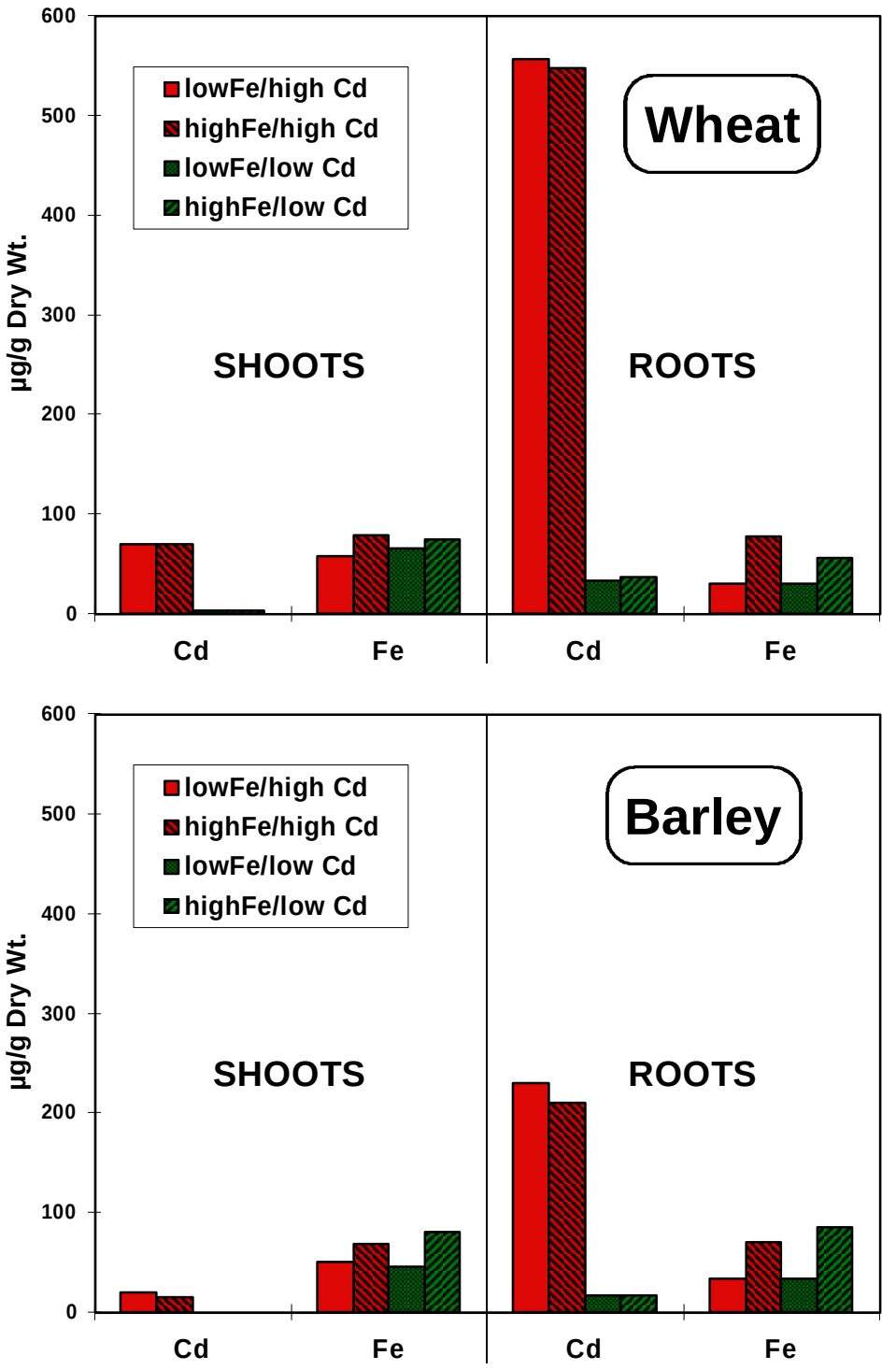


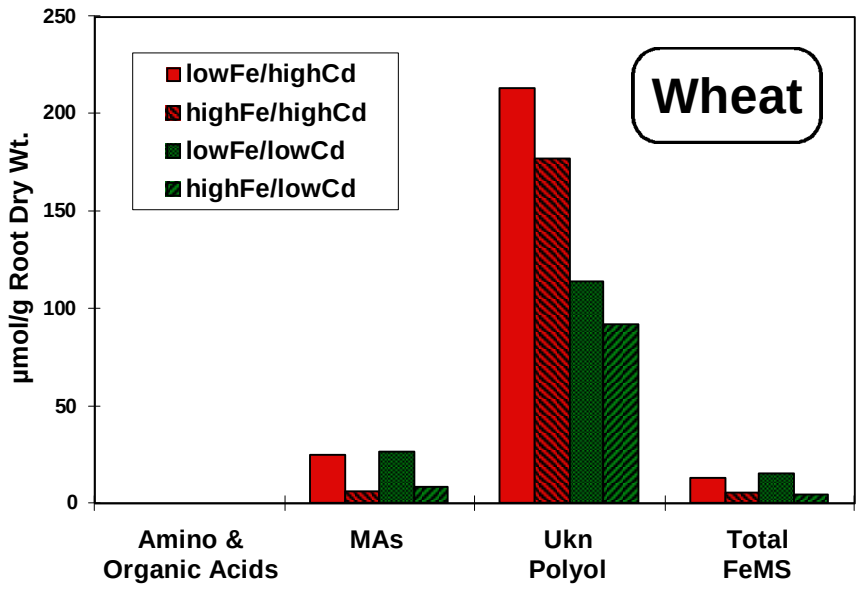
Fig. 5 Cd and Fe content of barley and wheat plants under combined Fe/Cd treatments. The Cd and Fe content of barley and wheat tissues was determined by ICP-AES. As expected, low Fe conditions resulted in a lower Fe status while high Cd treatments led to a high Cd content in shoot and root tissues of both plants. Also, the Cd content of roots was much greater than that of shoots in both plants with the Cd treatments. Interestingly, Fe deficiency did not affect the Cd content in either plants. Overall, wheat plants accumulated 2-4.6x more Cd (on a per gram basis) in both shoot and root tissues than barley plants.

Table 2. Ligand compositional changes in barley root exudate with Fe & Cd treatments[a].

	Treatment			
	low Fe/high Cd	**high Fe/high Cd**	**low Fe/low Cd**	**high Fe/low Cd**
lactate	2.88	2.83	3.15	2.69
pyruvate	7.59	5.72	6.06	5.98
Ala*	1.63	2.95	2.20	1.80
Gly*	0.24	0.38	0.27	0.23
Val	0.28	0.3	0.33	0.21
Leu	0.15	0.17	0.16	0.12
succinate	0.28	0.21	0.29	0.15
GAB*	1.03	1.35	1.16	0.74
Pro	0.16	0.18	0.15	0.13
fumarate	0.27	0.28	0.27	0.24
glyceraldehyde-3-P	0.13	0.11	0.14	0.08
Ser*	0.36	0.49	0.35	0.31
Thr*	0.38	0.53	0.32	0.31
Phe	0.07	0.08	0.07	0.06
malate	0.34	0.20	0.48	0.09
Asp*	0.19	0.27	0.14	0.11
Cys*	0.08	0.03	0.04	0.00
Glu*	0.19	0.35	0.22	0.19
Asn	0.17	0.19	0.15	0.13
Gln	0.11	0.14	0.23	0.16
α-glycerol-3-P	0.20	0.16	0.17	0.12
His	0.03	0.04	0.06	0.01
citrate	0.02	0.03	0.05	0.03
GB*	1.14	1.56	1.52	0.82
3-*epi*-OH-MA**	5.25	1.15	7.97	0.96
2'-DMA**	0.49	0.00	0.62	0.01
MA**	0.79	0.09	1.02	0.04
CMPD X**	0.64	0.72	1.14	0.92
Total FeMS (Cu-CAS)[b]	13.15	4.97	15.40	4.33
Total - 3 MAs	6.58	1.20	9.61	0.99
Total - all ligands	25.30	20.74	29.22	16.81
FeMS:3 MAs ratio	2.01	4.00	1.60	4.31

[a] All ligands are in µmole/g root dry weight; [b] Total Fe mobilizing substances determined from the Cu-chrome azurol S assay. The ligand concentrations were determined from GC-MS and NMR analysis as in Table 1.

As observed before, the 3 MAs showed the most marked increase in exudation under low Fe conditions, but this response was slightly diminished by the Cd treatments. A similar reduction was also observed for the polyhydroxylated Compound X**. Those ligands* that increased in exudation in response to high Cd were primarily amino acids and their derivatives and this occurred mostly under Fe sufficient conditions (as denoted in Red). As in Table 1, the higher FeMS:3 MAs ratio for the Fe sufficient treatments suggest that ligands other than the MAs contributed significantly to Fe chelation.**



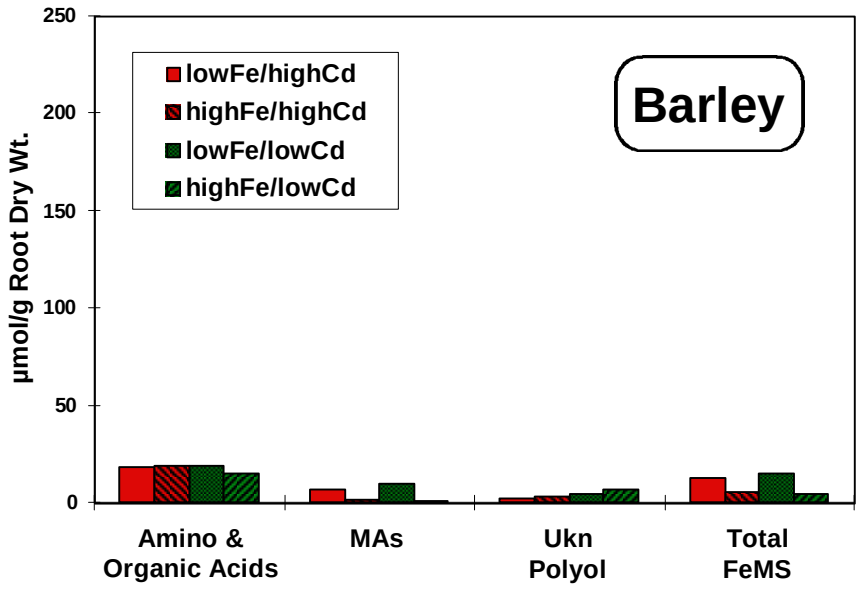
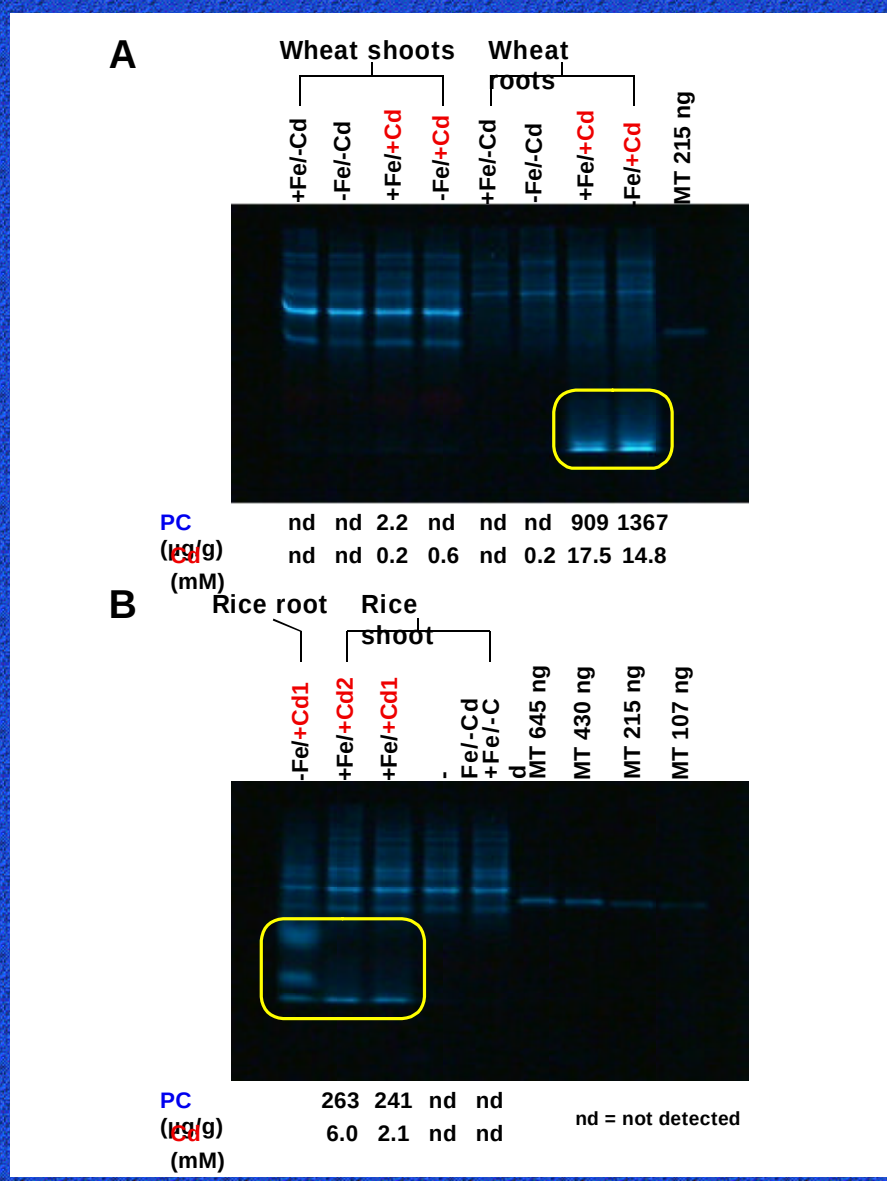


Fig. 6 Different ligands may be involved in Cd accumulation by wheat and barley plants. This figure and Fig. 5 together illustrate that the increase in exudation of mugineic acids did not enhance Cd accumulation in either wheat or barley plants. Thus, the MAs were probably not specifically involved in Cd(II) sequestration. However, the Cd accumulation in barley and wheat was correlated with the increase in exudation of certain amino acids (Table 2) and Compound X, respectively, indicating a role of these compounds in Cd(II) acquisition. Interestingly, since both roots and shoots of wheat acquired a much higher Cd content than those of barley (Fig. 5), Compound X may be more specifically involved in Cd(II) mobilization and translocation.



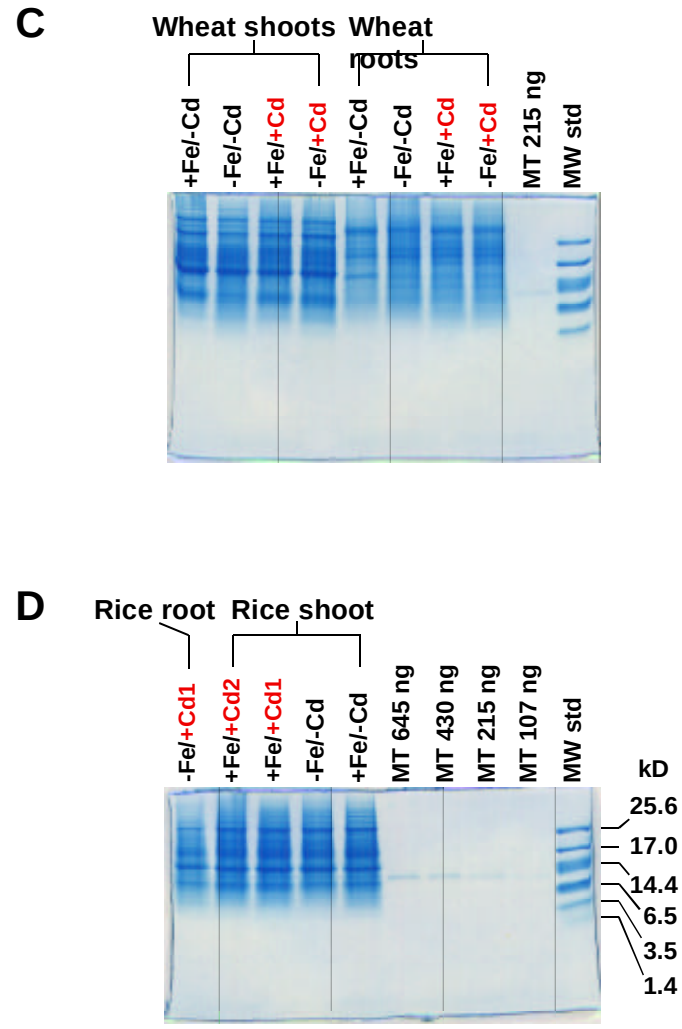


Fig. 7A-D SH-rich peptides are induced in Cd-treated wheat and rice plants. A convenient polyacrylamide gel electrophoretic (PAGE) method was developed for assaying SH-rich peptides (e.g. phytochelatins, PC) that may be involved in intracellular sequestration of Cd(II). Peptidic SH groups were derivatized by bromobimane, then separated on PAGE with fluorescence image analysis. Panels A-B show the analysis for wheat and rice plants after Fe/Cd treatments. The yellow boxes highlight the regions where SH-rich peptides (e.g. PC) are induced by Cd accumulation into tissues. Also illustrated is the analysis of a metallothionein (MT) standard, from which a calibration curve was used to determine tissue "PC" content (µg MT equivalent/g dry wt). The Cd content was obtained from X-ray fluorescence (XRF) analysis. Panels C-D show the Coomassie blue-stained peptide patterns of the same gels. The "PC" bands were lost with the staining procedure.

CONCLUSIONS

- The combined NMR and GC-MS approach allowed composition of whole root exudate to be determined without sample fractionation.

- Amino/organic acids appeared to be significant in Fe mobilization under mild Fe deficiency while the phytosiderophore mugineic acids became dominant under severe Fe deficiency.

- The mugineic acids were probably NOT the key to Cd(II) mobilization by barley or wheat plants.

- Amino acids & derivatives in barley and a polyhydroxylated compound in wheat may play important roles in Cd(II) mobilization.

- SH-rich peptides in wheat and rice plants may be important to Cd(II) sequestration into tissues

ACKNOWLEDGEMENTS

This study was supported, in part, by U.S. Dept. of Energy grant #DE-FG07-96ER20255, and U.S. EPA funded (#R819658) Center for Ecological Health Research. We thank Dr. M. Shenker for conducting the wheat and barley growth, metals analysis in Fig. 5, and FeMS analyses. We also wish to thank Dr. M. Gradwell for assistance with the HMBC experiment, and the U.K. Medical Research Council Biomedical NMR Centre for use of the NMR instrumentation.