Phytoremediation of Heavy Metal-Contaminated Soils using High Biomass Plants

By

LUO Chun Ling

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Department of Civil and Structural Engineering

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis entitled "Phytoremediation of Heavy Metal-Contaminated Soils using High Biomass Plants" is original and has not been submitted for other degrees or the like in this University or any other institutes. It does not contain any material, partly or wholly, published or written by others, except those references quoted in the text.

LUO Chun Ling

ABSTRACT

The contamination of soils with metals is a major environmental problem throughout the world. The emerging phytoremediation techniques, with their lower cost and environmental friendly nature, have received increasing attention in the last decades. The main aim of this research project is to study the phytoextraction of heavy metals from soils with the application of chelates. Screening high-biomass plant species more sensitive to the application of chelates and optimizing chelate application methods were investigated for a better combination of plants and chelate applications to increase the metal phytoextraction efficiency and reduce potential metal leaching to the surrounding environment.

Results from plant screening experiment showed that, of all of the plants that were tested, garland chrysanthemum (*Chrysanthemum coronarium* L.) was the species most sensitive to the application of EDTA (ethylenediaminetetraacetic acid), and had the highest enhancement of Cu and Pb concentrations in its shoots. For Cu and Pb, 9.5- and 69-fold increases in metal concentrations were achieved 7 d after the application of 3 mmol kg^{-1} of EDTA, respectively. The plant of garland chrysanthemum may be as a good candidate plant species in the area of chelate-enhanced phytoextraction.

Regarding chelate application, results showed that EDDS was more effective than EDTA at increasing the concentrations of Cu and Zn in corn (*Zea mays* L.) and beans (*Phaseolus vulgaris* L.). For Pb and Cd, EDDS was less effective than EDTA.

Understanding the mechanisms involved in enhancing metal accumulations in plants through chelate application will be helpful for optimizing chelate-induced phytoremediation. Results showed pretreatments on the roots of Indian mustard with MC, HCl, and hot water increased the concentration of Pb in shoots by 14-, 7-, and 15-fold, respectively, compared with the shoots that had not been pretreated. Using a pot experiment, the biodegradable chelating agent of EDDS was added in a hot solution at 90°C to the soil in which garland chrysanthemum and beans were growing. Results showed when 1 mmol kg^{-1} of EDDS as a hot solution was applied to soil, the concentrations of Cu, Pb, Zn and Cd and the total phytoextraction by the shoots of the two plant species exceeded or approximated those in the shoots of plants treated with 5 mmol kg^{-1} of normal EDTA solution. The concentrations of metals in the shoots of beans were significantly correlated with the relative electrolyte leakage rate of root cells, indicating that the root damage resulting from the hot solution might play an important role in the process of chelate-enhanced metal uptake.

Metal leaching study due to chelate application to soil was also carried out

immediately after harvesting of the plants in pot experiments. Results showed on an average, the leached metal amounts of Cu, Pb, Zn and Cd on the application of EDDS at the rate of 1 mmol kg^{-1} were reduced by 46%, 21%, 57% and 35% compared with that leached from the 5 mmol kg^{-1} of EDDS application, respectively. For the treatment of 1 mmol of EDDS, the leached metals decreased to the control group 14 days after the application of EDDS. Therefore, the application of biodegradable EDDS in hot solutions to soil can be a good alternative in chelate-enhanced phytoextraction.

The naturally enhanced phytoextraction using plant root exudates to induce metal uptake was also studied in the current project. Results showed when the plant of pea (*Pisum sativum* L.) were mixing-cultured with barley (*Hordeum vulgare* L.), the concentrations of Cu, Pb, Zn, Cd and Fe in the shoots of pea reached 1.5-, 1.8-1.4-, 1.4- and 1.3-fold of those grown in sole. Adding root exudates of barley to the pea plants grown in the pots resulted in significant increases of metal accumulation in the shoots of pea, which indicated that root exudates in the mixed culture system played an important role in solubilizing metals in soil and facilitating the metal uptake by plants. The results may provide a new potential direction on more economical and much safer phytoremediation methods.

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LIST OF ACRONYMS IN THIS THESIS

Abbreviation	Full Name
AAS	atomic absorption spectrophotometer
CA	citric acid
CCA	coated chelating agent
CDTA	trans -1, 2 -diaminocyclohexane -N, N, N', N'-tetraacetic acid
CEC	cation exchangeable capacity
DIW	deionized water
DM	dry matter
DMA	2'-deoxymugineic acid
DNP	2, 4-dinitrophenol
DOC	dissolved total organic carbon
DTPA	diethylenetriaminepentaaceticacid
DW	dry weight
EC	electrical conductivity
EDDHA	etylenediamine-di (*o*-hydroxyphenylacetic acid)
EDDS	(S,S-ethylenediaminedisuccinic acid)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol -bis (ß -aminoethyl ether), N, N, N', N-tetraacetic acid
GSH	glutathione
HBED	N,N-di(2-hydroxybenzyl)ethyleneamide N,N-prime-diacetic acid
HEDTA	N-hydroxyethylenediaminetriacetic acid
HEIDA	N-(2-hydroxyethyl)iminodiacetic acid
ICP-AES	inductively coupled plasma-atomic emission spectrometry
MC	methanol and trichloromethane
NTA	nitrilotriacetate
PCs	phytochelatins
PS	phytosiderophores
SRM	standard reference material
USEPA	US Environmental Protection Agency

TABLE OF CONTENT

CERTIFICATE OF ORIGINALITY.....II

ABSTRACT.....III

ACKNOWLEDGMETN.....VI

LIST OF ACRONYMS IN THIS THISIS.....VII

TABLE OF CONTENT.....VIII

LIST OF TABLES.....XV

LIST OF FIGURES.....XVIII

CHAPTER 1 – INTRODUCTION..... ...1

1.1 Background.....1

1.2 Objectives of the Present Study.....4

1.3 Scopes of Work.....5

1.4 Organization of the Thesis.....6

CHAPTER 2 – LITERATURE REVIEW.....8

2.1 Phytoremediation: Definition, Advantages and Limitations.....8

2.2 Phytoextraction of Metals.....13

2.2.1 Continuous Phytoextraction.....14

2.2.1.1 Mechanisms of Metal Hyperaccumulation.....15

2.2.1.2 Limitations of Using Hyperaccumulators to Remediate Contaminated Soils.....19

2.2.1.3 Potential Development of the Continuous Phytoextraction Technology.....20

2.2.2 Induced Phytoextaction.....24

2.2.2.1 Enhanced Pb Phytoextraction with the Application of Chelates.....26
2.2.2.2 Enhanced Cu, Zn, Cd and Ni Phytoextraction with the Application of Chelates.....31
2.2.2.3 Enhanced U Phytoextraction with the Application of Citric Acid.....34
2.2.2.4 Inorganic Chemicals Application in Phytoextraction of Metals.....35
2.3 The Future of Phytoremediation.....59

CHAPTER 3 – SCREENING PLANT SPECIES FOR EFFICIENT PHYTOEXTRACTION.....65

3.1 Introduction.....65
3.2 Materials and Methods.....66
3.2.1 Phytoextraction of Heavy Metals Contaminated Soils by 18 Different Plants with EDTA Application.....66
3.2.2 Comparison of EDTA and EDDS for Enhanced Phytoextraction of Heavy Metals by Garland Chrysanthemum and Corn, and Residual Effects of Chelates in Soils.....70
3.2.3 Comparison of EDTA and EDDS for Enhanced Phytoextraction of Cu from Cu-contaminated Soils by Garland Chrysanthemum and Corn, and Residual Effects of Chelates in Soils.....72
3.2.4 Plant and Soil Analysis.....73
3.2.5 Data Analysis.....75
3.3 Results and Discussion.....75
3.3.1 Effects of EDTA on the Growth of Different Plants.....75
3.3.2 Effects of EDTA on the Shoot Metal Concentrations and Phytoextraction by Different Plants.....77
3.3.3 Effects of EDTA and EDDS on the Growth of Garland

Chrysanthemum and Corn.....85
3.3.4 Effects of EDDS and EDTA on Shoot Metal Concentrations and Phytoextraction by Garland Chrysanthemum and Corn.....85
3.3.5 The Residual Effects of EDTA and EDDS on the Growth and Uptake of Metals by Corn.....100
3.3.6 Effects of EDTA and EDDS on the Growth of Garland Chrysanthemum and Corn Grown in the Cu-Contaminated Soil.....104
3.3.7 Effects of EDTA and EDDS on Cu Concentration and Phytoextraction by Garland Chrysanthemum and Corn Grown in the Cu-contaminated Soil.....106
3.3.8 The Residual Effects of EDTA and EDDS on the Growth and Uptake of Cu by Pea Grown in the Cu-contaminated Soil.....112

CHAPTER 4 – SELECTING CHELATES AND OPTIMIZING THE STRATEGY OF CHELATES APPLICATION.....117

4.1 Introduction 117
4.2 Materials and Methods.....118
4.2.1 Soil Characterization.....118
4.2.2 Comparison of EDTA, EDDS and Citric Acid.....119
4.2.3 The Combined Application of EDTA, EDDS and Citric Acid.....120
4.2.4 The Combined Application of EDTA and EDDS at Different Ratios.....121
4.2.5 Extracting Metals with Chelates.....121
4.2.5.1 Extracting Metals with EDTA and EDDS within Different Time.....121
4.2.5.2 Extracting Metals with Combined Application of EDTA and EDDS at Different Ratio.....122
4.2.6 Plant and Soil Analysis.....123
4.3 Results and Discussion.....125

4.3.1 Effects of EDTA, EDDS and Citric Acid on Plant Growth.....125
4.3.2 Effects of EDTA, EDDS and Citric Acid on Shoot Metal Concentrations and Phytoextraction.....127
4.3.3 Effects of the Combined Application of Chelates on the Growth of Corn.....137
4.3.4 Effects of the Combined Application of Chelates on Metal Concentrations and Phytoextraction in Corn.....139
4.3.5 Effects of the Combined Application of EDTA and EDDS at Different Ratios.....144
4.3.6 Effects of EDTA and EDDS on the Solubility of Metals in Soil.....150

CHAPTER 5 - EXPLORING THE MECHANISM OF CHELATE-ENHANCED PHYTOREMEDIATION and Optimizing the Combination of Plant and Chelate Application.....160

5.1 Introduction.....160
5.2 Materials and Methods.....162
5.2.1 Plant Culture.....162
5.2.2 Effects of EDTA on the Accumulation of Pb in Indian Mustard.....162
5.2.3 Effects of Pb and EDTA on the Relative Electrolytic Leakage Rate of Root Cells.....163
5.2.4 Effects of Root Damage on the Accumulation of Pb in Indian Mustard.....164
5.2.5 Hot EDTA and EDDS Treatments with Pot Experiment.....164
5.2.6 Hot Citric Acid and NTA Treatment.....166
5.2.7 Metal Leaching Study.....166
5.2.8 Root Pretreatment with Hot Water in Hydroponic Experiment.....167
5.2.9 Plant and Soil Analysis.....168
5.3 Results and Discussion.....169

5.3.1 Growth of Indian Mustard.....170

5.3.2 Effects of EDTA on the Concentrations of Pb in Indian Mustard....175

5.3.3 Relative Electrolyte Leakage Rate of Root Cells.....182

5.3.4 Effects of Root Damage Pretreatments on the Accumulation of Pb in Shoots.....185

5.3.5 Plant Growth in Pot Experiment.....189

5.3.6 Metal Concentrations and Phytoextraction in Hot EDTA and EDDS Treatments.....191

5.3.7 Metal Leaching Study after the Treatment with EDTA and EDDS.....199

5.3.8 Metal Accumulation in Treatments of Hot Citric Acid and NTA.....203

5.3.9 Effects of Pretreatment with Hot Water on the Accumulation of Pb in Beans.....205

CHAPTER 6 – METAL LEACHING STUDY AFTER THE APPLICATION OF EDDS.....209

6.1 Introduction.....209

6.2 Materials and Methods.....211

6.2.1 Soil Preparation.....211

6.2.2 Effects of EDDS Treatment on Plant Growth and Metal Uptake.....211

6.2.3 Pot Leaching Study with the Addition of Artificial Rainwater.....212

6.2.4 Chemical and Physical Analysis.....213

6.3 Results and Discussion.....213

6.3.1 Plant Growth.....214

6.3.2 Metal Concentrations and Phytoextraction with the Application of EDDS.....216

6.3.3 The Leaching of Metals from the Pots.....222

CHAPTER 7 – EXPLORING THE NATURALLY ENHANCED PHYTOREMEDIATION: EFFECTS OF ROOT INTERACTION ON PHYTOEXTRACTION OF METALS FROM SOILS.....233

7.1 Introduction.....233

7.2 Materials and Methods.....235

7.2.1 Mixing-culture Pea with Corn, Barley and Wheat.....235

7.2.2 Collection of Root Exudates.....236

7.2.3 Extraction of Metals from Soil.....238

7.2.4 Metal Accumulation in Pea with the Addition of Root Exudates from Barley.....238

7.2.5 Plant Analysis.....239

7.3 Results and Discussion.....240

7.3.1 Plant Growth and Metal Accumulation in Sole and Intercropping Systems.....240

7.3.2 Mobilization of Metals from Soil by Barley Root Exudates.....245

7.3.3 Metal Concentrations and Phytoextraction by the Shoots of Pea.....248

CHAPTER 8 – CONCLUSIONS AND RECOMMENDATIONS.....251

8.1 Major Findings.....251

8.2 General Conclusions.....256

REFERENCES.....263

PUBLICATIONS FROM THE RESEARCH PROJECT.....292

DATABASE OF ORIGINAL RESULTS IN ALL EXPERIMENTS.....293

LIST OF TABLES

Table 3.1 The physicochemical properties of the soils used in the study.....69

Tabel 3.2 The structures and log K values for metal complexation of EDTA and EDDS..... ..69

Table 3.3 The temperature setting of plant and soil samples for the acid digestion.....75

Table 3.4 Dry biomass yields (g $plant^{-1}$) of 18 different plants 7 and 14 days after the application of EDTA.....80

Table 3.5 Cu concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA.....81

Table 3.6 Pb concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA.....82

Table 3.7 Zn concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA.....83

Table 3.8 Cd concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA.....84

Table 3.9 The concentration and phytoextraction of metals in the shoots of garland chrysanthemum and corn with the application of EDTA and EDDS.....97

Table 3.10 Effects of chelate treatments on the translocation of metals from roots to shoots in garland chrysanthemum 7 d after the treatment.....98

Table 3.11 Effects of chelate treatments on the translocation of metals from roots to shoots in corn 7 d after the treatment.....99

Table 3.12 The concentration of metals in the shoots of corn grown in the second cropping.....103

Table 3.13 Effects of EDTA and EDDS treatments at rate of 3 mmol kg^{-1} on Cu translocation from roots to shoots in garland chrysanthemum 7 d

after the treatment.....110
Table 3.14 Effects of EDTA and EDDS treatments at rate of 3 mmol kg^{-1} on Cu translocation from roots to shoots in corn 7 d after the treatment.....111
Table 4.1 The physicochemical properties of the soils used in the study.....124
Table 4.2 Shoot metal concentrations in corn and beans grown in soil with and without the application of chelates (mg kg^{-1}).....133
Table 4.3 Effects of chelate treatments on the translocation of metals from the roots to the shoots of corn and beans 14 d after treatment.....136
Table 4.4 Effects of chelate treatments on the translocation of metals from roots to shoots in corn 14 d after treatment.....143
Table 4.5 Effects of chelate treatments on Pb translocation from roots to shoots in corn 14 d after treatment.....149
Table 4.6 Cu, Pb, Zn, Cd, and Ca concentrations (mg kg^{-1}) in soil solutions extracted with different chelates within two days.....157
Table 5.1 Effects of various Pb treatments on the root elongation and dry matter weights of Indian mustard.....172
Table 5.2 Effects of different EDTA treatments on the root elongation and dry matter weights of Indian mustard.....173
Table 5.3 Effects of different EDTA treatments on the net elongation of the roots and dry matter weights of Indian mustard exposed to 500 μmol L^{-1} of Pb.....174
Table 5.4 Effects of various pretreatments of Indian mustard roots on the uptake of Pb in 500 μmol L^{-1} of Pb + 250 μmol L^{-1} of EDTA, 500 μmol L^{-1} of Pb + 3000 μmol L^{-1} of EDTA or 500 μmol L^{-1} of Pb + 3000 μmol L^{-1} of EDTA + 100 μmol $L^{-1}$of DNP solutions for 2 d.....188
Table 5.5 Total phytoextraction (μg pot^{-1}) of Cu, Pb, Zn and Cd in the shoots of garland chrysanthemum and beans 7 d after the application of EDTA and EDDS at different concentrations (mmol kg^{-1} soil).....198
Table 5.6 Effects of the application of EDTA and EDDS at different rate (mmol

kg^{-1} soil) on metal solubility (mg kg^{-1} soil) 7 d after the application.....201

Table 5.7 Effects of the application of citric acid (CA) and NTA at different concentration (mmol kg^{-1} soil) on the shoot dry weight (g pot^{-1}) and concentration (mg kg^{-1}) of Cu, Pb, Zn and Cd in the shoots of beans.....204

Table 5.8 The correlation between the relative electrolyte leakage rate of roots and the concentrations of Cu, Zn, and Cd in the shoots of beans (R^2 was shown in the Table).....208

Table 6.1 Total phytoextraction (μg pot^{-1}) of Cu, Pb, Zn, and Cd in the shoots of corn and beans 7 d after the application of EDDS at different concentrations (mmol kg^{-1} soil).....221

Table 6.2 The leachate solution volume collected under the pots (ml).....228

Table 6.3 The pH of the leachate solutions.....229

Table 6.4 Total amounts of DOC and metals of in the leachate solutions 7 d after the application of EDDS 232

Table 7.1 The dry matter yields (g $plant^{-1}$) in the shoots of plants.....243

Table 7.2 The content of dissolved organic C (mg g^{-1} root DW) and the pH of the root exudates collected from barley in control (full nutrition solution) and Fe deficiency nutrition solution.....247

Table 7.3 The amounts of metals in the soil extracted with the addition of root exudates (mg kg^{-1} root DW of barley).....247

Table 7.4 The concentration of metals (mg kg^{-1} DW) in the shoots of pea.....250

Table 7.5 The total phytoextraction of metals (mg kg^{-1} root DW of barley) in the shoots of pea.....250

LIST OF FIGURES

Fig. 1.1 The relationship among different parts of the thesis.....7

Fig. 3.1 Effects of the application of EDTA and EDDS on the dry mass yields of shoots in garland chrysanthemum (a) and corn (b).....92

Fig. 3.2 Effects of the application of EDTA and EDDS on the concentration of Cu in the shoots of garland chrysanthemum (a) and corn (b).....93

Fig. 3.3 Effects of the application of EDTA and EDDS on the uptake of Cu in the shoots of garland chrysanthemum (a) and corn (b).....94

Fig. 3.4 Effects of the application of EDTA and EDDS on the concentration of Pb in the shoots of garland chrysanthemum (a) and corn (b).....95

Fig. 3.5 Effects of the application of EDTA and EDDS on the uptake of Pb in the shoots of garland chrysanthemum (a) and corn (b).....96

Fig. 3.6 The dry mass yields of corn grown in the second cropping.....102

Fig. 3.7 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1}on the dry matter yields of shoots in garland chrysanthemum (a) and corn (b) grown in different Cu-contaminated soil.....105

Fig. 3.8 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the concentration of Cu in the shoots of garland chrysanthemum (a) and corn (b) grown in different Cu-contaminated soil.....108

Fig. 3.9 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the phytoextration of Cu in the shoots of garland chrysanthemum (a) and corn (b) grown in different Cu-contaminated soil.....109

Fig. 3.10 The dry mass yields of pea grown in the second cropping.....114

Fig. 3.11 The concentration (a) and the total uptake (b) of Cu in the shoots of pea grown in the second cropping.....115

Fig. 3.12 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the solubilization of Cu in the different Cu-contaminated soil.....116

Fig 4.1 Effects of the application of chelates on the dry matter yields of shoots and roots in corn (a) and beans (b).....126
Fig. 4.2 Effects of the application of chelate on the uptake of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn and beans 7 d after the treatment.....134
Fig. 4.3 Effects of the application of chelates on the uptake of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn and beans 14 d after the treatment.....135
Fig. 4.4 Effects of the application of chelates on the dry matter yields of shoots and roots in corn 14 d after the treatment.....138
Fig. 4.5 Effects of the application of chelates on the concentrations of Cu (a), Pb (b), Zn (c), and Cd (d) in the shoots of corn.....141
Fig. 4.6 Effects of the application of chelates on the uptake of Cu (a), Pb (b), Zn (c), and Cd (d) in the shoots of corn.....142
Fig. 4.7 Effects of the combined application of EDTA and EDDS at different ratios on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn.....146
Fig. 4.8 Effects of the combined application of EDTA and EDDS at different ratios on the dry matter yields of shoots and roots in corn.....147
Fig. 4.9 Effects of the combined application of EDTA and EDDS at different ratios on the uptake of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn.....148
Fig. 4.10 Changes in soluble Cu (a), Pb (b), Zn (c) and Cd (d) concentrations in soil treated with chelates with time.....156
Fig. 4.11 Changes in the soluble concentrations of Pb (a), Ca (b), and pH (c) in soil treated with EDTA and EDDS at different concentrations.....158
Fig. 4.12 Changes in the soluble concentrations of Pb (a), Ca (b), and pH (c) in soil treated with the combined application of EDTA and EDDS at different ratios.....159
Fig. 5.1 Effects of various EDTA treatments on the concentrations of Pb in the

shoots (a) and roots (b) of Indian mustard exposed to 500 μmol L^{-1} of Pb in solution for 7 d.....179

Fig. 5.2 Effects of different EDTA treatments on the ratios of Pb concentration between shoots/roots (a) and the percentages of the Pb absorbed by shoots (b) exposed to 500 μmol L^{-1} of Pb in solution for 7 days.....180

Fig. 5.3 Relationship between the concentrations of EDTA and Pb in the shoots of Indian mustard exposed to 500 μmol L^{-1} of Pb and various concentrations of EDTA in solution for 7 days.....181

Fig. 5.4 Effects of various Pb treatments on the relative electrolyte leakage rates of root cells (a) and the correlation between the relative electrolyte leakage rate and the concentration of Pb in the shoots of Indian mustard (b).....183

Fig. 5.5 Effects of different EDTA treatments on the relative electrolyte leakage rates of root cells (a) and the correlation between the relative electrolyte leakage rate and the concentration of Pb in the shoots (b) of Indian mustard exposed to 500 μmol L^{-1} of Pb in solution for 7 days.....184

Fig. 5.6 Effects of the application of EDTA and EDDS on the dry matter yields of garland chrysanthemum (a) and beans (b).....190

Fig. 5.7 Effects of the application of EDTA and EDDS on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of garland chrysanthemum.....196

Fig. 5.8 Effects of the application of EDTA and EDDS on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of beans.....197

Fig. 5.9 Effects of the application of hot EDTA and EDDS at different concentrations on the solubility of Cu (a), Pb (b), Zn (c) and Cd (d).....202

Fig. 5.10 The correlation between the relative electrolyte leakage rate of roots and the concentration of Pb in the shoots of beans.....207

Fig. 6.1 Effects of the application of EDDS on the shoot dry matter yields of corn (a) and beans (b).....215
Fig. 6.2 Effects of EDDS application on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn.....219
Fig. 6.3 Effects of EDDS application on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of beans.....220
Fig. 6.4 Amounts of DOC, Cu, Pb, Zn, and Cd in the leachates from the soil without plants and the soil grown with corn and beans after the application of normal EDDS during the whole leaching study.....231
Fig. 7.1 The concentrations of Cd, Cu, Pb, Zn and Fe in the shoots of plants in different cropping system. The data for Cd and Pb shown here are 10 times of the original data.....244

Chapter 1 - Introduction

1.1 Background

There have been increasing concerns in Hong Kong and world wide about the metal contamination of the environment by urban wastes and by-products of industrial and mining industries (Body et al., 1991; Li et al., 2000). The majority of these contaminants are heavy metals, such as Cu, Pb, Zn and Cd, which can affect flora, fauna and human being living in the vicinity or downstream of the contaminated sites. The remediation of soils contaminated with toxic metals is a challenging task because metals are very difficult to degrade and the danger of them is aggravated by their almost indefinite persistence in the environment. Conventional cleanup technology is generally too costly, and often harmful to normal soil properties (i.e. texture, organic matter) for the restoration of contaminated sites (Holden et al., 1989; Smith et al., 1995). The emerging phytoremediation techniques with lower costs and environmental friendly nature have received increasing attention in the last two decades (Salt et al., 1998; Garbisu and Alkorta, 2001).

The success of phytoremediation process is dependent on the adequate plant yield and efficient transfer of metals from plant roots into shoots for effective metal removal. Most hyperaccumulators, such as *Thlaspi, Urtica, Chenopodium,*

Polygonum sachalase, and *Alyssim* have the characteristics of slow-growing, low-biomass production, which make these plants unfeasible for practical phytoextraction in the field (Mulligan et al., 2001; Puschenreiter et al., 2001). For this reason, more recent research projects on phytoextraction have focused on high biomass crop species, such as maize (*Zea mays*), pea (*Pisum sativum*), oat (*Avena sativa*), barley (*Hordeum vulgare*) and Indian mustard (*Brassica juncea*), and relevant plant husbandry and soil management practices to enhance the metal uptake by these high biomass species (Blaylock et al., 1997; Huang et al., 1997; Ebbs and Kochian, 1997; Shen et al., 2002). Although several conditions must be met in order for phytoremediation to be effective, the bioavailability of metals to plant roots is considered to be a critical requirement for plant uptake to occur (Kayser et al., 2000). Soil factors such as pH, cation exchange capacity, or organic matter content play an important role in successful soil remediation processes. To increase metal availability, a number of chelates such as EDTA (ethylenediaminetetraacetic acid), CDTA (trans -1, 2 -diaminocyclohexane -N, N, N', N'-tetraacetic acid), EGTA [ethyleneglycol -bis (ß -aminoethyl ether), N, N, N', N-tetraacetic acid], EDDHA [etylenediamine-di (*o*-hydroxyphenylacetic acid)]) have been used to desorb metals from the soil matrix into soil solution to facilitate metals transport into xylem, and increase metal translocation from roots to shoots of some fast-growing, high-biomass-producing plants (Blaylock et al., 1997; Huang et al., 1997; Cooper et al., 1999; Wu et al., 1999; Shen et al., 2002).

Of the chelates used in phytoremediation study, EDTA has been the most widely studied chelating agent because of its high extraction efficiency of many metals. It was reported that EDTA not only increases the amount of soil Pb taken up by plants, but also the transport of metals through the xylem and the translocation of Pb from roots to shoots (Huang et al., 1997; Epstein et al., 1999; Shen et al., 2002). Although EDTA is very effective in mobilizing metals in soils, EDTA and EDTA-heavy metal complexes can be toxic to plants and soil microorganisms and they can be also persistent in the environment due to its low biodegradability (Bucheli-Witschel and Egli, 2001; Grčman et al., 2003). This nature may increase potential off-site migration of metals, either in surface runoff or by leaching of metals into ground waters (Nowack, 2002). In EDTA-facilitated phytoremediation, the amount of heavy metals taken up by plants is minor compared to the amount mobilized from the soil and the large quantities that are leached out of the root zone (Madrid et al., 2003, Chen et al., 2004a, b).

In recent years, some easily biodegradable chelating agents, such as NTA (nitrilotriacetate) and EDDS (S,S-ethylenediaminedisuccinic acid), have been proposed to enhance the uptake of heavy metals in soil phytoremdeiation (Kulli et al., 1999; Kayser et al., 2000; Grčman et al., 2003; Kos and Leštan, 2003 a, b). However, the accumulation of Cu, Zn and Cd, in maize, Indian mustard and

other plants was only increased by a factor of 2 to 3, although the solubility of these metals in the soil increased by a factor of 9 to 21 (Kayser et al., 2000). It was concluded that even the highest concentrations of heavy metals in the harvestable plant tissues achieved, it was still far from effective using this procedure.

This study aims at screening several common high biomass plant species sensitive to chelates and exploring strategy of chelates application to enhance the phytoextraction and reduce the leaching of heavy metals. Meanwhile, the mechanisms underling the improved accumulation of heavy metals by plants with the addition of chelates have been studied to provide more information in developing phyotoextraction technique applicable in the field. Besides, some naturally enhanced phytoremedaion techniques were also explored in the current study. The findings in the present study will be useful for the improvement of chemically assisted phytoextraction technology in practical operations.

1.2 Objectives of the Present Study

The overall research aims are to optimize the combination of plant species and chelate application strategy to achieve the most efficient metal phytoextraction with least negative effects on the surrounding environment. The specific

objectives of this study are:

(1) To screen possible high-biomass plant species from normal crop plants and vegetables which are more sensitive to the application of chelates;

(2) To identify key soil amendments that efficiently increase metals transformation from soil compartments to soil solution, and trigger metal accumulation in plants;

(3) To identify the best synergistic combinations between soil amendments and plant selection to maximize total metal removal and to minimize total chelate usage;

(4) To elucidate the controlling mechanisms of enhanced uptake of metals by plants with the application of chelates;

(5) To explore the best management practices to avoid groundwater pollution arising from metal mobilisation by soil amendments; and

(6) To investigate the potential of naturally improved phytoextraction without the application of chelates for use in the future phytoremediation development.

1.3 Scopes of Work

This thesis focuses on the phytoremediation with the application of chemical agents. The experiment programs include the plant culture and the metal analysis of plant and soil samples. Using soil pot experiment and hydroponic experiments, plant species more sensitive to the chelate application were

screened and chelate application methods were explored for the higher metal removal efficiency and less metal leaching to ground water and the surrounding environment.

1.4 Organization of the Thesis

This thesis is divided into eight chapters. The present chapter covers the background, objectives, and scope of this research project. A detailed literature review is given in Chapter Two. Chapter Three focuses on the plant species screening experiment. Chapter Four concentrates on the selection of chelates and the study of chelate application methods. Chapter Five studies the potential mechanisms of metal uptake by plants and optimizing the combination of plant and chelate application. Metal leaching associated with the application of chelates is evaluated in Chapter Six. Chapter Seven explores the potential natural phytoremediation methods using the plant root exudates. Finally, Chapter Eight gives a general summary of the major findings in this study, and overall discussion and conclusion of chemically assisted phytoremediation techniques studied. The following flow chart briefly describes the overall organization of the thesis (Fig. 1.1).

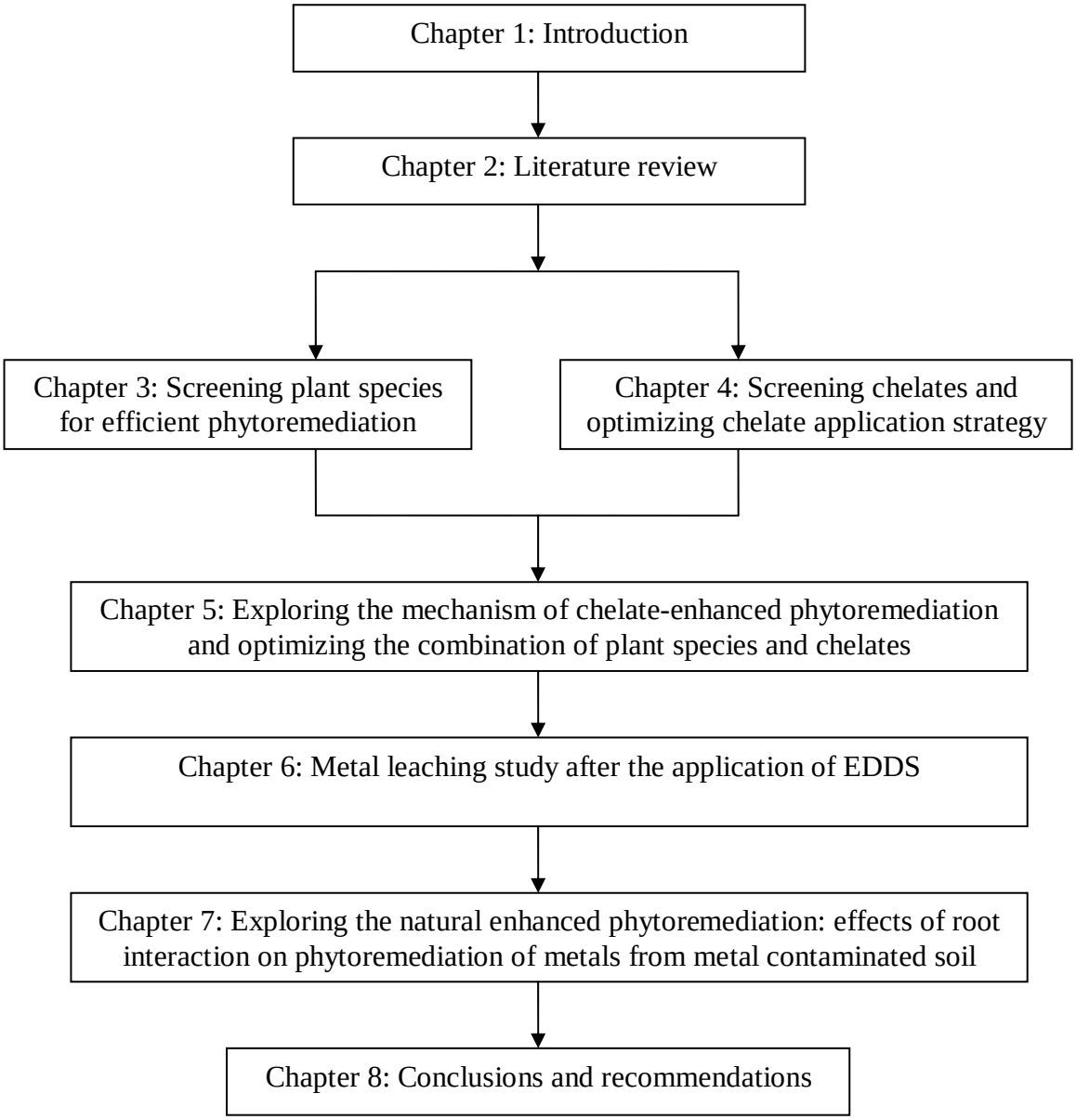


Fig. 1.1 The relationship among different chapters of the thesis.

Chapter 2 - Literature Review

This review mainly focuses on phytoextraction technologies for removing toxic metals from contaminated sites. In particular, the area of induced phytoextraction with the application of chelates is discussed in details. By comparing the chelate-assisted phytoextraction with that using the hyperaccumulating plants, the advantages, limitations and the areas to be improved in the phytoextraction technology are discussed. Besides, other phytoremediation technologies, such as phytofiltration, phytostabilization, and phytovolatilization, are also described in this chapter. Finally, the future development in the area of phytoremediation is discussed.

2.1 Phytoremediation: Definition, Advantages and Limitations

Rapid industrialization, increased anthropogenic activities, modern agricultural practices and faulty waste disposal methods have increased the concentrations of toxic metals in the environment, which may cause toxicity to living organisms, including human being. Contamination of radionuclides has also become problematic since the development of nuclear technology in the second half of the 20^{th} century. Other sources of radioactive contamination include naturally occurring radioactive materials such as uranium, thorium, radon and radium. Recognition of the ecological and human health hazards of the

pollutants has led to the development of several technologies for remediation. Among them, the emerging phytoremediation techniques have received increasing attention in the last two decades (Salt et al., 1998; Garbisu and Alkorta, 2001).

Phytoremediation, defined as the use of plants to remove pollutants from the environment or to render them harmless by stablisation processes (Cunningham and Berti, 1993; Raskin et al., 1994). Phytoremediation is often also referred as botanical bioremediation or green remediation (Chaney et al., 1997). It can be applied to both organic and inorganic pollutants, present in solid substrates (e.g. soil), liquid substrates (e.g. water), and the air. Phytoremediation is divided into the following areas (Salt et al., 1995a, 1998; Chaney et al., 1997; Raskin et al., 1997):

Phytoextraction: the use of plants to remove contaminants from soils. Pollutant-accumulating plants are utilized to transport and concentrate contaminants (metals or organics) from the soil into the above-ground shoots. The term is mostly used to refer to metal removal from soils. In some cases, roots can be harvested as well (Kumar et al., 1995).

Phytofiltration: the use of plant roots (rhizofiltration) or seedlings (blastofiltration) to absorb or adsorb pollutants, mainly metals, from water and

aqueous-waste streams. Plant roots or seedlings grown in aerated water absorb, precipitate and concentrate toxic metals from polluted effluents.

Phytostabilization: the use of plants to reduce the bioavailability of pollutants in the environment. Plants stabilize pollutants in soils, thus rendering them harmless and reducing the risk of further environmental degradation from leaching of pollutants into the ground water or airborne dispersion.

Phytovolatilization: the use of plants to volatilize pollutants. Plants extract volatile pollutants (e.g., selenium (Se) and mercury (Hg)) from soil and volatilize them from the foliage.

Phytodegradation: the use of plants and associated microorganisms (plant-assisted bioremediation) to degrade organic pollutants. Plant roots in conjunction with their rhizospheric microorganisms are utilized to remediate soils contaminated with organics.

Some authors also distinguish between indirect and direct phytoremediation (Stomp et al., 1994). In the case of indirect phytoremediation, plants participate in the detoxification of pollutants via their support of symbiotic, root-associated microorganisms that actually accomplish contaminant detoxification (this matches the previously named plant-assisted bioremediation). On the other hand,

plants could participate directly through contaminant uptake and subsequent contaminant immobilization or degradation within the plants.

Phytoremediation has gained popularity with government agencies and industry in the past 10-15 years. This popularity is based in part on the relatively low cost of phytoremediation, combined with the limited funds available for environmental cleanup. The costs associated with environmental remediation are staggering. Currently, $6–8 billion per year is spent for environmental cleanup in the United States, and $25–50 billion per year worldwide (Glass, 1999; Tsao, 2003). Because biological processes are ultimately solar-driven, phytoremediation is on average 10-fold cheaper than conventional engineering-based remediation methods such as soil excavation, soil washing or burning, or pump-and-treat systems (Glass, 1999). The fact that phytoremediation is usually carried out *in situ* contributes to its cost-effectiveness and may reduce exposure of the polluted substrate to humans, wildlife, and the environment. Phytoremediation also enjoys popularity with the general public as a “green clean” alternative to chemical plants and bulldozers. Thus, government agencies like to include phytoremediation in their cleanup strategies to stretch available funds, while corporations (e.g., electric power, oil and chemical industry) like to advertise their involvement with this environment-friendly technology, and environmental consultancy companies increasingly include phytoremediation in their package of offered technologies

(Pilon-Smits, 2005).

Phytoremediation has its advantages as well as limitations. In order to apply phytoremediation efficiently, the limitations of this technique also should be taken into account. These limitations include i) the time necessary for acceptable effects: phytoremediation is often slower than the more established engineering remediation methods like excavation, incineration, or pump-and-treat systems. Soil cleanup via plant accumulation often takes years or decades; ii) the limited depth of the root system: effectiveness of phytoremediation is also limited by root depth because the plants have to be able to reach the pollutant. Root depth is typically 50 cm for herbaceous species or 3 m for trees (Negri et al., 2003); iii) the slow growth of plants: most hyperaccumulating plants grow very slowly and produce little biomass yields (Salt et al., 1998); iv) the sensitivity toward some pollutants: the plants that mediate the cleanup have to be where the pollutant is and have to be able to act on it. Therefore, soil properties, toxicity level, and climate should allow plant growth; and v) the availability of metals: phytoremediation may also be limited by the bioavailability of the pollutants. If only a fraction of the pollutant is bioavailable, but the regulatory cleanup standards require that all of the pollutant is removed, phytoremediation is not applicable by itself (Flechas and Latady, 2003).

Nonbiological remediation technologies and bio/phytoremediation are not mutually exclusive. Because pollutant distribution and concentration are heterogeneous for many sites, the most efficient and cost-effective remediation solution may be a combination of different technologies, such as excavation of the most contaminated spots followed by polishing the site with the use of plants. Such an integrated remediation effort requires a multidisciplinary approach in clean up actions.

2.2 Phytoextraction of Metals

The term “phytoextraction” mainly concerns the removal of heavy metals or radionuclides from soil by means of the uptake capabilities of plants. Plants can accumulate heavy metals essential for growth and development such as Fe, Mn, Zn, Cu, Mg, Mo, and possibly Ni. In addition, some of them have the capacity to accumulate heavy metals with no known biological functions, such as Cd, Cr, Pb, Co, Ag, Se and Hg (Baker and Brooks, 1989; Raskin et al., 1994). The idea of using plants to remove metals from soils came from the discovery of different wild plants, often endemic to naturally mineralized soils, which accumulate high concentrations of metals in their foliage (Brooks et al., 1979; Baker and Brooks, 1989; Raskin et al., 1997). The prevention of herbivory and disease is thought to be the main function of this unique phenomenon of hyperaccumulation (Baker and Brooks, 1989; Ernst et al., 1990; Boyd et al.,

1994; Boyd and Martens, 1994, 1995). As pointed out by Salt et al. (1998) there are, at present, two strategies of phytoextraction: continuous phytoextraction using hyperaccumulators and chelate-assisted or induced phytoextraction.

2.2.1 Continuous Phytoextraction

Continuous phytoextraction depends on the natural ability of some plants to accumulate, translocate and resist high amounts of metals over the complete growth cycle. In this context, hyperaccumulators are the most suitable plants for the phytoextraction of metal-polluted soils. Metal hyperaccumulation is a rare phenomenon that occurs in terrestrial plants. To date, over 400 plant species have been identified as natural metal hyperaccumulators, representing < 0.2% of all angiosperms (Brooks, 1998; Baker et al., 2000; Reeves and Baker, 2000). Threshold values of metal concentrations have been used to define metal hyperaccumulation, including 10 000 mg kg^{-1} dry weight of shoots for Zn and Mn, 1000 mg kg^{-1} for Co, Cu, Ni, As and Se, and 100 mg kg^{-1} for Cd (Brooks et al., 1977; Baker and Brooks, 1989). These concentrations are two to three orders of magnitude higher than that in normal plant species growing on uncontaminated soils. Instead of these rather arbitrary values, the following common traits are now known to be shared by all hyperaccumulators: the bioconcentration factor is usually greater than 1, and in some cases reaching 50-100 (Ma et al, 2001; Zhao et al., 2003); the shoot-to-root ratio of metal

concentration is greater than 1, which is indicative of an efficient root-to-shoot transport; hyperaccumulators possess a much enhanced tolerance (hypertolerance) to metals in the medium and inside plant cells, indicating a strong internal detoxification mechanism. Understanding the biological mechanisms of hyperaccumulation may therefore help in the development of superior plants for the phytoremediation of metals.

2.2.1.1 Mechanisms of Metal Hyperaccumulation

Rhizospheric Interactions Hyperaccumulator species are able to accumulate higher metal concentrations in their shoots than surrounding nonaccumulator plants even from soils containing non-phytotoxic background levels of metals (Brown et al., 1994; McGrath et al., 1997). One possible mechanism to explain this enhanced metal accumulation could be enhanced ability to solubilize metals within the rhizosphere of the hyperaccumulator. This is supported by evidence suggesting that the zinc hyperaccumulator *Thlaspi careulescens* has an enhanced ability to extract zinc from the immobile fraction of the soil (McGrath et al., 1997). In soil, co-cultivation of the zinc hyperaccumulator *T. caerulescens* with the nonaccumulator *T. arvense* increased shoot zinc accumulation in the nonaccumulator (Whiting, 1997), again suggesting than the hyperaccumulator is able to modify the rhizosphere to enhance metal solubility. However, this effect was not observed for nickel when the nickel

hyperaccumulator *T. goesingense* was co-cultured hydroponically with the nonaccumulator *T. arvense* (Salt, 1999).

Root Uptake Enhanced root uptake of Zn and Cd was verified in the hyperaccumulator *T. caerulescens* (Lasat et al., 1996; Assuncáo et al., 2001; Lombi et al., 2001; Zhao et al., 2002). Roots of the zinc hyperaccumulator *T. caerulescens* appear to contain more zinc transporters per gram fresh weight than the non-accumulator *T. arvense* (Lasat et al., 1996). Several Zn transporter cDNAs have recently been cloned from *T. caerulescens* (Pence et al., 2000; Assuncáo et al., 2001). These transporters belong to the ZIP family (zinc-regulated transporter/iron-regulated transporter like proteins) (Maser et al., 2001). *ZNT1* and *ZNT2* are highly expressed in the roots of *T. caerulescens* and expression is barely responsive to the Zn status in the plants. Through functional complementation in yeast, *ZNT1* was shown to mediate high-affinity uptake of Zn^{2+} as well as low affinity uptake of Cd^{2+} (Pence et al., 2000). Specific alterations in Zn-responsive elements (e.g. transcriptional activators), possibly play an important role in Zn hyperaccumulation in this plant (Pence et al., 2000). In the case of Cd, the superior ability of the Southern French ecotype of *T. caerulescens* to accumulate Cd cannot be explained by the Zn transport pathway (Lombi et al., 2001; Zhao et al., 2002), but may be related to an enhanced expression of *IRT1* (Lombi et al., 2002). *IRT1* is essential for Fe acquisition by the roots of *Arabidopsis thaliana* and can also mediate

high-affinity Cd^{2+} uptake (Connolly et al., 2002; Vert et al., 2002). In the arsenic hyperaccumulator *P. vittata*, arsenate uptake is mediated by phosphate transporters (Wang et al., 2002), as is the case for arsenic non-hyperaccumulating plants.

Root-to-Shoot Metal Translocation Enhanced root-to-shoot transport is another key component of metal/metalloid hyperaccumulation. This may be achieved by a reduced sequestration of the metal in the root vacuoles (Lasat et al., 1998) or by enhanced xylem loading. However, there has been little progress in research on this aspect. In the Ni hyperaccumulator *Alyssum lesbiacum*, exposure to Ni elicited a large increase in the concentration of histidine in the xylem sap (Krämer et al., 1996). The response may explain the enhanced Ni tolerance of roots of *A. lesbiacum*, as well as the enhanced root-to-shoot transport of Ni.

Metal Sequestration and Complexation Hypertolerance is essential for the hyperaccumulation phenotype to occur in natural hyperaccumulators. Hypertolerance is achieved by internal detoxification and probably involves compartmentation and complexation. There is evidence that metals and metalloids are sequestered in leaf vacuoles in Zn, Cd, Ni and As hyperaccumulators (Vazquez et al., 1994; Küpper et al., 1999; Krämer et al., 2000; Küpper et al., 2001; Lombi et al., 2002). Metal transporter genes, which

encode putative vacuolar ion transport proteins, have been cloned from *T. caerulescens* (*ZTP1*) (Assuncáo et al., 2001) and from the Ni hyperaccumulator *Thlaspi goegingense* (*TgMTP*) (Persans et al., 2001).

With regard to complexation, Ni may be complexed by organic acids, particularly citrate in some Ni hyperaccumulators (Brooks, 1998), or by histidine in roots and xylem saps of *A. lesbiacum* (Krämer et al., 1996). In *T. caerulescens*, Zn was found to be coordinated with histidine in roots and with organic acids or uncomplexed in shoots (Salt et al., 1999). In *Arabidopsis halleri*, Zn was predominantly coordinated with malate in shoots, and with malate, citrate and phosphate in roots (Sarret et al., 2002). There is strong evidence that phytochelatins (PCs) are essential for constitutive tolerance to Cd in non-hyperaccumulator plants (Cobbett and Goldsbrough, 2002). However, recent studies showed that PCs are not involved in the hypertolerance to Cd in *T. caerulescens* (Ebbs et al., 2002; Schat et al., 2002). Detoxification of arsenate generally involves a reduction of arsenate to arsenite, followed by complexation with thiols, particularly PCs, in non-hyperaccumulator species (Meharg and Hartley-Whitaker, 2002). In *P. vittata*, however, current evidence indicates that the main storage form of arsenic in the shoots is inorganic arsenite, uncomplexed with thiols (Ma et al., 2001; Francesconi et al., 2002; Wang et al., 2002; Zhang et al., 2002), and is probably sequestered in the vacuoles (Lombi et al., 2002). In the Se hyperaccumulator *Astragalus bisculatus*, Se is assimilated

into Se-methyl-selenocysteine, which is incorporated into proteins, resulting in the hyperaccumulation of Se (Neuhierl and Bock, 1996).

2.2.1.2 Limitations of Using Hyperaccumulators to Remediate Contaminated Soils

Continuous phytoextraction relies on the natural ability of hyperacuumulator plants to accumulate, translocate and resist high amounts of metals over the complete growth cycle. Major disadvantages of using naturally occurring metal hyperaccumulators for continuous phytoextraction are their relatively low biomass, slow growth rates, the specificity of metal accumulation, and the lack of any hyperaccumulators for the most environmentally important metallic pollutants (e.g. Pb, Cd and U) (Salt et al., 1998; Reeves and Baker, 2000).

Nonetheless, hyperaccumulators can naturally accumulate elevated levels of Co, Cu, Mn, and possible Pb (Byers, 1935, 1936; Brooks et al., 1979; Reeves and Brooks, 1983; Bañuelos and Meeks, 1990; Chaney et al., 1995; Salt et al., 1995b, 1998). It is more important to emphasize that even the best metal accumulators, such as *T. caerulescens* (*Brassicaceae*) (Baker et al., 1994), take a relatively long time of continuous cultivation (13-14 years) to clean a site of Ni or Zn (Salt et al., 1995b). Most hyperaccumulators exhibit remarkable single-element specificity in their natural habitats. It is an advantage for the

clean up of specific contaminant. But it is a disadvantage for multi-element removal in many contaminated sites. Besides, most metal-accumulating plant species known today are discovered growing on soils containing high levels of heavy metals. These plants are often endemic to these types of soils, suggesting that metal accumulation is associated with heavy metal resistance (Baker and Brooks, 1989). The majority of hyperaccumulating species discovered so far are restricted to tropical areas (Baker and Brooks, 1989; Baker et al., 1993; Brooks et al., 1993). Therefore, the plant may not survive or can not hyperacuumulate metals in the areas other than beyond district, which implies the application of this technology may be not possible under some specific climates and regions (Macek et al., 2000; Salt and Krämer, 2000; Schmoger et al., 2000).

2.2.1.3 Potential Development of the Continuous Phytoextraction Technology

The ideal plants to be used in phytoextraction should have the following characteristics: be tolerant to high levels of the metal; accumulate high levels of the metal in its harvestable parts; have a rapid growth rate; have the potential to produce a high biomass in the field; have a profuse root system (Garbisu and Alkora, 2001). Considering these conditions, a two-component long-term strategy needs to be developed for continuous phytoextraction to succeed in the future.

Firstly, attempts to search for new high-biomass metal hyperaccumulators should be continued. The first hyperaccumulators characterized were members of the *Brassicaceae* and *Fabaceae* families. The number of metal-accumulating taxa identified is still growing. Therefore, a continuous search for novel phytoextracting plants adapted to particular ecosystems and climates will be required. There are fast-growing hyperaccumulators that can produce a large biomass. Examples are the Ni hyperaccumulators *Alyssum bertolonii* and *Berkheya coddii*, which produced 9 and 22 t $ha^{-1}$of shoot dry matter, respectively, in small-scale field experiments (Robinson et al., 1997a, b). The newly discovered arsenic hyperaccumulator *P. vittata* can also produce a relatively large biomass under favourable climates (Ma et al., 2001; Cai and Ma, 2002). It can accumulate up to 22 000mg As kg^{-1} in the frond dry weight, although phytotoxicity occurs when shoot arsenic is larger than about 10 000 mg kg^{-1} (Wang et al., 2002). When grown in pots on an arsenic-contaminated soil containing 98 mg As kg^{-1} for 20 weeks under greenhouse conditions, its bioconcentration factor reached 87 and the fronds produced removed 26% of the soil's initial arsenic (Tu et al., 2002). More recently, several other fern species, including *Pityrogramma calomelanos* (Francesconi et al., 2002), *Pteris cretica*, *Pteris longifolia* and *Pteris umbrosa* (Zhao et al., 2002), have been identified as arsenic hyperaccumulators.

Secondly, the biological processes involved in metal acquisition, transport, and shoot accumulation in both hyperaccumulating and nonaccumulating plants should be fully investigated. To improve the potential for metal phytoextraction, it is proposed to using modern genetics to transfer hyperaccumulation genes to fast growing, high biomass-producing nonaccumulating plants (Brown et al., 1995). Many genes are involved in metal uptake, translocation and sequestration and transfer of any of these genes into candidate plants is a possible strategy for genetic engineering of plants for improved phytoremediation traits. Depending on the strategy, transgenic plants can be developed which will be engineered to accumulate high concentrations of metals in harvestable parts. Transfer or overexpression of genes will lead to enhanced metal uptake, translocation, sequestration or intracellular targeting. Genetic engineering of plants for synthesis of metal chelates will improve the capability of plant for metal uptake (Karenlampi et al., 2000; Clemens et al., 2002; Pilon-Smits and Pilon, 2002). Classic genetic studies have shown that only a few genes (one to three) are responsible for metal tolerance (Macnair et al., 2000). Some of the possible areas of genetic manipulation are mainly focused on genes of metallothioneins phytochelatins and metal chelators (Zhu et al.,1999a, b; Ezaki et al., 2000; Takahashi et al., 2001), metal transportors (Van der Zaal et al., 1999; Hirschi et al., 2000; Song et al., 2003), alteration of metabolic pathways (Bizily et al., 2000; Dehankher et al., 2002), alteration of oxidative stress mechanism (Ezaki et al., 2000; Grichko et al., 2000), and

alteration in roots and biomass (Eriksson et al., 2000; Nedelkosaka and Doran, 2000; Eapen et al., 2003).

It was reported the bacterial genes merA, encoding a mercuric ion reductase, and merB, encoding an organomercurial lyase, were successfully transferred to higher plants including *A. thaliana*, tobacco and yellow poplar. The transformed plants show much increased tolerance to mercuric ions (merA plants) or methylmercury (merA/merB plants), as well as increased phytovolatilisation of Hg (Hg^0) (Meagher, 2000).

Dhankher et al. (2002) developed transgenic Arabidopsis plants, which could transport oxyanion arsenate to aboveground, reduce to arsenite and sequester it in thiol peptide complexes. *E. coli* Ars C gene encoding arsenate reductase (Ars C) which catalyzes the glutathione (GSH) coupled electrochemical reduction of arsenate to the more toxic arsenite. Arabidopsis plants transformed with Ars C gene expressed from a light induced soybean rubisco promoter (SRSIp) strongly expressed Ars C protein in leaves, but not in roots and were hypersensitive to arsenate. Arabidopsis plants expressing *E. coli* gene encoding γ-glutamyl cysteine synthetase (γ-ECS) with actin promoter was moderately tolerant to arsenic compared to control plants. Plants expressing SRSIp/ArsC and ACT 2p/γ-ECS together showed higher tolerance to arsenic. These transgenic plants accumulated 4- to 17-fold greater fresh shoot weight and accumulated 2- to

3-fold more arsenic per gram of tissue than wild plants or transgenic plants expressing γ-ECS or ArsC alone.

There are numerous studies in which a single gene (e.g. γ-*ECS* or the genes encoding glutathione synthetase or metallothionein) has been transferred to or overexpressed in plants. The transformed plants typically show an enhanced tolerance to metals like Cd or Cu, but do not accumulate these metals in shoots to levels that can be considered useful for phytoextraction. This is not surprising considering the multiple traits required for metal hyperaccumulation (McGrath and Zhao, 2003).

2.2.2 Induced Phytoextraction

Plants grown on heavy metal contaminated soils generally do not accumulate high levels of the targeted metals in the plant tissue with the exception of certain metal hyperaccumulators, such as Zn or Ni hyperaccumulators. As it has been discussed above, these hyperaccumulators are generally not suitable for practical phytoextraction because of the slow growth and lower biomass production of these plants. The goal of metal phytoextraction in the field is to reduce the levels of toxic metals in the contaminated soils within a reasonable timeframe (1-3 years). To achieve this goal, it needs to use plants that are able to accumulate greater than 1% of targeted metals in shoots and produce more

than 20 metric tons of shoot biomass/ha per year. Previous results from both greenhouse and field experiments clearly indicate that shoot metal concentrations for plants grown on the contaminated soils were far below the level targeted for commercial phytoextraction. However, with the application of soil amendments to the contaminated soils, it is able to achieve the targeted metal concentration for commercial phytoextraction (Huang and Cunningham, 1996; Blaylock et al., 1997; Huang et al., 1997). The application of soil amendments such as synthetic chelates and organic acids increase metal desorption from soil to soil solution and metal translocation from roots to shoots.

It has been demonstrated that most heavy metals are rapidly accumulated in the roots if the metals are bioavailable in the plant growth media; however, only a small portion of the absorbed metals is translocated to the shoots (Jones, 1973; Kumar et al., 1995; Huang and Cunningham, 1996; Ebbs and Kochian, 1997). Two major limitations to the phytoextraction of heavy metals are the low metal bioavailability in the soil and the poor metal translocation from roots to shoots. For example, for most Pb-contaminated soils that have been studied, Pb in soil solution is usually less than 0.1% of total soil Pb. Furthermore, for plants grown on the Pb-contaminated soils, Pb translocation from roots to shoots was less than 30% for the plant species showing the highest rate of Pb translocation from roots to shoots (Huang and Cunningham, 1996). A key to the success of metal

phytoextraction is to increase and maintain metal concentrations in the soil solution. Chelates and other chemical compounds have been used to increase the solubility of metals in plant growth media, and could significantly increase metal accumulation in plants (Wallace et al., 1977; Albasel and Cottenie, 1985; Norvell, 1991). Some studies demonstrated that chelates, organic acid and certain inorganic chemical compounds can be used to trigger metal hyperaccumulation in a number of agronomic crops with high biomass production.

2.2.2.1 Enhanced Pb Phytoextraction with the Application of Chelates

Lead is a major metal contaminant notorious for posing a significant risk to humans, especially for children. It has been estimated that in the USA alone Pb poising affects more than 800,000 children between the age of one and five (Pirkle et al., 1998). Although the total Pb concentration in many contaminated soils may be high, the phytoavailable Pb fraction (water soluble and exchangeable) is usually low due to the strong association of Pb with organic matter, Fe-Mn oxides, and clays, and precipitation as carbonates, hydroxides, and phosphates (McBride, 1994). Results from hydroponic studies indicate that shoot Pb concentrations in plants increased dramatically as Pb levels in the nutrient solutions increased (Kumar et al., 1995; Huang and Cunningham, 1996). This led to the later application of soil amendments to increase Pb desorption

from soil to soil solution. Several chelating agents such as EDTA (ethylenediaminetetraacetic acid), CDTA (trans -1, 2 -diaminocyclohexane -N, N, N', N'-tetraacetic acid), DTPA (diethylenetriaminepentaaceticacid), EGTA [ethyleneglycol -bis (ß -aminoethyl ether), N, N, N', N-tetraacetic acid], EDDHA [etylenediamine-di (*o*-hydroxyphenylacetic acid)], HEDTA (N-hydroxyethylenediaminetriacetic acid), and HEIDA [N-(2-hydroxyethyl)iminodiacetic acid] have been tested for this purpose (Blaylock et al., 1997; Huang et al., 1997; Cooper et al., 1999; Kulli et al., 1999; Wu et al., 1999; Kayser et al., 2000; Shen et al., 2002; Grčman et al., 2003; Kos and Leštan, 2003 a, b).

EDTA has been the most widely used chelating agent in studies of phytoremediation because of its high efficiency in extracting many metals. For the phytoextraction of Pb in the soil, EDTA was more efficient than other chemical agents (Huang et al., 1997; Shen et al., 2002). In pot experiments described in the literature, the concentrations of Pb in plant shoots was generally lower than 2000 mg kg^{-1} DW after the application of EDTA (Wu et al., 1999; Bricker et al., 2001; Grčman et al., 2001; Lombi et al., 2001; Barocsi et al., 2003; Grčman et al., 2003; Kos and Leštan, 2003a; Kos et al., 2003; Walker et al., 2003; Wenzel et al. 2003; Chen et al., 2004a; Lim et al., 2004; Meers et al., 2004), except for the results in a few experiments (Blaylock et al., 1997; Huang et al., 1997; Epstein et al., 1999; Shen et al., 2002). Blaylock et al. (1997)

reported that the concentrations of Pb in the shoots of Indian mustard increased from less than 100 to 15 000 mg kg^{-1} when the plants were grown in soil containing 600 mg kg^{-1} of Pb amended with 10 mmol EDTA kg^{-1} of soil. Huang et al. (1997) measured more than 10 000 mg kg^{-1} of Pb in the shoots of pea grown in soil containing 2 500 mg kg^{-1} of Pb with the addition of 1.3 mmol EDTA kg^{-1} of soil. Adding 3 mmol kg^{-1} of EDTA to a naturally multi-contaminated soils collected from an old mining site, the concentration of Pb in cabbage shoots increased 100 to 4620 mg kg^{-1} on Day 14 after EDTA application (Shen et al., 2002). The different efficiency of EDTA induced phytoextraction of Pb shown in the different studies may be attributed to factors from experimental setup, environmental conditions, soil type, different test plant species, the harvest of younger or older plants, metal concentrations and forms in the soil, the dosage and the methods of EDTA applied.

The improved phytoextraction of Pb with the chelate application was associated with greatly enhanced concentrations of soluble Pb-EDTA. A sequential extraction employed by Kirkham (2000) indicated that soil EDTA addition mobilized Pb associated with the ion-exchangble and carbonate soil fractions. Lead retained in the oxide and the organic fraction was less affected by EDTA addition (Elless and Blaylock, 2000). However, soluble Pb-EDTA easily percolates through soil profile, posing a high risk of groundwater contamination (Wu et al., 1999). Different chelates possess different ability to desorb Pb from

the soil matrix. The order of effectiveness in increasing Pb desorption from the soil was EDTA > HEDTA >DTPA > EGTA > EDDHA (Huang et al., 1997). Wu et al. (1999) found the application of HBED (N,N-di(2-hydroxybenzyl)ethyleneamide N,N-prime-diacetic acid) resulted in a greater stimulation for Pb accumulation in maize roots than EDTA. However, the effect of HBED application on Pb translocation from root to shoot was less.

Although EDTA is very effective in mobilizing metals in soils, EDTA and EDTA-heavy metal complexes can be toxic to plants and soil microorganisms and they can be also persistent in the environment due to their low biodegradability (Bucheli-Witschel and Egli, 2001; Grčman et al., 2003). This nature may also increase the potential off-site migration of metals, either via surface runoff or by the leaching of metals into groundwater (Nowack, 2002). The enhanced metal leaching associated with EDTA application will be elaborated later.

Another chelate of ethylenediaminedisuccinic acid (EDDS), a structural isomer of EDTA, can form stable hexadentate chelates with transition metals. But unlike EDTA, the [S,S]-stereoisomer of EDDS, is readily degraded in activated sludge systems (Schowanek et al., 1997; Takahashi et al., 1997; Nörtemann, 1999). The calculated half-life of EDDS in sludge-amended soil was 2.5 days (Jaworska et al., 1999). The superior biodegradability of EDDS and an overall

favorable environmental profile have prompted its use as a chelate to replace EDTA in consumer products. In recent years, researchers in the area of phytoremediation began to apply this easily biodegradable chelating agent of EDDS (S,S-ethylenediaminedisuccinic acid) to enhance the uptake of heavy metals in soil phytoremediation (Grčman et al., 2003; Kos and Leštan, 2003a, b; Kos and Leštan, 2004a).

Applying 2.5, 5, and 10 mmol kg^{-1} of EDDS to the soil column led to enhanced uptake of Pb by the shoots of Chinese cabbage. The highest Pb uptake appeared in the 10 mmol kg^{-1} of EDDS treatment, which was 50.1 times over the control treatment. However, the improved effect was still lower than the same addition of EDTA, which produced 96.8-fold Pb concentration of the control group (Kos and Leštan, 2003a). Similarly, a significantly lower Pb uptake in the shoots of *Brassica rapa* was found in the treatment of EDDS than the same amount application of EDTA (Kos and Leštan, 2003b). However, Grčman et al. (2003) reported when EDDS was applied at a single doses of 10 mmol kg^{-1}, the concentration of Pb in cabbage leaves was enhanced 102-fold compared with the control group without the EDDS application (Grčman et al., 2003). The application of EDTA at the same resulted in only 94.2-fold higher Pb than the control group. Applying 10 mmol of EDDS to the soil column, the concentration of Pb in the shoots of hemp was enhanced 1926-fold compared to the treatment with no EDDS addition (Kos and Leštan, 2004a). Therefore, the

application of EDDS may provide a good alternative for the phytoremediation of Pb-contaminated soils. Further study should be carried out to evaluate the efficiency and safety of this technology before it is applied in the field.

2.2.2.2 Enhanced Cu, Zn, Cd and Ni Phytoextraction with the Application of Chelates

Besides Pb, EDTA can enhance the concentrations of other heavy metals in the soil solution and in the biomass of several plants, but generally to smaller extents. Luo et al. (2001) showed that applying 2.4 mmol EDTA kg^{-1} to Cu-polluted paddy soil (158 mg Cu kg^{-1}) increased Cu concentrations in the leaf tissue of India mustard from 18 to 54 mg kg^{-1}. Outstanding results were obtained by Deram et al. (2000) whereby the application of 3.6 mmol EDTA kg^{-1} to a soil contaminated with high amounts of Cu and especially Ni (187 and 6300 mg kg^{-1}, respectively) resulted in 38- and 160-fold increase in Cu and Ni concentrations, 7.5 g Cu kg^{-1} and 1.3 g Ni kg^{-1}, respectively. In a highly Zn-contaminated soil, the addition of 6.7 mmol EDTA kg^{-1} resulted in 450-fold increase in the soluble Zn concentration in the soil and 2-fold increase in the Zn concentration in the shoots of Indian mustard. Blaylock et al. (1999) reported after spiking soils with high amounts of heavy metals followed by 2.5 mmol kg^{-1} of EDTA, Indian mustard shoot concentrations of Zn and Cu were at or above 1000 mg kg^{-1} and Cd and Ni concentrations were 480 and 200 mg kg^{-1},

respectively. The application of 2 mmol kg^{-1} of EDTA to the soil containing 400 mg kg^{-1} of Cu, the concentration of Cu in the shoots of *Agrostis tenuis* growing in repacked soil increased from 30 to 300 mg kg^{-1}, but the same application to an intact core only brought about an increase from 10 to 60 mg kg^{-1} (Thayalakumaran et al., 2003). The effect of EDTA application on the uptakes of Cu, Zn, Cd, Ni was always lower than Pb uptake (Grěman et al., 2001; Grěman et al., 2003; Liphadzi et al., 2003; Wenzel et al., 2003; Chen et al., 2004a, b; Meers et al., 2005), which might be attributed to the higher logarithms of stability constants for Pb-EDTA complex than Cu-, Zn-, Cd-, and Ni-EDTA complexes (Bucheli-Witschel and Egli, 2001).

Compared with EDTA addition, a lower, but still significant phytoextraction enhancement has been observed by several authors after adding NTA to the soil. In a pot experiment with a calcareous, mainly Cu-contaminated soil (530 mg kg^{-1}), even low dosage of 2.7 mmol NTA kg^{-1} (split into two applications) increased the concentrations of Cd, Zn and Cu 1.4- to 1.9-fold in lettuce and perennial ryegrass (Kulli et al., 1999). Adding 9.3 mmol NTA kg^{-1}, the Cd and Zn concentrations of perennial ryegrass doubled compared with the control plants with average Cd and Zn concentrations of 2.7 ad 505 mg kg^{-1}, respectively. A field experiment to test the effect of increased rates of NTA on heavy metal accumulation was conducted by Kayser et al. (2000). Nine injections of NTA led to a total of 4.6 and 9.2 g NTA m^{-2}, which significantly

increased heavy metal accumulation by several plants. At 9.2 g NTA m^{-2}, the Cd, Zn, and Cu concentrations in most crops were doubled. Dry mass yields of the crops did not change significantly after NTA treatment. Results from a hydroponic experiment showed when the plant of tobacco was exposed to 126 µM Cu and 500 µM NTA in nutrient solutions without and with 10 g L^{-1} montmorillonite, NTA increased Cu uptake and translocation into shoots of tobacco by a factor of 3.5 from the nutrient solution and by a factor of 26 from the montmorillonite nutrient solution (Wenger et al., 2003).

Using a soil column experiment, Grěman et al. (2003) found the application of EDDS at the rate of 10 mmol kg^{-1} increased the concentrations of Zn and Cd in the shoots of Chinese cabbage to 4.7- and 3.5-fold of the control group. The enhanced effect was comparable to that caused by the addition of EDTA, which caused 4.3- and 3.8-fold increase than the control group. A much higher enhancing effect on the concentrations of Zn and Cd was found in the in the shoots of hemp on the applying of 10 mmol kg^{-1} of EDDS, which was 7.5 and 11 times higher than the control group (Kos and Leštan, 2004a). In another soil column experiment, Kos and Leštan (2004b) investigated the effect of 5 mmol kg^{-1} soil addition of citric acid, EDTA, DTPA and EDDS on phytoextraction of Cu from a vineyard soil with 162 mg kg^{-1} Cu, by the test plant of *Brassica rapa* var. *pekinensis*. Result showed the application of EDDS was most effective in increasing Pb uptake by the plant, in which plant Cu concentration reached 38

mg kg^{-1} and increased by 3.3-times over the control group.

Copper is usually much more bioavailable in soils, and thus particularly toxic to many plant species (Pahlsson, 1989). Few studies have been performed with biodegradable chelates as the ligand to enhance the phytoextraction of Cu from contaminated soils and the enhanced effects on Cu phytoextraction were always poor (Kulli et al., 1999; Kayser et al., 2000). In the future, the application of EDDS in the phytoextraction of Cu-contaminated may become a desired alternative.

2.2.2.3 Enhanced U Phytoextraction with the Application of Citric Acid

Surface soil contamination with U has resulted from the development of the nuclear industry, which involved the mining, milling, and fabrication of various U products. Because there are large areas of U contaminated soils in the world, engineering-based remediation such as excavation requires millions of tons of soils to be disposed of as low-level radioactive waste. To search for ideal soil amendments to enhance soil U desorption from soil to soil solution, the U-contaminated soils were treated with a number of selected soil amendments including synthetic chelates, inorganic and organic acids, and sodium and potassium bicarbonates. Among the different options, citric acid was most effective in enhancing U desorption from soil to soil solution (Huang et al.,

1998). The strong mobilization of U by citric acid was due to the formation of citrate-uranyl complexes rather than to the decreased pH.

The addition of citric acid and its salts selectively increase U mobility in soil and subsequently plant uptake (Ebbs et al., 1998 a, b; Huang et al., 1998). The application of citric acid at the rate of 20 mmol kg^{-1} to a U-contaminated soil (total soil U 280 mg kg^{-1}) increased U concentration in soil solution from 1.2 to 240 mg L^{-1}, which represented a 200-fold increase of U in the soil solution. In this study with more highly contaminated soil (750 mg U kg^{-1}), the U concentration in the tissue of 4-wk-old Indian mustard increased by 1000 folds from < 5mg U kg^{-1} up to 5200 mg U kg^{-1} within 3 d after adding citric acid (Huang et al., 1998). When other organic acids such as malic acid and acetic acid were added at the same concentrations, the U concentrations were 2100 and 1700 mg U kg^{-1}, respectively.

2.2.2.4 Inorganic Chemicals Application in Phytoextraction of Metals

The widely used inorganic agents in the phytoextraction include sulfur, ammonium sulfate, chloride salts and calcium carbonate. The addition of different amounts of S in a series of pot experiments lowered the soil pH from 5.7 to values below 4 (Tichy et al., 1997). The highest increase of Cd removal by common mustard (*Sinapis alba* L.) from an artificially highly polluted soil

was 1.3-fold at a pH of 5. In a pot experiment with contaminated calcareous soil (calcium carbonate content 6.4%), adding 12.8 g sulfur kg^{-1} lowered the pH from 7.2 to 3.6 and enhanced Cd mobility by 10 folds (Kayser et al., 1999a). The Cd concentrations in Indian mustard and tobacco increased 27- and 14-fold, respectively. In a field experiment conducted by Kayser et al. (2000), when 1.15 kg S m^{-2} were mixed into the rooting zone of tobacco, sunflower, corn, and willow, the concentrations of Cd and Zn in the crops increased 1.4- to 2.2-fold, but the Cu concentrations did not change. The most Cu (510 g ha^{-1}) and Zn (3.4 kg ha^{-1}) were accumulated by sunflower and the most Cd (41 g ha^{-1}) was accumulated by tobacco.

The effect of ammonium sulfate fertilization on phytoextraction was especially tested for enhanced Cd and Zn accumulation because these heavy metals can easily be solubilized at those pH values that are predominant in agricultural soils. After the application of ammonium sulfate (equal to 100 mg N kg^{-1}) to a soil moderately contaminated with 1.9-2.4 mg Cd kg^{-1} and planted with willow, the Cd and Zn concentrations in willow increased up to 2.2-fold (up to 45 and 400 mg kg^{-1}, respectively), compared with potassium nitrate fertilization.

Because Cl^- ions are known to form stable inorganic Cd complexes, most experiments that examined the effect of salt addition to contaminated soil used Cl salts, mostly NaCl (Bingham et al., 1983; Smolders et al., 1998; Norvell et

al., 2000). Smolders et al. (1998) found the Cd concentration in the leaves of Swiss chard was doubled in a soil of pH 4.6 with a concentration of 0.2 mg EDTA-extractable Cd kg^{-1}, when the NaCl concentration in the saturation extract was 60 mM. In another study, it was reported 66% of the variability of Cd in durum wheat grain could be explained by both the chloride concentration in the soil and the DTPA-extractable (roughly plant-available) Cd pool. However, it has been proved enhanced heavy metal accumulation after NaCl application is not a general rule. It was found the solution concentration of free metal Cd^{2+} was not significantly affected by NaCl and concluded that the enhancing effect of NaCl on Cd concentration in the crop is due to chloride complexation of Cd.

Liming is a common method to decrease the heavy metal mobility in soils and their accumulation in plants. However, applying calcium carbonate to contaminated noncalcareous soils has been observed to both reduced (Sims and Kline, 1991; Hooda and Alloway, 1996; Krebs et al., 1998) and increased metal mobility (Sims and Kline, 1991; Maier et al., 1997). In the field, liming reduced Cd and Zn concentrations in field pea [*Pisum sativum* L. subsp. *sativum* var. *arvense* (L.) Poir] (Krebs et al., 1998) to 50 and 60%, respectively, but increased the Cd content of potato (*Solanum tuberosum* L.) tubers 1.5-fold (Maier et al., 1997). In the long term, liming of acidic soils can decrease metal mobility by increasing soil pH. On the other hand, the calcium cation is a

competitive exchanger for cations that are unspecifically bound to sorption sites in soil. In slightly acidic soils, the addition of Ca^{2+} may therefore enhance metal availability.

Cunnane et al. (1993) and Elless and Lee (1998) reported that, because of the formation of relatively highly soluble U-carbonate complexes after liming of carbonate-rich, uranium-contaminated soils, the mobility of U in these soils increased and the underlying ground water was polluted. Vangronsveld and Cunningham (1998) summarized that in most cases heavy metals in soils can be effectively stabilized with iron or manganese oxides. Especially at low soil pH, liming is recommended as a supporting measure because heavy metal complexation on the surface of those minerals has its optimum at pH 7 (Mench et al., 1998).

Mechanism of Chemical-Enhanced Metal Accumulation by Plants Synthetic chelates, such as EDTA, have been used to supply plants with micronutrients in both soil and hydroponics. The predominant theory for metal-chelate uptake is the split-uptake mechanism, where only free metal ions are absorbed by plant roots leaving the chelate in soil solution (Chaney et al., 1972; Marschner et al., 1986; Shen et al., 1998). Fe-EDTA is known to dissociate before plant uptake (Marschner et al., 1986). Zinc was predominantly present as Zn phosphate dihydrate in the roots and leaves of *Phaseolus vulgaris* regardless of Zn form in

solution ($ZnSO_4$ or Zn-EDTA), suggesting the split-uptake of Zn-EDTA complexes at the root level (Sarret et al., 2001). Another theory has been suggested that some of the purported intact metal chelates are taken up by plants (Wallace, 1983; Bell et al., 1991; Laurie et al., 1991). Several studies on the Pb accumulation in plants showed that both Pb and EDTA were present in the shoots, suggesting that the metal was absorbed and transferred as a Pb-EDTA complex (Vassil et al., 1998; Epstein et al., 1999). Sarret et al. (2001) showed that both Pb and EDTA could be absorbed by plant, and parts of Pb present in the leaves of *P. vulgaris* were complexed to EDTA. The complexes of Pb-EDTA can not split through reduction or oxidation of Pb. Also, it is not likely that diffusion of Pb-EDTA or EDTA across the plasma membrane at any significant rate as they are too large and polar to move the plasmalemma lipid bilayer. Bell et al. (1991) suggested that the plant uptake of metal chelate complexes occur at the breaks in the root endodermis and Casparian strip. EDTA could damage the membrane of root cells by chelating Zn^{2+} and Ca^{2+} cations that stabilize the membrane, thus allowing free equilibration between hydroponic or soil solutions and the xylem sap (Vassil et al., 1998). Wu et al. (1999) found that there were significant increases in the Pb uptake and translocation for corn transplanted into soil, and then treated with EDTA, in comparison with the plants germinated and grown in Pb-contaminated soil to which EDTA was subsequently applied. However, the physiological basis of the uptake of Pb-EDTA complexes, particularly the possibility for these negatively charged large molecules to cross

the membrane, is still not well characterized so far.

In contrast to complexation processes, the solubilization of heavy metals through inorganic agents relies mainly on desorption (Brümmer et al., 1986). Heavy metal solubility in soils is mainly controlled by the soil reaction (pH), the amount and kind of sorption sites, and the total amount of heavy metals in the soil (Brümmer et al., 1986; Hornburg and Brummer, 1993; Gray et al., 1999). Most inorganic agents used for phytoextraction reduce the soil pH. Adding reduced sulfur with subsequent oxidation by soil microbes, or by physiological acidification of the rhizosphere soil after ammonium fertilization can decrease soil pH, due to a surplus of cation uptake relative to anion uptake (Marschner, 1986). Once heavy metals are transferred from their sorption sites into the soil solution and eventually removed from the soil, further proton attack will dissolve several soil minerals (Tessier et al., 1979; Zeien and Brümmer, 1989). The extent of this process is dependent on the capacity of soil minerals. When salts are added to soils, they dissociate in the soil solution to positively charged metal ions and negatively charged anions. Depending on their concentration and type, these cationic components can exchange heavy metals from sorption sites in the soil. As an additional effect, soluble metal-chloride salts, especially the relatively stable Cd-chlorides, followed by Pb-chlorides, can be formed (Doner, 1978; Zeien and Brümmer, 1989).

Enhanced Leaching Associated with the Application of Chelates

In companion with the enhanced phytoextraction, the *in situ* application of organic agents may pose a potential risk of surface and ground water pollution through the uncontrolled solubilization and migration of metals. In EDTA-facilitated phytoremediation, the amount of heavy metals taken up by plants is minor compared to the amount mobilized from the soil and the large quantities that are leached out of the root zone (Madrid et al., 2003, Chen et al., 2004b). The risk of Pb leaching definitely increases with increasing chelate application. Together with the high persistence of Pb-chelate complexes, the use of these agents in contaminated fields could pose severe environmental problems.

In soil column experiments conducted with several soils of differing calcium carbonate content and concentrations of heavy metals, considerable amounts of heavy metals were leached after the addition of 50 mmol EDTA kg^{-1} (Sun et al., 2001). The fractions of the total heavy metal amounts leached after 8 d were: Zn, 9 to 51%; Cd, 12 to 45%; Pb, 3 to 31%; and Cu 3 to 57%. By conducting sequential heavy metal extractions, it was found that EDTA also extracted Zn and Pb from the residual fraction (which is considered to the silicate-bound heavy metals) of some of the tested soils. The result suggested that the lability of metals in soil, the kinetics of metal desorption/dissolution and the mode of

EDTA addition were the main factors controlling the behavior of metal leaching with EDTA (Sun et al., 2001).

In a soil column phytoextraction experiment, cabbage was planted on a Pb-, Zn- and Cd-contaminated soil (1100, 800 and 5.5 mg kg, respectively) and 10 mmol EDTA kg^{-1} were added to each soil (Grčman et al., 2001). Three weeks after the EDTA treatment, up to 37.9, 10.4 and 56.3% of the initial total Pb, Zn and Cd in soil were leached from the soil. After calculating a mass balance, it was found that 315 times more Pb, 200 times more Zn, and 245 times more Cd were leached than taken up by the crop. It also showed a correlation between the amount of water added in this column study and the amount of heavy metals accumulated and leached after EDTA application. More metals were leached and less accumulated when the total of 3 L water per column was increased to 4.2 L. The ratio of leached to accumulated Pb, Zn and Cd increased from 315 to 1000, from 200 to 400, and from 245 to 425, respectively.

Using short soil-leaching columns (9.0-cm diameter, 20-cm height) by the percolation of artificial rainfall, the application of 5 mmol kg-1 of EDTA led to about 3.5, 15.8, 13.7 and 20.6% of soil Pb, Cu, Zn and Cd leached out of the soil, respectively. The growth of sunflowers in the soil columns had little effect on the amount of metals that were leached out (Chen et al., 2004b). This was probably due to the shallowness of the layer of soil, the short time-span of the

uptake of metals by the plant and the plant's simple root systems.

Much lower heavy metal leaching was found by Schremmer et al. (1999), who tested effect of ammonium sulfate vs. calcium nitrate as N fertilizers (equal to 100 kg N ha^{-1}) on heavy metal accumulation in willow. In a noncalcareous soil (pH = 6.4) with 1.9 mg Cd kg^{-1} and 250 mg Zn kg^{-1}, ammonium sulfate fertilization doubled the Cd concentration in the rhizosphere soil and increased the Zn concentration 2.7-fold. As a consequence, in contrast to nitrate fertilization, the amount of Cd and Zn leached per pot increased from 1.4 to 2.6 µg and from 0.22 to 0.53 mg Zn pot^{-1}. A mass balance showed that after ammonium sulfate fertilization, 11 and 7 times more Cd and Zn were accumulated by plants than leached from soil, respectively, whereas after nitrate fertilization, this ratio was only 4.7 and 4, respectively.

The prerequisites for heavy metal leaching are that (i) heavy metal can generally only be transported downward in the soil when they are in a water-soluble state (ionic and complexed) and (ii) the soil solution percolates. Heavy rainfalls no doubt wash soluble heavy metals into deeper soil layers as ions or, to a less extent, as colloids; in soils that tend to show preferential flow characteristics (in contrast to matrix flow), fast transport is possible. When on the field scale the mobilization is limited to the upper soil layer or to the main rooting zone, it can be assumed that metals will be transported into deeper soil

horizons through downward water flow, metals can be moved to the ground water quickly (Camobreco et al., 1996).

After the vegetation period, evapotranspiration is generally reduced due to reduced irradiation and plant growth. This promotes the downward movement of water in soils and, therefore, promotes leaching. This leaching has to be prevented in contaminated soils when the water balance is positive and/or strong rainfalls can cause percolation. It is, therefore, an important objective of research on enhanced phytoextraction that, especially after the vegetation period, the concentrations of heavy metals in the soil solution (which were previously enhanced during plant growth) are now reduce to their initial values if possible. When persistent organic agents, such as EDTA, are used to enhance phytoextraction, however, and especially as they are applied shortly before harvest, this objective clearly cannot be achieved.

The degradation rate of metal chelates basically depends on their stability constants, the microbial activity of the soil (which is mainly dependent on the soil water content, soil temperature, and redox potential), and on the concentration of free, ionic metals (Tebatabai and Bremner, 1975; Xun et al., 1996). Organic acids were degraded within 2 wk (Krishnamurti et al., 1998) and the half-lives of several Zn complexes in soils with oxalic, citric, and acetic acid were below 2 d (Wenger et al., 1998). Because the enzymes of microorganisms

are not adapted to mineralizing artificial organic matter, their degradation is rather slow (Tiedje, 1975; Bolton et al., 1993; Stumpf, 1996) and metals remain soluble for longer time. Complexes of several metals (Na, Ca, Mg) with EDTA were degraded aerobically within 4 h, but Fe(II)EDTA remained in the synthetic waste water solution as a relatively inert molecule (Henneken et al., 1998). In soil, only 1% EDTA and 6% DTPA were mineralized within 120 d (Bolton et al., 1993). The heavy metal forms of chelates are usually degraded much slower than other metal chelates (Tiedje and Mason, 1974; Tiedje, 1975)

It was reported Na_3NTA was completely mineralized in several soils after 10 d (Tabatabai and Bremner, 1975), but Wenger et al., (1998) showed that after the addition of 0.95 g NTA kg^{-1}, the decomposition of Zn-NTA complexes did not start until 20 d after formation. When 4.8 g NTA kg^{-1} were added, no decomposition was observed within 50 d, perhaps due to a toxic effect of NTA to soil microbes.

The application of biodegradable chelate of EDDS could lead to a reduction in metal leaching compared with the treatment with EDTA at the same application dosage. In the study of Grěman et al. (2003), weekly additions of 10 mmol kg^{-1} of EDTA to the soil columns led to an average of 22.7, 7.0, and 39.8% of initial total Pb, Zn, and Cd leached through the soil profile. The same amount of EDDS caused much lower leaching of Pb and Cd, which only accounted for 0.8

and 1.5 of initial total concentrations. Leaching of Zn, 6.2% of the total concentration, was comparable with the EDTA treatment. A biotest with red clover indicated a greater phytotoxic effect of EDTA than EDDS addition. EDDS was also less toxic to soil fungi and caused less stress to soil microorganisms (Grěman et al., 2003).

Possible Methods to Reduce the Leaching

Environmental and economic concerns require that the chelate addition should be minimized. This suggests that further improvements in the chelate selection and application process should be made in parallel with the selection of plant species. For the plants, screening for more sensitive species/cultivars and optimizing plant growth conditions will be helpful to reduce the chelate dosage for a given phytoextraction efficiency. In addition to hyperaccumulators, some fast-growing high-biomass plant species have been also evaluated for their potential use in chemically enhanced phytoextraction (Kumar et al., 1995; Ebbs et al., 1997; Stoltz and Greger, 2002). In the chelate-enhanced phytoextraction, efforts should also focus on identifying high biomass plant species that can accumulate significant amounts of heavy metals in response to chelate treatments to soil. Screening plant species that are more sensitive to chelate treatments will not only help minimize the amount of chelates applied in the field, but also decrease the environmental risk of mobilised metals.

Wu et al., (1999) found that the Pb concentration in corn plants differed if the seedlings were transplanted to or directly germinated in the contaminated soil. Lead concentration of 4500 mg kg^{-1} (which is 45-fold the control concentration) could be achieved when corn plants were transplanted 10 d after germination, whereas only 6-fold increase over the control concentration was measured when plants germinated in the contaminated soil. Thus, compared with plants that germinated on the contaminated soil, the metal translocation efficiency, expressed as shoot-to-root ratio of Pb concentration, increased from 0.23 to 1.57. In the transplanted seedlings, the Pb accumulation would probably have been smaller if they had been germinated on contaminated soil. Because transplantation is not a viable option for field remediation work, this finding implied that those pot experiments which may lead to later field work should not be conducted with transplants.

The efficiency of phytoremediation depends on plant yields and high metal concentrations in plant shoots. Therefore, increasing plant dry biomass yields can be helpful in increasing the total uptake by plants. It has been suggested that the use of foliar-applied P to plants grown in Pb-contaminated soils can overcome P deficiencies and avoid the necessity to add P fertilizer to soils. Hang and Cunningham (1996) reported that foliar P application not only increased plant biomass four-fold in goldenrod, but also increased total plant Pb

uptake by 115%.

In order to reduce metal leaching, some studies were designed to test the different application methods of chemicals. Kayser et al. (2000) observed by placing NTA 15 cm deep, the amount needed to enhance heavy metal accumulation was lower than when mixing this agent into the entire field. Using a pot experiment, it was found by placing ammonium sulfate at a depth of 15 cm, compared with mixing ammonium sulfate to the soil, the leaching of Cd and Zn were halved or even reduced to one-third.

Applying EDTA at several smaller dosages (versus one application) can lead to enhanced phytoextraction of Pb (Grčman et al., 2001; Puschenreiter et al., 2001; Shen et al., 2002). Grčman et al. (2001) studied the effect of agent application frequency on Pb accumulation efficiency. They found that a single dose of 2.9 g EDTA kg^{-1} enhanced Pb accumulation of cabbage grown in a greenhouse 105-fold, as compared with a 44-fold increase if the same amount of EDTA was split and added in four dosages. In the study of Puschenreiter et al. (2001), the Pb concentration in corn grown on a soil with 5600 mg total Pb kg^{-1} increased 8-fold to 49 mg kg^{-1} after adding 1 g EDTA kg^{-1} 3 wk before harvesting. This value increased to 18-fold when the EDTA application was split into three dosages over a period of 3 wk. Also Cd, Ni, Zn, and Cu concentrations in the plants increased after splitting the EDTA. Shen et al. (2002) found the

application of EDTA in three separate doses was most effective in enhancing the accumulation of Pb in the shoots of cabbage and decreased mobility of Pb in the soil compared with one- and two-dose application methods, which meant this approach could help to minimize the amount of chelate applied in the field and to reduce the potential risk of soluble Pb movement into ground water.

In several experiments, it was found that the application of glyphosate enhanced Pb accumulation of crops. The mechanism of enhanced metal accumulation after glyphosate application was explained by a disruption of plant metabolism, leading to enhanced transport of heavy metals from root to shoot (Ensley et al., 1999). In a field experiment, when 0.6 g kg^{-1} EDTA were applied to a highly contaminated calcareous soil, the Pb concentration in the soil solution increased 100-fold and in the tissue of Indian mustard 5-fold to 600 mg kg^{-1} (Kayser et al., 1999b). Combining EDTA and glyphosate increased the Pb concentration in the tissue of this crop even 15-fold, when glyphosate was added shortly before harvest. None of the measures undertaken by Kayser et al. (1999b) hampered the growth of Indian mustard. With approximately 20 t ha^{-1} of harvestable dry matter, it was calculated that 12 or 33 kg of Pb could be removed from 1 ha without and with glyphosate addition, respectively. In a pot experiment with the same soil used by Kayser et al. (1999b), 1 wk after EDTA application Indian mustard was sprayed with glyphosate and the plants died 3 d later (Mathis and Kayser, 2001). Applying EDTA doubled the Pb concentration to 90 mg kg-1,

whereas after glyphosate application this concentration was enhanced to 680 mg kg-1. Both in the field and pot experiments of this research group, a combination of EDTA and glyphosate yielded higher Pb accumulation of Indian mustard than these agents alone.

In a field experiment with Pb-contaminated soil, Kayser et al. (1999b) showed that, in combination with the addition of NTA, glyphosate increased the Pb concentration in Indian mustard more effectively than NTA application alone. The combination resulted in a 2.5-fold increase to 280 mg Pb kg^{-1} (NTA alone, 1.2-fold). This increase was somewhat lower than when glyphosate was combined to the application of EDTA (Kayser et al., 1999b).

Maxted et al. (2001) found smaller Cd concentrations as compared with the control in corn plants after glyphosate application of 1 L ha^{-1}. In a pot experiment with the same soil as used by Kayser et al. (1999b), 1 wk after EDTA application Indian mustard was sprayed with glyphosate and the plants died 3 d later (Mathis and Kayser, 2001). The application of EDTA had no significant effect on Cu concentrations, but after glyphosate application, the Cu concentration increased 10 times to 400 mg kg^{-1}.The reported differences in the effeiciency of glyphosate for metal accumulation by different plants might be due to observed different absorption and translocation rates (Green et al., 1992) and also to the rate of glyphosate applied.

Blaylock et al. (1997) demonstrated that the efficiency of organic agents on metal accumulation by plants could be enhanced by lowering the soil pH. In their field study, applying 5 mmol kg^{-1} of EDTA increased Pb accumulation of Indian mustard shoots 28-fold to 785 mg Pb kg^{-1}; however, when 5 mmol acetic acid was additionally applied, the concentration increased to 1471 mg kg^{-1}. This result was explained by lower cell wall retention of the Pb as lead carbonate at lower rhizosphere pH.

Electrodic and electrokinetic remediation is another alternative to remove heavy metals, radionuclides, and organic contaminants from contaminated soil and ground water (Acar and Alshawabkeh, 1993; Reed et al., 1995; Alshawabkeh et al., 1999a, b; Ko and Schlautman, 2000; Li and Li, 2000; Maini et al., 2000; Yong, 2001). Lim et al. (2004) reported the addition of an electric field around the plants in combination of the application of EDTA could enhance the uptake of Pb by Indian mustard compared with the EDTA addition only. In comparison with the EDTA addition alone, the accumulation of Pb in the shoots of Indian mustard was increased by 2- to 4-fold when 0.5 mmmol kg^{-1} of EDTA was applied with the parallel application of electrodics.

A new slow-release chelating agent was developed by coating solid EDTA with a layer of silicate to slow down metal mobilization in the soil in order to match

plant uptake, and thus prevent excessive mobilization (Li et al., 2005). Adding this coated chelating agent (CCA), solid EDTA, and EDTA solution to the soils contaminated with Pb and Zn grown by corn, results showed at the same application dosage of the three kinds of EDTA, shoot Pb contents were highest with solid EDTA, intermediate with CCA, and lowest with EDTA solution, and the data were always significantly higher than that in the controls. For the contents of Zn, no significant enhancement was found in the EDTA treatment compared with the controls. On Day 10 after the application of chelates, when most of the chelating agent had been released from the CCA, soil organic carbon levels remained relatively constant and similar to those in the control. The Pb level in the exchangeable and carbonate-bound fraction was significantly lower in the soil treated with CCA than that with solid EDTA and EDTA solution (Li et al., 2005). All these results indicated that slow release of CCA improved the bioavailability metals in the soil to match plant uptake of these metals and could reduce the risk of metal leaching from the soil.

Using deep-rooted, higher water-use plants or trees to reduce metal leaching may be as another good approach. Chen et al. (2004a) observed although the deep-rooted plant of vetiver grass could not accumulate higher concentrations of metals with the application of EDTA, 98, 54, 41, and 88% of the initially applied Pb, Cu, Zn, and Cd could re-adsorbed in the soil due to the effects of vetiver grass, which may reduce the risk of heavy metals migrating downwards

and entering the groundwater. Therefore, if other high metal-tolerant plants, such as Indian mustard, were intercropped with the plant of vetivar, on one hand the metals could be accumulated by the shoots of thus plants, and on the other hand the leached metals could be reduced by the by resorption in deep soil layers by the roots of vetiver grass.

As mentioned above, using easily degradable chelate could reduce the risk of metal leaching. Besides, Kos and Leštan (2003b, 2004b) reported that the use of EDDS in combination of permeable barrier might lead to environmentally sound induced Pb phytoextraction and the *in situ* washing of Pb. Applying of 10 mmol kg^{-1} of EDDS to the soil (1350 mg Pb Kg^{-1}) led to 20% of the initial Pb leached from columns with no barrier, while barriers with vermiculite or hydrogel and apatite decreased leaching by more than 60 times, to 0.35% (Kos and Leštan, 2003b). In another study with the same EDDS application, placing barriers 30 cm deep in the soil column reduced leaching of Pb, Zn, and Cd by 435, 4 and 53 times, respectively, compared with columns with no barrier. Lower positioned barriers were almost equally effective in preventing leaching of Pb than barriers positioned closer to the soil surface, less effective for Cd, and did not prevent leaching of Zn (Kos and Leštan, 2004b).

Which One is Better: Chemical-enhanced Phytoextraction or Hyperaccumulator?

As mentioned above, phytoextraction of metal-contaminated soil requires either hyperaccumulating plants or chemical addition to enhance metal uptake by high biomass crop plants. The main differences between the two technologies are that the former can have considerably higher heavy metal concentrations in their tissue, and that the latter can produce a higher dry matter biomass. Because heavy metal removal is the product of both parameters, it is useful to determine which group performs better. The aim of phytoextraction research is its implementation on contaminated fields. Therefore, the processing of the metal-rich biomass after harvest is also an issue.

If non-hyperaccumulating plants are used for phytoremediation of polluted soils, two prerequisites must be fulfilled for the creation of sustainable agronomic systems in which high heavy metal extraction rates are combined with the prevention of heavy metal leaching: (i) agronomic and climatic suitability of the particular contaminated site, enabling high dry biomass production in the frame of a suitable crop rotation system, and (ii) tolerance for heavy metals in the plant species used. In addition to relying on the choice of appropriate crop species and varieties, phytoextraction requires good crop management to optimize growth conditions for given climates and soil and management practices. Various soil amendments should be optimized to reduce the dosage as possible.

Considering the potential risk to the surrounding environment associated with the application of chemicals, pot experiments are usually carried out before the technology can be used in the field. The effects of enhanced phytoextraction of heavy metals by plants usually differ greatly in laboratory and field studies. In pot experiments, heavy metal concentrations in plant increased more than in field experiments. For example, Kayser et al. (2000) showed that in pots filled with the same soil as later used for a field study, three times more Cd was found in tobacco and sunflower and even seven times more in Indian mustard than in the field. On average, the field heavy metal removal was only 20% of what was originally expected from the pot results. For this phenomenon, several explanations have been presented: (i) in some pot experiment, the soil used was artificially enriched with heavy metals salts, hence increasing the variability of results and the probability of achieving extraordinary high heavy metal concentrations; (ii) the efficiency of soil amendments is higher in pot experiments because plant roots explore potted soil very intensely and are always in contact with the soil affected by amendments; and (iii) pot experiments have been conducted over short time periods, because plants were harvested in an early growth stage or their growth was curtailed. Restricted plant growth was reported in most experiments, probably due to toxic level of plant-available heavy metals in the soil solution. As a consequence, the reported concentrations were comparatively high. Due to low biomass accumulation, the

total removal remained low. For the development of new phytoremediation strategies, no doubt, pot or column studies are valuable tools. More importantly, the difference between the initial tests and field experiments should be considered.

Field trials demonstrate that the capacity of both plant groups to remove heavy metals is similar, or that those plants which achieve a high biomass during the growing season have an even higher rate of metal removal than hyperaccumulators. In pot experiments, Ebbs et al. (1997) found that the dry matter biomass of rape and Indian mustard were 13 times the biomass of *T. caerulescens*. Cadmium removal was the same for these plants, but four times more Zn was removed by the *Brassicaceae*. In another pot experiment, Lombi et al. (2001) determined that *T. caerulescens* had the same biomass as corn and extracted 8 to 63 times more Cd and 4 times more Zn than corn due to its higher heavy metal tissue concentrations. On average, the uptake of Pb did not differ in two soils of different contamination levels between *T. caerulescens* and corn. The *T. caerulescens* plants had a maximum dry matter biomass of 12.7 g pot^{-1} (1 kg soil) soil after approximately 30 d of growth time. Earlier, the same research group found a dry matter biomass of only 1 g pot^{-1} (600 g soil), probably due to unfavorable growth conditions. After 90 d of growth, Perronnet et al. (2000) measured 3 g dry mater per pot, filled with 500 g of contaminated soil. In field studies, *T. caerulescens* reached dry matter biomass of 2 t ha^{-1}

(Kayser et al., 2000) and of 3.7 to 5.7 t ha^{-1} (McGrath, 1998). In the former study, it removed roughly the same amounts of Cd and Zn as corn or sunflower (with dry matter biomasses of 19 and 25 t ha^{-1}, respectively), but when 1.16 kg S m^{-2} or 4.6 g NTA m^{-2} were applied to the non-hyperaccumulating crops, Cd and Zn removals were 3 to 6 and 2 times higher, respectively. More comparative field studies in several climatic zones are needed to assess whether hyperaccumulating plants or metal-tolerant biomass crops have higher Cd and Zn uptake rates.

Metal-tolerant plants could tolerate high concentrations of metals in soils and showed greater accumulation when metal availability was increased by soil manipulation. Therefore, these plants can be preferences under some situations. With enhanced phytoextraction, willow showed higher remediation capacity for Zn and Cd-contaminated soils (Riddell-Black et al., 1997; Riddell-Black, 1999; Kayser et al., 2000; Schremmer et al., 1999). Indian mustard, perennial French ryegrass, corn, and sunflower showed higher Pb concentrations in the shoots (Blaylock et al., 1997; Huang et al., 1997; Cooper et al., 1999; Kayser et al., 1999a, b; Wu et al., 1999; Deram et al., 2000; Liphadzi et al., 2003). However, only the combination of heavy metal concentrations, crop dry matter yields, and heavy metal leaching rates will show if these crops can be part of a sustainable phytoextraction management. Sunflower was a better choice for the enhanced phytoremediation of Cu-polluted land (Kayser et al., 2000).

Efficient phytoextraction should include the operation cost, potential environmental effects (positive and negative effects), and reasonable remediation time span. Usually, phytoextraction using plants for metal accumulation and subsequent physical removal of the contaminated biomass from the site is not an option on severely polluted soils due to a very long remediation time and the risk of plant toxicity. This technology is therefore more ideal to be applied to the remediation of low- or moderate-pollution soils, and additional benefits will be provided such as so called “regrowing resources” (Riddell-Black et al., 1997; Obernberger et al., 1997). It is very hard to say which technique (hyperaccumulator or chemical-enhanced phytoextraction) is better due to the limited comparative results in the current literature. In general, continuous phytoextraction with hyperaccumulating plant should be in combination with the induced phytoextraction using some chemicals to achieve the highest efficiency in the removal of contaminants from soils and to minimize negative environmental impacts. To optimize the ecological and economic efficiencies, some further researches are still required. Field experiment must be conducted to prove that the measures of enhanced phytoextraction are practicable. Tests should be conducted using easily degradable chemicals and with improved application methods to maximize the efficiency. For the continuous phytoextraction, exploring high-biomass hyperaccumulators and gene engineering study should be continued in the

future.

2.3 The Future of Phytoremediation

In the past decades phytoremediation has gained acceptance as a technology and has been acknowledged as an emerging area of research. There has already been a substantial increase in our knowledge of the mechanisms that underlie the uptake, transport, and detoxification of pollutants by plants and their associated microbes. Still, large gaps in our knowledge await further research, as indicated above. Phytoremediation efficiency is still limited by a lack of solid knowledge of many basic plant processes and plant-microbe interactions. There is also a need for more phytoremediation field studies to demonstrate the effectiveness of the technology and increase its acceptance (Heitzer and Sayler, 1993; Widada et al., 2002).

Identification of metal hyperaccumulator species has been an impetus for phytoremediation research (Lasat, 2002). Despite significant research efforts, phyremediation is still an emerging technology. Investigators as well as science administrators have strived to identify and address research needs in an effort to close knowledge gaps and make phytoremediation a reliable cleanup technology. It is possible, however, that in addition to scientific issues this transition would also require a new research approach. Complex interactions that take place

under site specific conditions require that metal phytoextraction must be handled as a multidisciplinary research efforts. Success will ultimately depend on the employment of a holistic approach to integrate the work of plant biologist, soil microbiologists, agronomists, and environmental engineers. These experts will have to work together focusing on identifying and solving numerous and diverse scientific issues posed by metal phytoextraction. For example, for Pb phytoextraction soil chemists will have to work to enhance Pb bioavailability for uptake while avoiding groundwater contamination. To ensure a research continuum, biologists will have to identify solutions to optimize the plant's ability to take up and store Pb. Agronomists will have to further provide solutions to applied issues such as, how to incorporate amendments including synthetic chelates in soil, and when and how to harvest. Such an integrated approach will allow the formulation of a comprehensive research plan, ensure research continuity, and avoid redundance (Lasat, 2002).

Phytoremediation is an emerging technology potentially effective and applicable to a number of different contaminants and site conditions. At this stage, additional experience and information are needed to consolidate phytoextraction into a decision matrix or cost model. Expectations for phytoremediation should also be revised and more appropriately managed. For example, phytoextraction, which is likely to require multiple growing seasons, is a lengthy process compared with traditional engineering approaches. In

addition, the potential for cleanup depends upon chemical speciation of the metal, with some chemical species simply unavailable for plant uptake and removal. It should also be added that evaluation of field performance is complicated by the difficulty to characterize the mass balance of metal contaminants. Wide variation of metal concentration (both vertically and horizontally) precludes these efforts. In addition, the problem is compounded by metal leaching away from the original sources (e.g. following the application of synthetic chelates) and /or alteration of contaminant concentration due to soil tillage (Lasat, 2002).

The cost associated with the implementation of phytoextraction is difficult to estimate because of a lack of economic data. It is likely, however, that the cost will be site specific. Existing data suggest the cost of initial demonstrations may be high (due to monitoring requirements) with a rapid cost decline as we gain more experience and improve the efficiency of the technology (Lasat, 2002).

Future field phytoremediation projects should benefit from (more) collaboration between research groups and industry so that they can be designed to address hypotheses and gain scientific knowledge in addition to meeting cleanup standards. Future field phytoremediation projects will also benefit from coordinated experimental design across projects so that results can be better compared (Pilon-Smits, 2005).

An interesting development in phytoremediation is its integration with landscape architecture. Remediation of urban sites (i.e., parks and nature areas) may be combined with an attractive design so that the area may be used by the public during and after the remediation process while minimizing risk (Kirkwood, 2001). Other sites that are phytoremediated may be turned into wildlife sanctuaries, like the Rocky Mountain Arsenal in Denver, once one of the most polluted sites in the United States (McIntyre, 2003).

Another new development in phytoremediation is the use of transgenic plants. Knowledge gained from plant molecular studies in the past 10 years has led to the development of some promising transgenics that show higher tolerance, accumulation, and/or degradation capacity for various pollutants, as described above. So far, these transgenics have mainly been tested in laboratory studies using artificially contaminated medium rather than soils from the field, let alone field studies. However, this is going to change. One field phytoremediation study using transgenic Indian mustard plants that overexpressed enzymes involved in sulfate/selenate reduction and in accumulation of GSH was just completed (Pilon-Smits et al., 1999; Zhu et al., 1999a, b). Three types of transgenic Indian mustard plants that overexpressed enzymes involved in sulfate/selenate reduction and in accumulation of GSH showed enhanced Se accumulation in the field when grown on soil polluted with Se, B, and other

salts (Pilon-Smits et al., 2005).

In the coming years, mining of the genomic sequences from *Arabidopsis thaliana* and rice and availability of new genomic technologies should lead to the identification of novel genes important for pollutant remediation, including regulatory networks (e.g., transcription factors) and tissue-specific transporters. The expression of these genes may then be manipulated in highbiomass species for use in phytoremediation. Other new developments in plant genetic engineering are tailored transgenics that overexpress different enzymes in different plant parts (e.g., root-specific expression of one gene and shoot-specific expression of another) or that express a transgene only under certain environmental conditions (Dhankher et al., 2002). Also, genetic engineering of the chloroplast genome offers a novel way to obtain high expression without the risk of spreading the transgene via pollen (Ruiz et al., 2003).

As transgenics are being tested in the field and the associated risks assessed, their use may become more accepted and less regulated, as has been the case for transgenic crops. Also, as more information becomes available about the movement of pollutants in ecosystems and the associated risks, the regulatory guidelines for cleanup targets may be adjusted depending on future use of the site, bioavailability of the pollutants, and form of the pollutants. Because

phytoremediation only remediates the bioavailable fraction of the pollution, stringent cleanup targets limit the applicability of this technology. If targets can be adjusted to focus on the bioavailable (i.e., toxic) fraction of the pollutant, phytoremediation could become more widely applicable. This would reduce cleanup costs and enable the cleanup of more sites with the limited funds available (Pilon-Smits, 2005).

Chapter 3 - Screening Plant Species for Efficient Phytoextraction

3.1 Introduction

The early phytoremediation studies were often conducted using hyperaccumulator species, which were able to accumulate unusually high levels of metals in their aboveground harvestable parts. However, most hyperaccumulaor species are not suitable for phytoremediation application in the field due to their small biomass and slow growth. As an alternative, efforts to develop phytoextraction using high biomass plant species that can accumulate heavy metals are necessary. Therefore, efforts have been focused on identifying high biomass plant species that can accumulate significant amounts of heavy metals in response to chelate treatments to soil. Screening plant species that are more sensitive to chelate treatments will not only help minimize the amount of chelates applied in the field, but also decrease the environmental risk of mobilised metals.

Copper can be particularly toxic to many species of plants (Pahlsson, 1989). The threshold concentration of Cu toxicity for crops is about 30 mg kg^{-1} DM (Marschner, 1995). Copper toxicity might be a limiting factor in the phytoremediation of multi-metal contaminated soils (Lombi et al., 2001). It would be difficult to produce enough biomass before the application of chelates if the soil were heavily contaminated with metals such as Cu. The

chelate-enhanced uptake of Cu in plant shoots has generally been minimal (Kulli et al., 1999; Kayser et al., 2000; Lombi et al., 2001; Römkens et al., 2002; Shen et al., 2002; Thayalakumaran et al., 2003; Wenzel et al., 2003).

The objectives of the present study were: (i) to study the potential use of the 18 different species and cultivars of plants in the chemically enhanced phytoextraction of heavy metals (Cu, Pb, Zn, and Cd) from artificially contaminated soils; (ii) to evaluate the relative effectiveness of non-biodegradable chelate EDTA and biodegradable chelate EDDS in enhancing the accumulation of heavy metals in selected species of plants grown in multi-metal contaminated soil, particularly in soil with high concentrations of Cu; and (iii) to compare the residual effects of EDTA and EDDS in soil after the first cropping with the application of these two chelates.

3.2 Materials and Methods

3.2.1 Phytoextraction of Heavy Metals Contaminated Soils by 18 Different Plants with EDTA Application

The experiment was conducted in Nanjing Agricultural University, Nanjing, China. Soil samples were collected from a farm in Nanjing. After being air-dried, the samples were crushed to pass through a 1-cm diameter sieve. The soils were artificially amended with multi-metals: Cu (500 mg kg^{-1} of soil) as $CuSO_4 \cdot 5H_2O$ (copper sulfate); Pb (500 mg kg^{-1} of soil) as $Pb(NO_3)_2$ (lead nitrate); Zn (400 mg kg^{-1} of soil) as $ZnSO_4 \cdot 7H_2O$ (zinc sulfate); and Cd (1 mg

kg^{-1} of soil) with $Cd(NO_3)_2 \cdot 4H_2O$ (cadmium nitrate). These metal concentrations were based on the national guidelines for contaminated soil in China (Xia et al., 1995). The basal fertilizers applied to the soil were 250 mg N kg^{-1} of dry soil (as NH_4NO_3), 100 mg P kg^{-1} of dry soil, 250 mg K kg^{-1} of dry soil as KH_2PO_4, and 60 mg S kg^{-1} of dry soil (as $MgSO_4$). The soil was treated to five cycles of saturation with de-ionized water and air-dried before being used for pot experiments. The electrical conductivity (EC) of the soil was measured using a conductivity meter on the soil extract, obtained by shaking soil with double-distilled water at a water-to-soil ration of 1:2 (w/v). The soil pH was measured by 0.01 mol $CaCl_2$ at a 1:5 ratio (w/v) using a pH meter. The cation exchangeable capacity (CEC) of the soil was determined using the ammonium acetate saturation method. The soil texture, organic matter content, total N and field capacity were measured by the procedures described by Avery and Bascomb (1982). The soil texture was analyzed by a hydrometer method, and the organic matter content was measured by weighing the loss after the ignition of the soil. Total N was determined using LECO CNS-2000. Field water capacity was determined by adding excessive water to 500 g of fully dried soil. The pots were assumed to be at a field water capacity when the formation of further droplets at the bottom of the pot was fully ended after the free percolation. The pots were then re-weighed, and the field capacity was estimated. The total metal concentrations were determined by ICP-AES (Perkin-Elmer Optima 3300 DV) after strong acid digestion (1:4 concentrated HNO_3 and $HClO_4$ (v/v)) (Li et al., 2001).The selected physical and chemical properties of the soil are presented in Table 3.1.

Eighteen different plant species/cultivars of common crop plants and vegetables, including thirteen dicotyledons and five graminaceous monocotyledons, were used in this study: corn (*Zea mays* L. cv. Nongda No. 108), wheat (*Triticum aestivum* L. cv. Weimai No. 8, *Triticum aestivum* L. cv. Shangnong No. 93-52 and *Triticum aestivum* L. cv. Zimai No. 12), sorghum (*Sorghum bicolor* L. cv. Moench-S. vulgare Pers), Chinese mustard (*Brassica juncea* L. Czern. Et Coss. cv. Liyangkucai), greengrocery (*Brassica chinensis* L. cv. Xiaoairen and *Brassica chinensis* L. cv. Xiaoza No. 56), cabbage (*Brassica pekinensis Rupr* L.), cole (*Brassica campestris* L. cv. Suyou No. 1 and *Brassica campestris* L. cv. Qinyou No. 8), lettuce (*Lactuca sativa* L.), soy bean (*Glycine max* L. Merrill), garland chrysanthemum (*Chrysanthemum coronarium* L.), caraway (*Coriandrum sativum* L.), carrot (*Daucus carota* L. var. sativa DC.), radish (*Raphanus sativus* L.), and spinach (*Spinacia oleracea* L.). The seeds were purchased from Nanjing Agricultural University and sown directly in the soil. After germination, the plants were thinned to 6 plants per pot (2.5 kg dried soils, 20 cm i.d. x 20 cm height) for all species and varieties.

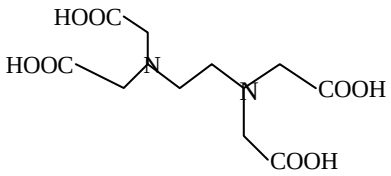
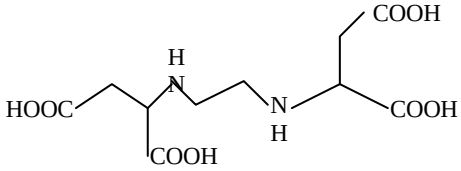
On the 56^{th} day after sowing, EDTA (from BDH Laboratory Supplies Poole, minimum assay: 99.5%) was added in a 250 ml 30mM Na_2EDTA salt solution with the pH of 4.8 to the surface of the soil to make up the amount of EDTA to 3 mmol EDTA kg^{-1} of soil. All of the experiments were conducted in a greenhouse under natural light. The air temperature ranged from 27 to 35 ºC. Each treatment was replicated three times, and was in a completely randomized block design. Three plants were harvested by cutting the shoots 0.5 cm above the surface of the soil, and the roots were removed from the pots 7 and 14 d

after the application of EDTA. The shoots were washed with tap water and rinsed with DIW (deionsed water), dried at 70 ºC in an oven to a constant weight for dry weight measurement.

Table 3.1 The physicochemical properties of the soils used in the study

	Soil used in 3.2.1	**Soils used in 3.2.2**
pH ($CaCl_2$)	6.57	7.12
Electrical conductivity at 25°C ($\mu S\ cm^{-1}$)	287	262
Sand (%) > 0.05 mm	57	79.5
Silt (%) 0.05-0.001 mm	28	13
Clay (%) < 0.001 mm	15	7.5
N_{Total} (%)	0.10	0.15
Organic matter (%)	1.5	2.7
Cation exchange capacity ($cmol\ kg^{-1}$)	3	4.2
Field water capacity (%)	42.3	39.7
Background total metal content ($mg\ kg^{-1}$)		
Pb	25	80
Cu	15	80
Zn	80	200
Cd	1.9	1.6

Table 3.2 The structures and log K values for metal complexation of EDTA and EDDS

	structure	logK Cd	logK Cu	logK Zn	logK Pb
EDTA		16.5	18.8	16.5	17.9
EDDS		10.8	18.4	13.4	12.7

3.2.2 Comparison of EDTA and EDDS for Enhanced Phytoextraction of Heavy Metals by Garland Chrysanthemum and Corn, and Residual Effects of Chelates in Soils

The experiment was conducted in the Hong Kong Polytechnic University, Hong Kong. Soil samples were collected from a disused agricultural field in the Yuen Long area of Hong Kong. The samples were sieved to pass through a 2 mm sieve and air-dried for one week. The soils were artificially contaminated with multi-metals: Cu (400 mg kg^{-1} of soil) as $CuCO_3$ (copper carbonate); Pb (500 mg kg^{-1} of soil) as $Pb_3(OH)_2(CO_3)_2$ (lead hydroxide carbonate) and PbS (lead sulfide – galena, a common lead mineral in mining areas) at a Pb concentration ratio of 1:1; Zn (500 mg kg^{-1} of soil) as $ZnCO_3$ (zinc carbonate) and ZnS (zinc sulfide) at a Zn concentration ratio of 1:1; and Cd (15 mg kg^{-1} of soil) with $Cd(NO_3)_2 \cdot 4H_2O$ (cadmium nitrate). The basal fertilizers applied to the soil were 80 mg P kg^{-1} of dry soil, and 100 mg K kg^{-1} of dry soil as KH_2PO_4 (Shen et al., 2002). After the addition of heavy metals, the soils were equilibrated for two months, undergoing seven cycles of saturation with de-ionized water and air-drying processes. The soil property parameters (pH, texture, CE) were measured in the same way as above. The selected physical and chemical properties of the soil are presented in Table 3.1.

The air-dried soils (500 g) were placed in plastic pots (12 cm i.d. x 12 cm height). The moisture of the soil was maintained at near field water capacity by adding DIW daily. Seeds of garland chrysanthemum (*Chrysanthemum coronarium* L.) and corn (*Zea mays* L. cv. Nongda No. 108) were sown directly

in the soils. Garland chrysanthemum was chosen in this part because the results from above screening experiment showed that garland chrysanthemum was the most sensitive species to the EDTA application, and produced the highest metal uptake efficiency. For the corn, the initial screening showed its fastest growing rate among the tested plants. In order to acquire uniform seedlings, garland chrysanthemum was sown three weeks before corn was sown. After germination, the seedlings were thinned to four plants per pot. On the 35^{th} day after the sowing of garland chrysanthemum, EDTA (the same as the mentioned above) and EDDS (from Fluka Chemie GmbH) were applied to the surface of the soils at rates of 0 (control), 0.5, 1.0, 1.5, 3.0, and 5.0 mmol kg^{-1} soil in 50 ml Na_2EDTA and EDDS-Na_3 salt solutions. To make up the different amounts of chelate treatments, EDTA and EDDS were diluted from 50 mM Na_2EDTA (pH 4.8) and EDDS-Na_3 (pH 10.1) salt solutions. Three replications were used for each treatment. All of the experiments were conducted in a greenhouse under natural light. The air temperature ranged from 18 to 23 ºC. The plants were harvested 7 d after the application of chelates. Their shoots and roots were separated for drying and further analysis.

In order to determine the residual effects of the applied chelates on the second cropping, the pots were kept at two-thirds of the field moisture capacity after the harvest of the first crop of corn. Six months after the harvest, the remaining soils were mixed thoroughly, and the corn seeds were again sown directly in the soils. Corn was selected in this experiment due to its less sensitivity to chelates treatment in soils than garland chrysanthemum. The seedlings were thinned to four plants per pot after germination. The experiment was conducted in the

same greenhouse under natural light. The air temperature ranged from 22 to 28 ºC. The shoots of the corn were harvested 14 d after growing in the pots and dried for dry biomass measurements and analysis of metal concentration.

3.2.3 Comparison of EDTA and EDDS for Enhanced Phytoextraction of Cu from Cu-contaminated Soils by Garland Chrysanthemum and Corn, and Residual Effects of Chelates in Soils

The original soils used in this part is the same as those in part 3.2.2 (see Table 3.1). After sieved to pass through a 2 mm sieve and air-dried for one week, the soils were artificially contaminated with $CuCO_3$ (copper carbonate) at concentrations of 0, 200, 400, 600 and 800 mg kg^{-1} of soil. The basal fertilizers applied to the soil were 80 mg P kg^{-1} of dry soil, and 100 mg K kg^{-1} of dry soil as KH_2PO_4 (Shen et al., 2002). After the addition of Cu, the soils were equilibrated for two months, undergoing seven cycles of saturation with de-ionized water and air-drying processes.

About 500 g air-dried soils were placed in plastic pots (12 cm i.d. x 12 cm height). The moisture of the soil was maintained at near field water capacity by adding DIW daily. Seeds of garland chrysanthemum (*Chrysanthemum coronarium* L.) and corn (*Zea mays* L. cv. Nongda No. 108) were sown directly in the soils. Garland chrysanthemum was sown three weeks before corn. After germination, the seedlings were thinned to four plants per pot. On the 35^{th} day after the sowing of garland chrysanthemum, EDTA and EDDS were applied to the surface of the soils at the rate of 3.0 mmol kg^{-1} soil in 50 ml Na_2EDTA and

EDDS-Na_3 salt solutions. EDTA and EDDS were also diluted from 50 mM Na_2EDTA (pH 4.8) and EDDS-Na_3 (pH 10.1) salt solutions. Three replications were used for each treatment. All of the experiments were conducted in a greenhouse under natural light. The air temperature ranged from 18 to 23 ºC. The plants were harvested 7 d after the application of chelates. Their shoots and roots were separated for drying and further analysis.

After the harvest of the first crop of corn, the pots were kept at two-thirds of the field moisture capacity. Six months after the harvest, the remaining soils were mixed thoroughly, and the pea (*Pisum sativum* L. cv. Qinxuan No. 2) seeds were sown directly in the soils. The seedlings were thinned to four plants per pot after germination. The experiment was conducted in the same greenhouse under natural light. The air temperature ranged from 22 to 28 ºC. The shoots of the pea were harvested 14 d after growing in the pots and dried for biomass measurements and chemical analysis.

After harvesting the pea, soils were thoroughly mixed and 4.0 g of soils (based on dry weight) were placed in a 50-mL polypropylene centrifuge tube and extracted with DIW at a soil-to-water ratio of 1:5. The suspension was shaken for 30 min, and was centrifuged at a speed of 3000 g min^{-1} for 15 min. After centrifugation, the supernatant will be filtered through a 0.45 μm paper filter (Whatman [Maidstone, UK] 42), digested with concentrated HNO_3 and analyzed for metal concentrations by ICP-AES.

3.2.4 Plant and Soil Analysis

All the samples, including the plants and soils, were digested using strong acid digestion according to the method of USEPA (1999) with some modifications for different types of samples in order to determine the “total” concentrations of trace metals and major elements. All the samples were weighted and placed into acid-washed Pyrex test tubes. The weight of the plant and soil samples to be digested were about 0.2 g, 0.1 g and 0.25 g for plant shoots, roots and soils, respectively. In general, 4:1 portion of analytical grade concentrated nitric acid and perchloric acid were added to each tube of sample. Each mixture was gently shaken using Vortex and then placed in an aluminium heating block (FOSS TECATOR 2000). The plant and soil samples were heated according to the temperature settings shown in Table 3.2. After the test tubes were cooled, 10 ml of 5% nitric acid was added into the residue and heated at 70 ºC for half an hour. The heated mixture was shaken gently and poured into polyethylene bubes for metal concentration analysis. The metal concentration analysis was subjected to the following quality control procedure: reagent blanks, duplications, and standard reference materials (NIST SRM 1515, apple leaves for the plant samples and NIST 2709 for the soil samples), representing 10%, 20%, and 10% of the samples respectively, were randomly inserted in each batch of the acid digestion. The recovery rates for most of the trace metals were around 90% - 110%. The relative standard deviations of trace metal concentrations in the duplicate samples were generally less than 10%.

The concentrations of metals were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Perkin Elmer Optima

3300DV). Quality standard was used during the determination of the elemental concentrations at the interval of every ten samples for the ICP-AES analysis to detect the contamination and drifting during the measurement. The variation in detection of drift was less than 10%.

Table 3.3 The temperature setting for the acid digestion of plant and soil samples

Temperature (ºC)	Plant Samples (hours)	Soil Samples (hours)
50 ºC	1	3
75 ºC	2	1.5
100 ºC	2	1.5
125 ºC	2	1.5
150 ºC	2	6
175 ºC	3	2
190 ºC	Until dryness	Until dryness

3.2.5 Data Analysis

Statistical analyses of the experimental data, such as correlation and significant differences, were performed using SPSS® 11.0 statistical software. The means and standard deviations of all the data were analyzed using Microsoft Excel 2000 software on a personal computer.

3.3 Results and Discussion

3.3.1 Effects of EDTA on the Growth of Different Plants

It has been demonstrated that high concentrations of EDTA and Pb-EDTA complexes were toxic to plants (Vassil et al., 1998; Epstein et al., 1999; Geebelen et al., 2002; Piechalak et al., 2003). All the 18 species/cultivars of plants developed normally without any visual symptoms of metal toxicity before EDTA was applied to soil. Results from Table 3.3 showed that the application of 3 mmol kg^{-1} of EDTA significantly depressed the growth of the plants, and decreased the yields of shoot dry matter (DM) in most of the plants tested ($P < 0.05$). Compared with the dicotyledon species, the five graminaceous monocotyledon plants showed relatively less response to the application of EDTA, with less chlorosis and smaller reductions of shoot biomass. Monocotyledon species are usually more tolerant to heavy metals than dicotyledon species (Marschner, 1995). These patterns were similar to that reported by Chen et al. (2004b). Of the five graminaceous monocotyledon plants, wheat (*Triticum aestivum* L. cv. Weimai No. 8, *Triticum aestivum* L. cv. Shangnong No. 93-52 and *Triticum aestivum* L. cv. Zimai No. 12) appeared to be the least sensitive plant to EDTA application, followed by corn (*Zea mays* L. cv. Nongda No. 108) and sorghum (*Sorghum bicolor* L. cv. Moench-S. vulgare Pers).

Most of 13 dicotyledon plants tested in the study showed severely toxic symptoms in terms of dry weight reductions after the application of 3 mmol kg^{-1} of EDTA, except for cole (*Glycine max* L. Merrill), soybean (*Brassica campestris* L. cv. Suyou No. 1 and *Brassica campestris* L. cv. Qinyou No. 8) and caraway (*Coriandrum sativum* L.). Spinach (*Spinacia oleracea* L.) and

garland chrysanthemum (*Chrysanthemum coronarium* L.) showed the toxic effects on the second day after receiving the 3 mmol kg^{-1} of EDTA treatment. On Day 7 after the EDTA treatment, the shoot DM yields of spinach and garland chrysanthemum were decreased by 40% and 44%, respectively, in comparison with the control groups. After a further 7 d growth, all spinach plants treated with EDTA died. Compared with those in the first harvest at Day 7, the decreases of shoot DM of all plants treated with EDTA became more pronounced in the second harvest (14^{th} day).

3.3.2 Effects of EDTA on the Shoot Metal Concentrations and Phytoextraction by Different Plants

In the control group, there were large variations of the concentration of metals in the shoots of different plant species/cultivars (Tables 3.4 – 3.7). The concentrations of Cu, Pb, Zn and Cd were in the range of 11 to 62, 1.4 to 7.5, 51 to 258, and 0.1 to 2.7 mg kg^{-1} in the plants, respectively. The application of EDTA at the rate of 3 mmol kg^{-1} dramatically increased the concentrations of Cu, Pb and Zn in the shoots of plants. The enhancement was more pronounced for the dicotyledon plants than for graminaceous monocotyledon plants (Tables 3.4 - 3.7). There were considerable differences of metal concentration increases by the EDTA treatment. Application of 3 mmol kg^{-1} of EDTA led to an average increase of 5.6- and 18.3-fold for the concentrations of Cu and Pb compared with the control group, respectively. Less than 3-fold enhancing effect was found for Zn in the shoots of all species/cultivars with the exception of garland chrysanthemum. Except for the garland chrysanthemum, EDTA application had

no significant effect on the concentrations of Cd in the shoots of plants. In garland chrysanthemum the concentrations of Zn and Cd showed 6.4- and 7.2-fold increase 7 d after the EDTA application. Among all the plants tested, garland chrysanthemum showed the most prominent sensitivity to the application of EDTA, with the highest enhancement of Cu, Pb, Zn and Cd concentrations in the shoots. In particular, the concentrations of Cu and Pb reached 46- and 135-fold increases 14 d after the application of 3 mmol kg^{-1} of EDTA, which was much higher than those in other plants. Chen et al. (2004b) also reported that the growth of and metal accumulation by mung bean and buckwheat had a higher sensitivity to the EDTA treatment in soils than the growth of and metal accumulation by other eight plant species. Further experiments would be needed to elucidate the mechanisms involved in the chelate-induced metal accumulation in plants.

With regard to the total amounts of metals phytoextracted from the soil, the application of EDTA also produced significant enhancing effects in the plants, although these effects were smaller than that on the metal concentrations in the shoots due to the reduction in shoot biomass as a result of the toxic effects of EDTA on the plants. The highest amounts of Cu and Pb extracted were achieved by garland chrysanthemum, with 1.85 mg Cu kg^{-1} soil and 0.68 mg Pb kg^{-1} soil, respectively. For Zn, the highest extraction (1.8 mg Zn kg^{-1} soil) was achieved by the cabbage (*Brassica campestris* L. cv. Suyou No. 1). Similar to the effect on the concentration of Cd in the shoots, the application of 3 mmol kg^{-1} of EDTA did not significantly improve the phytoextraction of Cd by all species of plants. Noticeably, on Day 14 of the chelate application the total

phytoextraction of Cu, Pb, Zn and Cd in garland chrysanthemum shoots reached 1540, 560, 1100 and 3.8 μg plant^{-1}, which were 15, 45, 5.5 and 1.4 times of the control group, respectively, although the biomass of the shoots decreased by 68% in comparison with the control group. According to Blaylock et al. (1997), the first step in phytoremediation is to produce high plant biomass at contaminated sites, which can be accomplished through intensive cultivation of high-biomass crops. The second step is the induction of the metal accumulation in the shoots, which can be achieved with the addition of synthetic chelates such as EDTA. Thus, for a practical application in field, the death of a plant prior to harvest does not preclude successful phytoextraction of heavy metals if substantial plant biomass has been established prior to the chelate application (Huang and Cunningham, 1996).

It has been reported that members of *Brassicae* have an ability to take up heavy metals from contaminated soils and transport these metals to the shoots (Kumar, et al., 1995; Salt et al., 1995; Ebbs et al., 1997; Ebbs and Kochian, 1997; Pandey and Sharma, 2002; Shen et al., 2002; Carrier et al., 2003). Compared to the graminaceous monocotyledon tested in the present study, the shoots of *B. juncea*, *B. chinensis*, *B. pekinensis* and *B. capestris* had higher concentrations of Cu, Pb, Zn and Cd in the treatments with and without EDTA (Tables 3.4 – 3.7). Similar results were obtained by Kos et al. (2003) and Chen et al. (2004b). However, the concentrations of metals in these *Brassicae* plants were still lower than that in garland chrysanthemum, particularly for Cu and Pb.

Table 3.4 Dry biomass yields (g plant^{-1}) of 18 different plants 7 and 14 days after the application of EDTA

Crop plants	EDTA (mmol kg^{-1} soil)	Shoot dry weight (g plant^{-1})	
		7 d	14 d
Monocotyledon			
Zea mays L. cv. Nongda No. 108	0	3.8 ± 1a	6.8 ± 1.54a
	3	3.4 ± 0.25a	6.1 ± 0.94a
Triticum aestivum L. cv. Weimai No. 8	0	2.7 ± 0.33a	4.5 ± 0.19a
	3	2.6 ± 0.24a	4.1 ± 0.74a
Triticum aestivum L. cv. Shangnong No. 93-52	0	2.0 ± 0.69a	2.9 ± 1.34a
	3	2.0 ± 0.03a	3.0 ± 0.23a
Triticum aestivum L. cv. Zimai No. 12	0	2.6 ± 0.84a	4.0 ± 1.34a
	3	2.7 ± 0.2a	3.8 ± 0.71a
Sorghum bicolor L. cv. Moench-S. vulgare Pers.	0	1.2 ± 0.15a	3.1 ± 0.2a
	3	1.0 ± 0.05a	2.3 ± 0.38b
Dicotyledon			
Brassica juncea L. Czern. Et Coss. Cv. Liyangkucai	0	3.5 ± 0.2a	5.9 ± 0.88a
	3	2.5 ± 0.45b	3.8 ± 0.82b
Brassica pekinensis Rupr. L. cv. Xiaoairen	0	3.8 ± 0.46a	5.0 ± 0.74a
	3	2.9 ± 0.67b	3.0 ± 0.27b
Brassica chinensis L. cv. Xiaoza56	0	5.0 ± 0.5a	6.1 ± 0.7a
	3	3.5 ± 0.81b	3.8 ± 1.2b
Brassica chinensis L. cv. Sijiucaixin	0	3.4 ± 0.4a	4.3 ± 0.69a
	3	2.3 ± 0.52b	2.3 ± 0.82b
Brassica capestris L. cv. Suyou No. 1	0	2.8 ± 1.68a	5.5 ± 1.54a
	3	2.6 ± 1.04a	5.5 ± 0.27a
Brassica capestris L. cv. Qinyou No. 1	0	2.4 ± 0.97a	3.9 ± 2.13a
	3	1.9 ± 0.29a	4.3 ± 1.62a
Lactuca sativa L.	0	1.5 ± 0.6a	3.9 ± 1.24a
	3	1.1 ± 0.57a	1.4 ± 1.0b
Glycine max L. Merrill	0	3.9 ± 0.37a	5.6 ± 0.19a
	3	3.7 ± 0.58a	5.3 ± 0.69a
Chrysanthemum coroium L.	0	1.8 ± 0.51a	3.4 ± 1.34a
	3	1.0 ± 0.37b	1.1 ± 0.43b
Coriandrum sativum L.	0	0.8 ± 0.16a	1.8 ± 0.56a
	3	1.0 ± 0.13a	1.6 ± 0.5a
Daucus carota L. var. sativa DC.	0	0.9 ± 0.27a	2.1 ± 0.14a
	3	0.7 ± 0.24a	1.3 ± 0.57b
Raphanus sativus L.	0	4.4 ± 1.11a	5.5 ± 1.02a
	3	3.6 ± 0.93a	4.3 ± 0.77a
Spinacia oleracea L.	0	2.0 ± 0.43a	4.0 ± 0.54
	3	1.2 ± 0.05b	Nd

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level. Nd denotes no data because the plant species did not survive.

Table 3.5 Cu concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA

	Cu concentration (mg kg^{-1})				Cu phytoextraction (μg kg^{-1} soil)			
	7 d		14 d		7 d		14 d	
EDTA (mmol kg^{-1} soil)	**0**	**3**	**0**	**3**	**0**	**3**	**0**	**3**
Crop plants								
Monocotyledon								
Zea mays L. cv. Nongda No. 108	19.6 ± 0.1	43.7 ± 12	11.1 ± 1.8	36.1 ± 2.9	88.5 ± 9.7	179 ± 21	90 ± 11	266 ± 32
Triticum aestivum L. cv. Weimai No. 8	26.4 ± 3.1	40.2 ± 5.5	15.6 ± 1.2	38.5 ± 4.6	83.9 ± 6.9	126 ± 15	84.3 ± 9.3	190 ± 21
Triticum aestivum L. cv. Shangnong No. 93-52	15.8 ± 2	72.1 ± 3.7	17.8 ± 3.2	70.1 ± 8.5	37.3 ± 4.1	171 ± 22	62.9 ± 7.4	255 ± 26
Triticum aestivum L. cv. Zimai No. 12	17.5 ± 2.1	40.1 ± 5.2	17.6 ± 2.5	51.8 ± 6.9	55.1 ± 6.8	128 ± 14	83.7 ± 9	239 ± 24
Sorghum bicolor L. cv. Moench-S. vulgare Pers	35.9 ± 2.7	68.3 ± 5.5	23.1 ± 3.8	51 ± 6	52.8 ± 5.2	78 ± 8.5	85.4 ± 11	139 ± 15
Dicotyledon								
Brassica juncea L. Czern. Et Coss. Cv. Liyangkucai	30.7 ± 3	112 ± 18	22 ± 3	120 ± 15	128 ± 13	341 ± 37	156 ± 17	546 ± 63
Brassica chinensis L. cv. Xiaoairen	41.2 ± 5.3	125 ± 15	32.9 ± 4.6	237 ± 28	186 ± 22	438 ± 69	197 ± 22	858 ± 87
Brassica chinensis L. cv. Xiaoza No. 56	27.2 ± 3.8	116 ± 25	23.1 ± 2	164 ± 12	162 ± 19	491 ± 53	169 ± 13	740 ± 74
Brassica pekinensis Rupr. L.	22.2 ± 3	100 ± 8.5	19.9 ± 2.8	126 ± 14	91 ± 11	276 ± 36	102 ± 14	339 ± 34
Brassica campestris L. cv. Suyou No. 1	25.7 ± 3	98.6 ± 11	21.6 ± 3	151 ± 12	86.1 ± 9.3	308 ± 56	143 ± 12	999 ± 12
Brassica campestris L. cv. Qinyou No. 8	26.6 ± 1	108 ± 7.5	17.9 ± 1.2	154 ± 10	77.9 ± 7.8	251 ± 34	83.3 ± 5.9	800 ± 57
Lactuca sativa L.	22.8 ± 1.7	107 ± 8	19.3 ± 2.1	252 ± 20	41.9 ± 5.6	136 ± 17	91 ± 6.7	420 ± 27
Glycine max L. Merrill	13 ± 0.8	42 ± 3.5	38.2 ± 3	64.7 ± 5.8	60.5 ± 5.7	188 ± 21	257 ± 32	414 ± 54
Chrysanthemum coronnarium L.	61.9 ± 7.2	585 ± 43	30 ± 2.1	1371 ± 17	136 ± 14	709 ± 83	121 ± 13	1850 ± 98
Coriandrum sativum L.	34.8 ± 4.9	96.8 ± 7.9	30.9 ± 4	152 ± 12	34.4 ± 46	119 ± 24	68.3 ± 5.3	296 ± 31
Daucus carota L. var. sativa DC.	28.6 ± 3.2	83.2 ± 7	19.6 ± 2.5	97.1 ± 9	30.7 ± 31	65.9 ± 6.9	49 ± 3.6	152 ± 21
Raphanus sativus L.	24.3 ± 2.5	50.3 ± 4.6	29 ± 3.8	89.2 ± 9	127 ± 16	216 ± 15	189 ± 32	462 ± 34
Spinacia oleracea L.	29.9 ± 2.9	187 ± 21	98.9 ± 14	Nd	71.7 ± 9	269 ± 45	475 ± 67	Nd

		Cu concentration	Cu phytoextraction
ANOVA	Plant Species	***	***
	EDTA Treatment	***	***
	Treatment time	*	**
	Species × Treatment × Time	*	**

Values are means ± S.D. (n = 3); ***, P < 0.001; **, P < 0.01; *, P < 0.05, NS, not significant.

Table 3.6 Pb concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA

	Pb concentration (mg kg^{-1})				Pb phytoextraction (μg kg^{-1} soil)			
	7 d		14 d		7 d		14 d	
EDTA (mmol kg^{-1} soil)	**0**	**3**	**0**	**3**	**0**	**3**	**0**	**3**
Crop plants								
Monocotyledon								
Zea mays L. cv. Nongda No. 108	4.2 ± 0.5	29.3 ± 3	3.5 ± 0.4	21.8 ± 3.2	19 ± 2.5	120 ± 13	28.6 ± 3.5	161 ± 17
Triticum aestivum L. cv. Weimai No. 8	2.4 ± 0.4	10.4 ± 1.5	1.4 ± 0.1	12.8 ± 2.3	7.6 ± 1.2	32.4 ± 1.3	7.5 ± 0.9	63.3 ± 5
Triticum aestivum L. cv. Shangnong No. 93-52	3.3 ± 0.2	32.5 ± 4.5	1.8 ± 0.2	26.3 ± 3.4	7.7 ± 0.9	77.3 ± 8.6	6.5 ± 1.1	95.8 ± 10
Triticum aestivum L. cv. Zimai No. 12	2.6 ± 0.3	12.3 ± 1.5	1.4 ± 0.2	15.6 ± 2	8.1 ± 0.6	39 ± 4.5	6.8 ± 1.7	71.6 ± 8.9
Sorghum bicolor L. cv. Moench-S. vulgare Pers	2.2 ± 0.3	37.9 ± 0.9	1.5 ± 0.6	27.1 ± 2.1	3.3 ± 0.2	43.3 ± 5.6	5.6 ± 0.8	73.8 ± 6
Dicotyledon								
Brassica juncea L. Czern. Et Coss. Cv. Liyangkucai	3.8 ± 0.7	49.7 ± 5.2	4.6 ± 0.3	46.9 ± 3.9	16 ± 1.7	151 ± 18	32.4 ± 3.5	214 ± 14
Brassica chinensis L. cv. Xiaoairen	4.5 ± 0.3	62.4 ± 0.7	5.2 ± 0.3	114 ± 13	20.2 ± 3	219 ± 25	31 ± 0.5	414 ± 47
Brassica chinensis L. cv. Xiaoza No. 56	3.6 ± 0.4	53.5 ± 4.5	4.7 ± 0.6	53.7 ± 5	21.5 ± 2.9	226 ± 27	34 ± 2.7	242 ± 26
Brassica pekinensis Rupr. L.	3.2 ± 0.3	46.4 ± 6.5	4.2 ± 0.3	56.8 ± 6	13 ± 2.1	128 ± 15	21.7 ± 3.0	154 ± 24
Brassica campestris L. cv. Suyou No. 1	3.1 ± 0.6	50.9 ± 6	2.8 ± 0.3	69 ± 7.2	10.5 ± 1.5	159 ± 23	18.8 ± 1.2	456 ± 48
Brassica campestris L. cv. Qinyou No. 8	3.3 ± 0.4	42.6 ± 3.9	1.9 ± 0.2	57.2 ± 4.8	9.7 ± 1.0	99 ± 15	8.9 ± 0.9	298 ± 36
Lactuca sativa L.	7.5 ± 0.9	65.5 ± 7.3	7.2 ± 0.9	154 ± 16	13.8 ± 1.7	83.6 ± 10	33.8 ± 2.5	257 ± 46
Glycine max L. Merrill	2.1 ± 0.1	16.3 ± 0.2	2.2 ± 0.3	25.3 ± 2.1	9.8 ± 2.1	72.8 ± 8.5	14.6 ± 2.4	162 ± 26
Chrysanthemum coronnarium L.	5.6 ± 0.7	385 ± 41	3.7 ± 0.4	502 ± 45	12.7 ± 1.4	467 ± 54	15 ± 1.8	677 ± 87
Coriandrum sativum L.	7.1 ± 0.9	72.4 ± 6.3	6.2 ± 5.5	55.2 ± 7.9	7.1 ± 0.8	89.2 ± 9.7	13.8 ± 2	108 ± 15
Daucus carota L. var. sativa DC.	3.2 ± 0.2	31.1 ± 0.5	3.1 ± 0.4	40.6 ± 5	3.4 ± 0.5	24.6 ± 3.7	7.8 ± 0.9	63.5 ± 7.8
Raphanus sativus L.	6.0 ± 0.8	14.9 ± 2.5	6.9 ± 0.5	23.2 ± 1.5	31.3 ± 4.3	64.1 ± 7.8	45.4 ± 2.7	120 ± 11
Spinacia oleracea L.	3.5 ± 0.5	146 ± 16	6.6 ± 0.5	Nd	8.4 ± 0.3	211 ± 25	31.7 ± 3.2	Nd

		Pb concentration	Pb phytoextraction
ANOVA	Plant Species	**	***
	EDTA Treatment	***	***
	Treatment time	*	**
	Species × Treatment × Time	*	**

Values are means ± S.D. (n = 3); ***, P < 0.001; **, P < 0.01; *, P < 0.05, NS, not significant.

Table 3.7 Zn concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA

	Zn concentration (mg kg^{-1})				Zn phytoextraction (μg kg^{-1} soil)			
	7 d		14 d		7 d		14 d	
EDTA (mmol kg^{-1} soil)	**0**	**3**	**0**	**3**	**0**	**3**	**0**	**3**
Crop plants								
Monocotyledon								
Zea mays L. cv. Nongda No. 108	147 ± 16	173 ± 12	104 ± 21	136 ± 17	664 ± 75	709 ± 87	845 ± 78	1000 ± 95
Triticum aestivum L. cv. Weimai No. 8	138 ± 19	186 ± 21	100 ± 15	186 ± 25	440 ± 57	579 ± 65	540 ± 55	920 ± 56
Triticum aestivum L. cv. Shangnong No. 93-52	96.1 ± 7.8	157 ± 14	98 ± 11	190 ± 28	226 ± 32	374 ± 43	346 ± 45	692 ± 78
Triticum aestivum L. cv. Zimai No. 12	148 ± 16	191 ± 23	112 ± 23	200 ± 35	466 ± 45	609 ± 72	533 ± 67	922 ± 76
Sorghum bicolor L. cv. Moench-S. vulgare Pers	211 ± 35	255 ± 47	168 ± 27	259 ± 45	310 ± 34	291 ± 31	622 ± 70	705 ± 34
Dicotyledon								
Brassica juncea L. Czern. Et Coss. Cv. Liyangkucai	141 ± 42	210 ± 19	123 ± 23	210 ± 17	586 ± 67	638 ± 67	871 ± 78	954 ± 87
Brassica chinensis L. cv. Xiaoairen	209 ± 17	188 ± 29	179 ± 31	405 ± 54	940 ± 47	1010 ± 85	1070 ± 87	1470 ± 120
Brassica chinensis L. cv. Xiaoza No. 56	130 ± 19	241 ± 39	120 ± 17	295 ± 54	773 ± 89	1020 ± 112	878 ± 90	1330 ± 125
Brassica pekinensis Rupr. L.	147 ± 18	246 ± 31	137 ± 12	294 ± 37	604 ± 67	677 ± 54	706 ± 76	793 ± 85
Brassica campestris L. cv. Suyou No. 1	123 ± 14	214 ± 22	86.6 ± 9	272 ± 35	411 ± 56	667 ± 45	574 ± 60	1800 ± 123
Brassica campestris L. cv. Qinyou No. 8	129 ± 15	201 ± 27	80.1 ± 7.3	263 ± 64	378 ± 46	466 ± 56	372 ± 42	1370 ± 150
Lactuca sativa L.	99 ± 12	187 ± 19	99 ± 14	365 ± 45	183 ± 25	239 ± 23	468 ± 21	609 ± 70
Glycine max L. Merrill	79 ± 9.5	91 ± 11	90.5 ± 11	114 ± 23	367 ± 45	405 ± 37	609 ± 68	727 ± 80
Chrysanthemum coronnarium L.	92 ± 16	586 ± 78	59.3 ± 8	983 ± 125	201 ± 23	711 ± 78	240 ± 13	1330 ± 125
Coriandrum sativum L.	105 ± 13	159 ± 21	103 ± 12	191 ± 25	104 ± 20	196 ± 23	227 ± 34	372 ± 45
Daucus carota L. var. sativa DC.	56 ± 7.5	91 ± 11	50.6 ± 7.9	132 ± 20	60 ± 7	72 ± 7	126 ± 15	206 ± 14
Raphanus sativus L.	132 ± 17	177 ± 25	136 ± 17	261 ± 36	688 ± 56	761 ± 45	889 ± 65	1350 ± 110
Spinacia oleracea L.	190 ± 41	341 ± 27	258 ± 37	Nd	457 ± 61	492 ± 56	1240±100	Nd

		Zn concentration	Zn phytoextraction
ANOVA	Plant Species	*	**
	EDTA Treatment	*	*
	Treatment time	*	*
	Species × Treatment × Time	*	*

Values are means ± S.D. (n = 3); ***, P < 0.001; **, P < 0.01; *, P < 0.05, NS, not significant.

Table 3.8 Cd concentration in the shoots and total phytoextraction by the different plants 7 and 14 days after the treatment of EDTA

	Cd concentration (mg kg^{-1})				Cd phytoextraction (μg kg^{-1} soil)			
	7 d		14 d		7 d		14 d	
EDTA (mmol kg^{-1} soil)	**0**	**3**	**0**	**3**	**0**	**3**	**0**	**3**
Crop plants								
Monocotyledon								
Zea mays L. cv. Nongda No. 108	1.0 ± 0.2	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.01	4.7 ± 0.6	1.9 ± 0.2	3.6 ± 0.4	1.4 ± 0.2
Triticum aestivum L. cv. Weimai No. 8	0.2 ± 0.01	0.3 ± 0.02	0.7 ± 0.1	0.5 ± 0.02	0.6 ± 0.1	0.8 ± 0.1	3.9 ± 0.3	2.7 ± 0.3
Triticum aestivum L. cv. Shangnong No. 93-52	0.6 ± 0.1	0.4 ± 0.05	0.6 ± 0.03	0.3±0.1	1.3 ± 0.2	0.9 ± 0.1	2.3 ± 0.2	1.2 ± 0.1
Triticum aestivum L. cv. Zimai No. 12	1.2 ± 0.1	0.2 ± 0.02	0.6 ± 0.1	0.2 ± 0.02	3.6 ± 0.3	0.6 ± 0.1	3.1 ± 0.3	1.1 ± 0.1
Sorghum bicolor L. cv. Moench-S. vulgare Pers	1.5 ± 0.2	0.8 ± 0.1	1.8 ± 0.2	1.0 ± 0.1	2.2 ± 0.1	0.91± 0.1	6.5 ± 0.7	2.7 ± 0.3
Dicotyledon								
Brassica juncea L. Czern. Et Coss. Cv. Liyangkucai	1.9 ± 0.3	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	7.7 ± 0.4	2.9 ± 0.3	7.2 ± 0.3	4.1 ± 0.3
Brassica chinensis L. cv. Xiaoairen	2.7 ± 0.3	1.1 ± 0.2	1.9 ± 0.2	1.8 ± 0.2	12.3 ± 1.3	3.8 ± 0.3	11.6 ± 0.9	6.6 ± 0.7
Brassica chinensis L. cv. Xiaoza No. 56	1.4 ± 0.2	1.4 ± 0.2	2.1 ± 0.2	1.7 ± 0.2	8.1 ± 0.5	5.8 ± 0.2	15.6 ± 1.5	7.8 ± 0.8
Brassica pekinensis Rupr. L.	1.5 ± 0.1	1.1 ± 0.2	1.6 ± 0.2	1.0 ± 0.1	6 ± 0.3	3.1 ± 0.4	8.3 ± 0.7	2.6 ± 0.3
Brassica campestris L. cv. Suyou No. 1	1.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	5.5 ± 0.4	2.5 ± 0.2	6.7 ± 0.4	7.8 ± 0.7
Brassica campestris L. cv. Qinyou No. 8	1.4 ± 0.1	0.7 ± 0.1	0.7 ± 0.08	0.7 ± 0.1	3.9 ± 0.2	1.6 ± 0.1	3.1 ± 0.2	3.8 ± 0.4
Lactuca sativa L.	1.8 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	2.4 ± 0.3	3.3 ± 0.4	1.2 ± 0.1	5.5 ± 0.6	4 ± 0.1
Glycine max L. Merrill	0.4 ± 0.1	0.1 ± 0.01	0.6 ± 0.1	0.6 ± 0.08	1.9 ± 0.2	0.5 ± 0.1	3.7 ± 0.5	3.9 ± 0.2
Chrysanthemum coronnarium L.	0.2 ± 0.03	1.3 ± 0.2	0.8 ± 0.1	3.4 ± 0.4	0.4 ± 0.1	1.5 ± 0.1	3.2 ± 0.2	4.6 ± 0.6
Coriandrum sativum L.	2.2 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	2.0 ± 0.2	2.2 ± 0.2	1.5 ± 0.1	2.4 ± 0.3	3.9 ± 0.3
Daucus carota L. var. sativa DC.	0.1 ± 0.01	0.6 ± 0.04	0.8 ± 0.1	0.4 ± 0.08	0.1 ± 0.05	0.4 ± 0.1	2.1 ± 0.1	0.6 ± 0.1
Raphanus sativus L.	1.9 ± 0.2	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.3	9.8 ± 0.7	4.6 ± 0.3	7.6 ± 0.8	6.3 ± 0.3
Spinacia oleracea L.	1.8 ± 0.2	1.9 ± 0.2	1.3 ± 0.2	Nd	4.3 ± 0.2	2.7 ± 0.3	6.3 ± 0.4	Nd

		Cd concentration	Cd phytoextraction
ANOVA	Plant Species	*	*
	EDTA Treatment	NS	NS
	Treatment time	*	*
	Species × Treatment × Time	NS	NS

Values are means ± S.D. (n = 3); ***, P < 0.001; **, P < 0.01; *, P < 0.05, NS, not significant.

3.3.3 Effects of EDTA and EDDS on the Growth of Garland Chrysanthemum and Corn

In this experiment, garland chrysanthemum and corn were chosen for a further study of their potential use in the chelate-enhanced phytoextraction of heavy metals from contaminated soil. The treatments with 0.5 mmol kg^{-1} soil EDTA and EDDS significantly affected the growth of garland chrysanthemum, leading to a respective 23% and 18% decrease in shoot dry matter yields compared with the control group (Fig. 3.1). Increasing the application dosage of EDTA and EDDS, the depressed effects on the dry biomass yields of garland chrysanthemum became more pronounced. The effects of EDDS and EDTA on the growth of corn were less significant than on the garland chrysanthemum (see Fig. 3.1).

3.3.4 Effects of EDDS and EDTA on Shoot Metal Concentrations and Phytoextraction by Garland Chrysanthemum and Corn

The concentrations of Cu in the shoots of garland chrysanthemum and corn significantly increased with the increasing level of EDDS and EDTA applied to the soil (Fig. 3.2). Compared with EDTA, EDDS was more effective at increasing the concentration of Cu in the shoots of the two species. The result was consistent with previously reported data in other plants (Meers et al., 2004, 2005). Between the two species, a larger increase was observed in garland chrysanthemum than in corn. On Day 7 after the application of 5 mmol kg^{-1} of EDDS, the concentration of Cu in the shoots reached 2340 mg kg^{-1} DW in

garland chrysanthemum, which was 69-fold the amount found in the control (without the application of chelates). The maximum concentration of Cu in the shoots of corn was 330 mg kg^{-1} DW, representing an 8.2-fold increase over that seen in the control group.

The total amount of Cu accumulated in shoots also increased with the rate of application of EDDS and EDTA to soil (Fig. 3.3). A higher level of Cu phytoextraction was always found in the EDDS treatment, in which with the application of 5 mmol kg^{-1} of EDDS, the phytoextraction of Cu was 38 and 5 times that of the control for garland chrysanthemum and corn, respectively. The percentages of Cu phytoextracted by garland chrysanthemum for 42 days and corn for 21 days with the EDDS treatment in one phytoextraction cycle were 0.11-1.81 % and 0.06-0.11% of the total Cu in the soil (400 mg kg^{-1}), respectively. The values by garland chrysanthemum in this study were higher than the data on the phytoextraction of Cu with NTA (Kulli et al., 1999; Kayser et al., 2000), with EDDS (Kos and Leštan, 2004a, b), and with EDTA (Thayalakumaran et al., 2003; Wenzel et al., 2003). The results suggest that EDDS can be regarded as a better candidate chelate for the phytoextraction of Cu in soils. EDDS and EDTA have an almost equal chemical affinity for Cu (log K_s = 18.4 and 18.8, respectively) (Martell et al. 2001). But for Fe, Pb, Zn, and Cd, EDDS has a much lower chemical affinity than EDTA. Thus, EDDS would be more effective at solubilizing soil Cu than EDTA (Tandy et al., 2004). Metal chelate complexes may enter the root through breaks in the root endodermis and Casparian strips, and be rapidly transported to the shoots (Römheld and Marschner, 1981; Bell et al., 1991). Also, it is likely that Cu may

enter the roots of plants and be transported to their shoots as a Cu-EDDS complex. Thus, some environmental stresses, such as excessive toxic metals, high temperatures, and droughts, could affect the effectiveness of phytoextraction with the application of chelate.

In most cases, the EDTA treatment was superior in terms of solubilizing soil Pb for root uptake and translocation into the aboveground biomass due to its strong chemical affinity for Pb (log K_s = 17.88) (Martell et al., 2001). The accumulation of Pb in plant shoots was correlated with the formation of the Pb-EDTA complex, and Pb-EDTA was the major form of Pb absorbed and translocated by plants (Vassil et al., 1998; Epstein et al., 1999). The results from the current study showed that the concentrations of Pb in the shoots of garland chrysanthemum and corn increased with the level of EDTA applied to the soil (Fig. 3.4). The application of EDDS at rates of 0.5-1.5 mmol kg^{-1} had no significant effect on the Pb concentration in the shoots of the two plant species. However, at the application rate of 5 mmol kg^{-1}, EDDS was much more effective than EDTA at increasing the uptake of Pb by the plants, particularly in garland chrysanthemum. The concentration of Pb in the shoots of garland chrysanthemum treated with 5 mmol kg^{-1} of EDDS reached 628 mg kg^{-1}, which was 4.7- and 126- fold the level in the plants treated with 5 mmol kg^{-1} of EDTA and the control group, respectively. Grčman et al. (2003) reported that single addition of 10 mmol kg^{-1} EDDS was similarly effective as EDTA for the phytoextraction of Pb from soil.

The enhancing efficiencies of total Pb phytoextraction by the two plant species

through EDDS and EDTA application (Fig. 3.5) were consistent with the increased concentrations of Pb in the shoots (Fig. 3.4), although the treatments with the chelates had a significant effect on the shoot biomass production of garland chrysanthemum and corn (Fig. 3.1). The maximum amount of Pb that was phytoextracted was found in garland chrysanthemum treated with 5 mmol kg^{-1} of EDDS, which reached 1.95 mg Pb kg^{-1} soil. This represents an increase of 70 times over the control groups. The highest percentages of Pb extracted by garland chrysanthemum and corn in one phytoextraction cycle were calculated to be 0.39% and 0.01% that of the total Pb (500 mg kg^{-1}) present in the soil, respectively. These values by garland chrysanthemum were higher than the data reported by Kos and Leštan (2003a) and Grčman et al. (2003) for the extraction of Pb by corn with EDDS and EDTA.

It has been suggested that a threshold concentration of EDTA is required to induce the accumulation of metals in plant shoots (Vassil et al., 1998). Blaylock et al. (1997) observed that the application of a threshold concentration of chelate of between 1 and 5 mmol kg^{-1} of EDTA induced a dramatic accumulation of Pb in the shoots of Indian mustard grown in soil containing 600 mg Pb kg^{-1}. At this threshold concentration (5 mmol kg^{-1} of EDTA) and above, synthetic chelates including EDTA could damage the membrane of root cells, which normally function to control the uptake and translocation of solutes, by chelating the Zn^{2+} and Ca^{2+} cations that stabilize the membrane (Vassil et al., 1998). Therefore, in chemically enhanced phytoextraction, the rate of application of chelates needs to be high enough to cause the breakdown of the cell membrane and to enable the unrestricted uptake and translocation of metals

into shoots. In the present study, a dramatic increase in the concentrations of Cu, Pb, and Zn in plant shoots occurred between the 3 and 5 mmol kg^{-1} EDDS treatments. However, this increase in the accumulation of the metals in shoots was not found in the EDTA treatments ranging from 0.5 to 5 mmol kg^{-1} soil. It was speculated that less than 5 mmol kg^{-1} of EDTA application was insufficient to break down plant uptake barriers under the conditions of the present experiment. This observation was consistent with the observation that EDTA was less toxic to garland chrysanthemum than EDDS (Fig. 3.1). It could partially explain why EDDS had a higher efficiency than EDTA in increasing the accumulation of metals in shoots at the application rate of 5 mmol chelate kg^{-1} to soil.

In both pot experiments, the EDTA-enhanced phytoextraction of Pb by corn was much less effective than by garland chrysanthemum. In the EDTA treatment, the concentration of Pb in the shoots was in the range of 3.4 - 43.8 mg kg^{-1} DW. This value was much lower than the data reported by Huang et al. (1997), and was comparable to those reported by Lombi et al. (2001) and Kos and Lestan (2003a). Lombi et al. (2001) showed that EDTA was far more efficient at overcoming the limitation in the diffusion of metals to the surface of roots than at breaking the barrier to their translocation from the roots to the shoots of corn plants. In the pot experiments conducted by Huang et al. (1997), the approach of transplanting seedlings rather than planting with seeds was used, and the breaks in the Casparian strip were thought to arise from mechanical damage to the roots (Wallace and Hale, 1962). A significant increase in the uptake and translocation of Pb has been reported for corn transplanted into soil, then treated

with EDTA, in comparison with plants that were germinated and grown in Pb-contaminated soil to which EDTA was subsequently applied (Wu et al. 1999).

The concentrations of Zn and Cd were enhanced through the application of chelating agents to soil (Table 3.8). However, the concentrations of Zn and Cd in the shoots treated with the chelates never exceeded those of the controls by more than 1.85 times, except for Zn in the 5 mmol kg^{-1} of EDDS treatment. The total amount of Zn extracted did not exceed that of the control groups by more than 1.5 times. No significant stimulating effect from the chelates was found on the phytoextraction of Cd in these plants. The lower ability of EDTA to enhance the uptake of Zn and Cd compared with Pb has also been reported previously (Blaylock et al., 1997; Shen et al., 2002). Grčman et al. (2001) observed increases in the concentrations of Pb, Cd, and Zn of 105, 2.3, and 3.2-fold, respectively, in the leaves of Chinese cabbage (*Brassica rapa*) grown on EDTA (10 mmol kg^{-1}) treated soil. Chen and Cutright (2001) found that both EDTA and HEDTA significantly enhanced the concentration of Cd, but decreased the total uptake of metal due to a severe decrease in biomass.

The distribution of metals in the shoots and roots of garland chrysanthemum and corn was also significantly affected by the application of EDTA and EDDS (see Tables 3.9 and 3.10). In the control group, the concentrations of Cu, Pb, Zn and Cd in the roots of the two plant species were significantly higher than those in the shoots. Most of the Cu and Pb absorbed by the plants were concentrated in the roots. The application of EDTA and EDDS significantly increased the

shoot-to-root ratios of the concentrations of Cu, Pb, Zn and Cd in both plant species. An increased trend for shoot-to-root ratios of the metal concentrations could be seen with the increasing application of EDTA and EDDS. Compared with EDDS, EDTA was more effective in stimulating the translocation of Pb from roots to shoots when the application rate was within 3 mmol kg^{-1}. But for the translocation of Cu and Zn, the most effective agent was EDDS. When 5 mmol kg^{-1} of EDTA and EDDS were applied, the mean percentage of absorbed Pb translocated from the roots to the shoots of both species of plants increased from 9.7% in the control samples to 54% and 62%, respectively; and from 21.1% to 45% and 81% for Cu.

In the present study, the sum of the phytoextraction of Cu, Pb, Zn, and Cd by garland chrysanthemum reached 14.7 mg kg^{-1} soil with the application of 5 mmol kg^{-1} of EDDS, which accounted for 1.04% of the total amount of Cu, Pb, Zn, and Cd in the soil. Those values were 2.9-fold those of the EDTA treatments. These results indicated that EDDS was superior to EDTA in the phytoremediation of contaminated soils with multiple heavy metals.

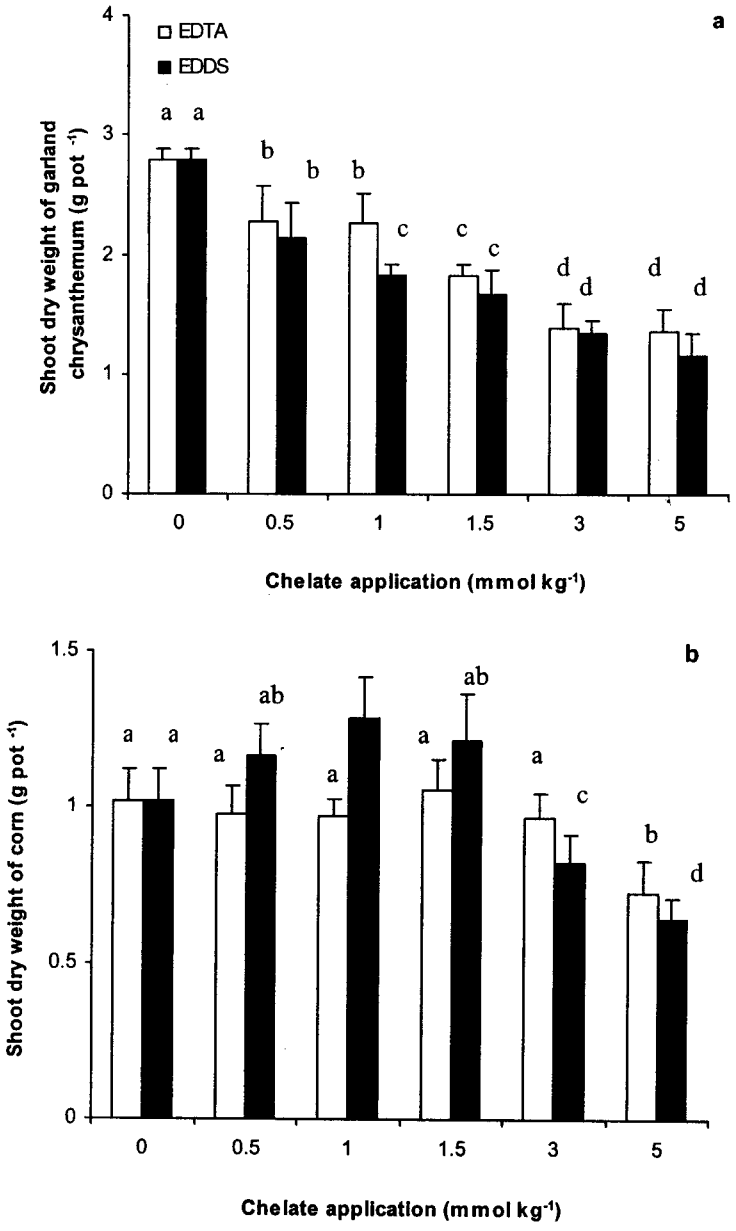


Fig. 3.1 Effects of the application of EDTA and EDDS on the dry mass yields of shoots in garland chrysanthemum (a) and corn (b). Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

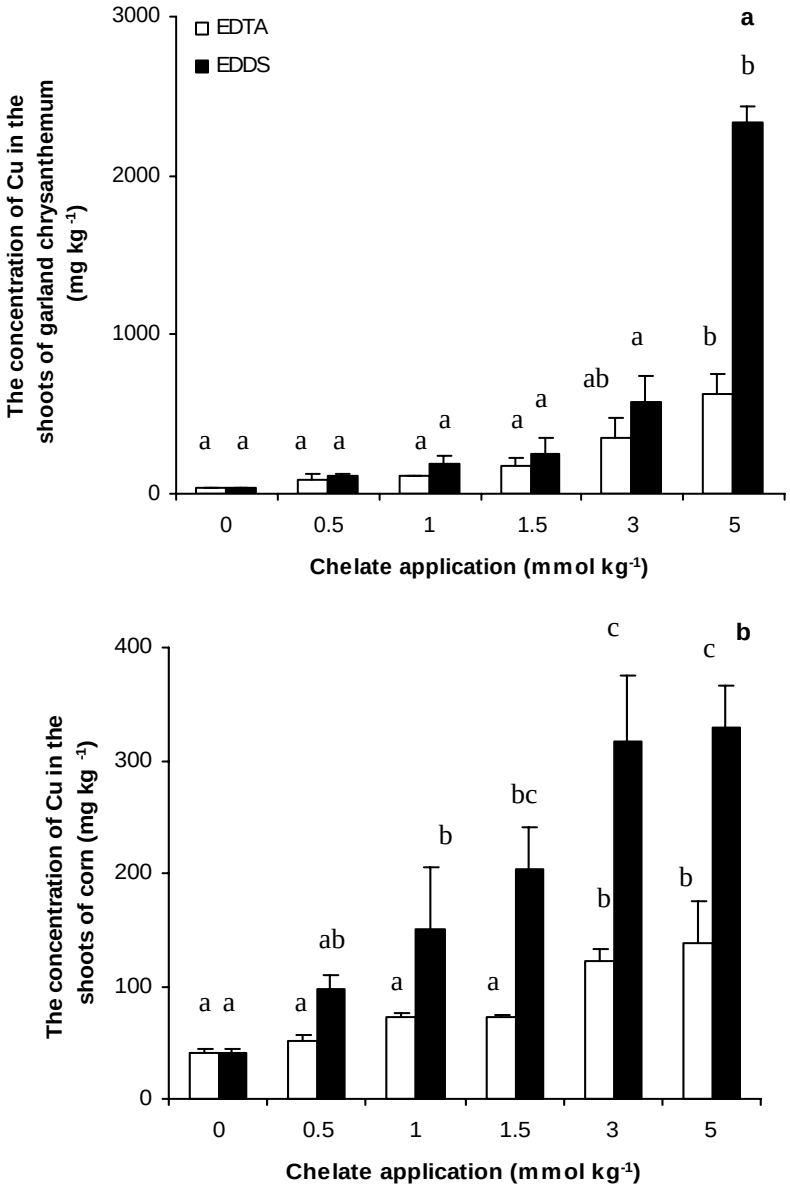


Fig. 3.2 Effects of the application of EDTA and EDDS on the concentration of Cu in the shoots of garland chrysanthemum (a) and corn (b). Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

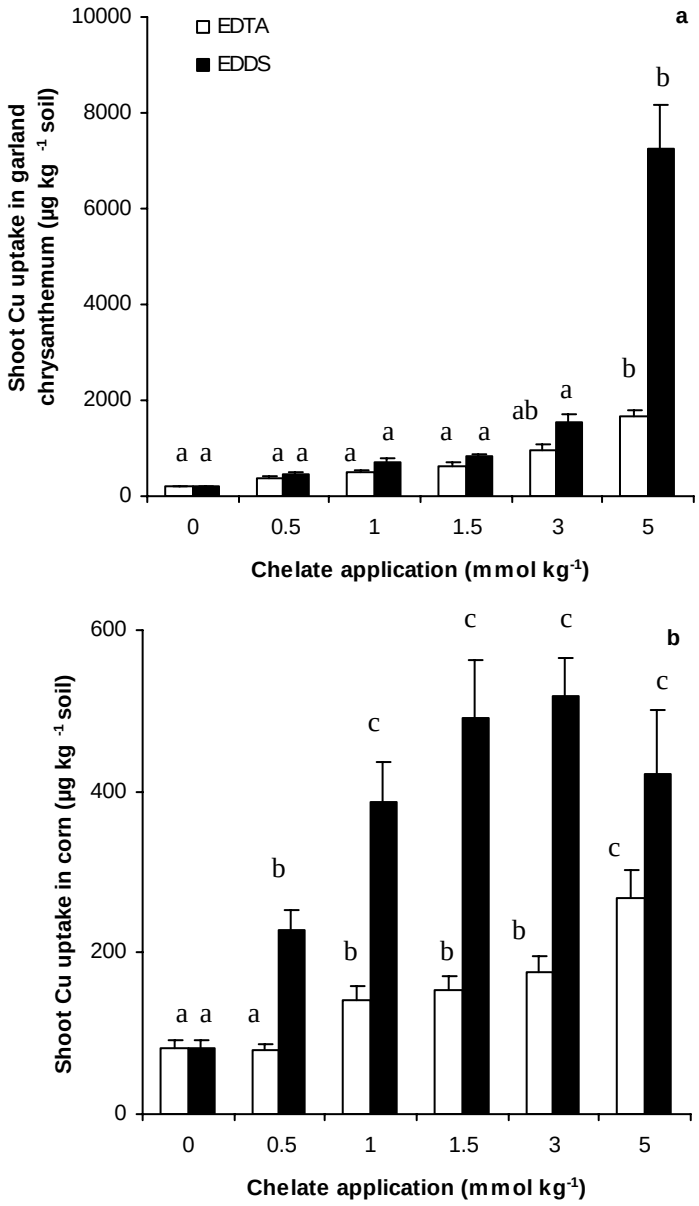


Fig. 3.3 Effects of the application of EDTA and EDDS on the uptake of Cu in the shoots of garland chrysanthemum (a) and corn (b). Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

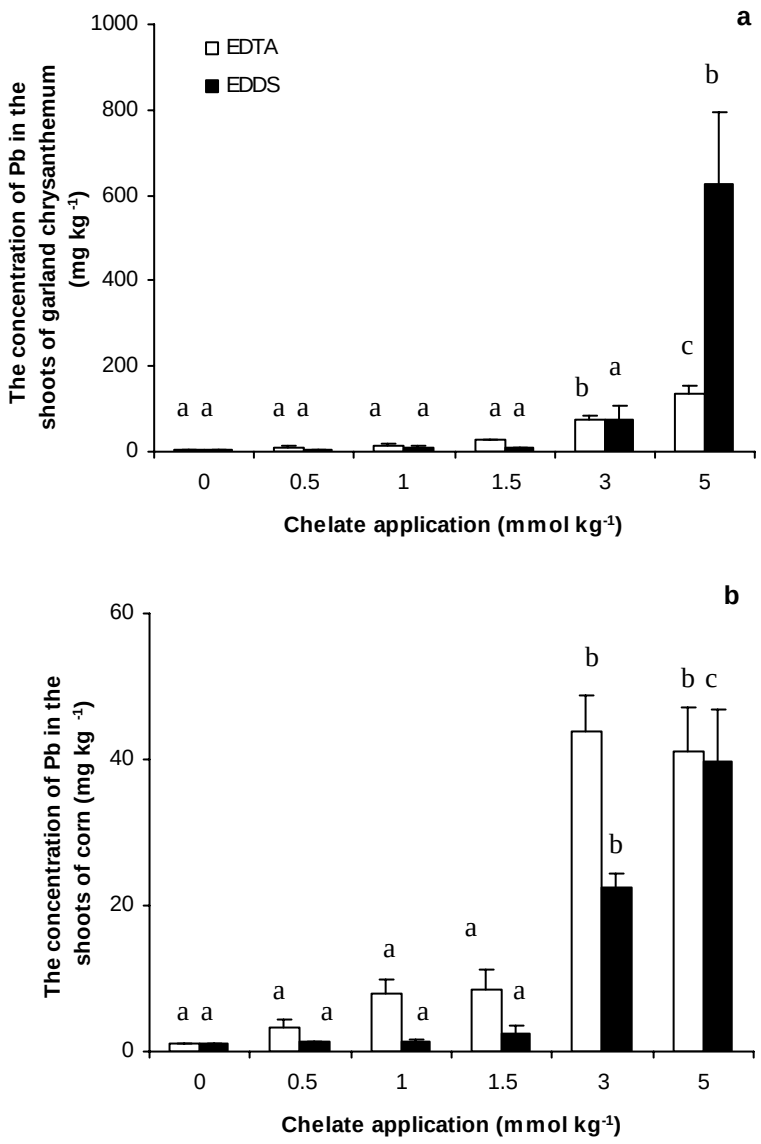


Fig. 3.4 Effects of the application of EDTA and EDDS on the concentration of Pb in the shoots of garland chrysanthemum (a) and corn (b). Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

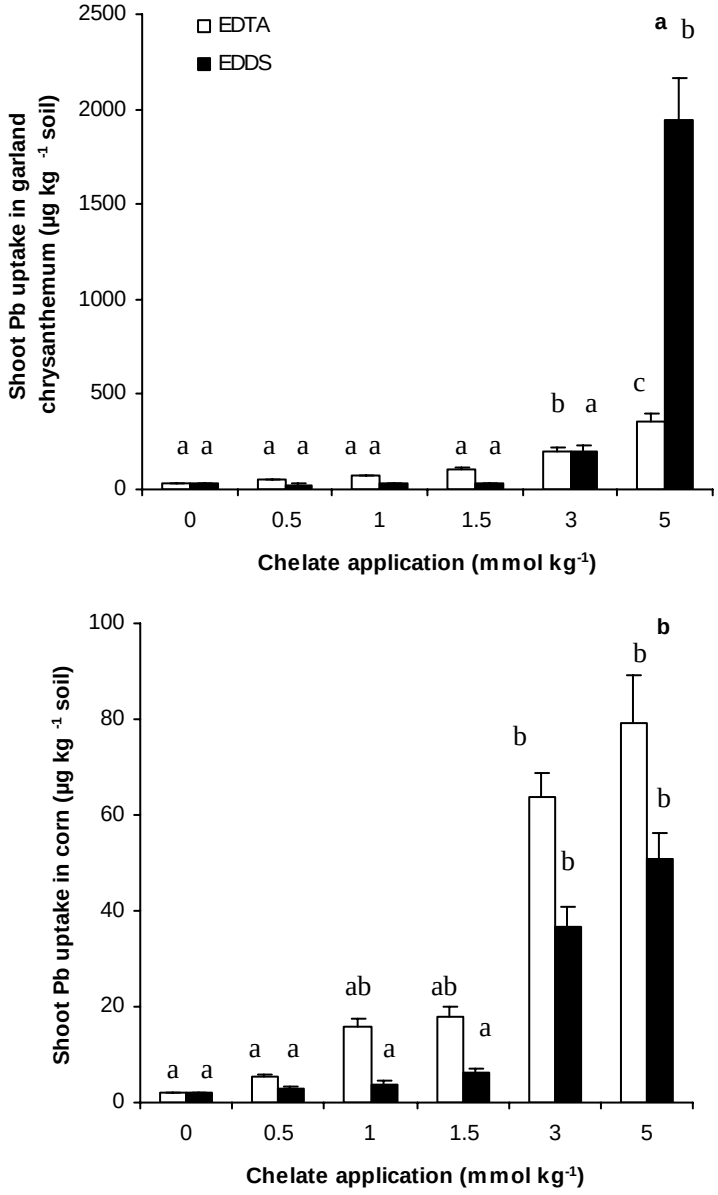


Fig. 3.5 Effects of the application of EDTA and EDDS on the uptake of Pb in the shoots of garland chrysanthemum (a) and corn (b). Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 3.9 The concentration and phytoextraction of metals in the shoots of garland chrysanthemum and corn with the application of EDTA and EDDS

	Garland chrysanthemum				Corn			
	EDTA		EDDS		EDTA		EDDS	
	Concentration (mg kg^{-1})							
	Zn	Cd	Zn	Cd	Zn	Cd	Zn	Cd
0	624 ± 70 a	34.8 ± 4 a	624 ± 70 a	34.8 ± 4 a	212 ± 20 a	24.5 ± 2 a	212 ± 20 a	24.5 ± 2 a
0.5	726 ± 22 a	47 ± 1.3 a	685 ± 96 a	36.6 ± 5 a	183 ± 28 a	17.5 ± 1.6 a	202 ± 33 a	22.5 ± 3.3 a
1	619 ± 61 a	42.6 ± 5.9 a	900 ± 200 b	46.6 ± 8 a	205 ± 17 a	24.3 ± 3.7 a	208 ± 3 a	24.3 ± 2.1 a
1.5	778 ± 120 a	55 ± 8.8 b	703 ± 76 b	39.4 ± 7.8 a	225 ± 25 a	24.2 ± 2.4 a	263 ± 17 a	20.8 ± 2.3 a
3	924 ± 75 b	63.2 ± 8 b	964 ± 68 b	44.7 ± 3.8 a	221 ± 41 a	29.4 ± 1 b	325 ± 67 b	35.6 ± 4.7 b
5	1050 ± 117 b	52.6 ± 8.2 b	1700 ± 83 c	55.4 ± 2.3 b	297 ± 20 b	26.3±1.6 b	383 ± 32 b	38.9 ± 7.9 b
	Phytoextraction (μg kg^{-1} soil)							
0	3470 ± 420 b	193 ± 20 a	3470 ± 420 a	193 ± 20 a	432 ± 56 b	50 ± 6 a	432 ± 56 a	50 ± 6 a
0.5	3300 ± 246 b	214 ± 26 a	2930 ± 314 a	156 ± 16 a	282 ± 34 a	27 ± 4 a	471 ± 68 a	52.6 ± 8 a
1	2800 ± 242 a	193 ± 24 a	3260 ± 372 a	169 ± 8 a	399 ± 48 a	47 ± 3.6 a	535 ± 64 a	62.6 ± 8 a
1.5	2830 ± 220 a	200 ± 30 a	2330 ± 278 a	131 ± 18 a	475 ± 76 b	51 ± 5.2 a	636 ± 92 a	50.5 ± 6 a
3	2570 ± 134 a	176 ± 18 a	2590 ± 280 a	120 ± 14 a	321 ± 40 a	42.8 ± 6 a	533 ± 72 a	58.3 ± 10 a
5	2820 ± 242 a	142 ± 12 a	5280 ± 692 b	172 ± 22 a	574 ± 62 b	50.9 ± 12 a	490 ± 54 a	49.8 ± 7.4 a

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 3.10 Effects of chelate treatments on the translocation of metals from roots to shoots in garland chrysanthemum 7 d after the treatment

Chelate application (mmol kg^{-1} soil)	EDTA				EDDS			
	Shoot to root ratio of metal concentration							
	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
0	0.08 ± 0.01a	0.04 ± 0.01a	0.83 ± 0.09a	0.39 ± 0.05b	0.08 ± 0.01a	0.04 ± 0.01a	0.83 ± 0.05a	0.39 ± 0.02a
0.5	0.14 ± 0.01a	0.10 ± 0.01a	0.97 ± 0.05a	0.42 ± 0.02b	0.14 ± 0.01a	0.04 ± 0.01a	0.93 ± 0.1a	0.50 ± 0.06b
1.0	0.11 ± 0.02a	0.11 ± 0.01a	0.60 ± 0.08a	0.24 ± 0.03a	0.24 ± 0.03a	0.06 ± 0.01a	1.04 ± 0.15a	0.37 ± 0.03a
1.5	0.20 ± 0.01a	0.22 ± 0.02b	0.90 ± 0.1a	0.36 ± 0.05b	0.31 ± 0.02a	0.05 ± 0.01a	1.0 ± 0.1a	0.36 ± 0.05a
3.0	0.34 ± 0.04b	0.47 ± 0.04c	1.06 ± 0.1b	0.36 ± 0.03b	0.60 ± 0.04b	0.49 ± 0.03b	1.62 ± 0.2b	0.32 ± 0.01a
5.0	0.53 ± 0.06c	0.67 ± 0.05c	1.19 ± 0.15b	0.42 ± 0.04b	2.17 ± 0.3c	2.92 ± 0.2c	2.57 ± 0.2c	0.51 ± 0.07b
	Metal absorbed by shoot/metal absorbed by entire plant (%)							
	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
0	29.4 ± 3.5a	16.6 ± 2.5a	81.7 ± 9a	67.8 ± 7.6b	29.4 ± 3a	16.6 ± 2a	81.7 ± 8.5a	67.8 ± 7.5a
0.5	34.5 ± 2.7a	28.2 ± 3b	78.7 ± 6.8a	61.4 ± 4.8b	30.1 ± 5.a1	10.1 ± 3a	74.6 ± 5.8a	61.3 ± 5.9a
1.0	33.6 ± 4.2a	33.9 ± 2.8b	73.2 ± 5.2a	52.4 ± 5.8a	39.7 ± 3.4a	14.8 ± 2.5a	74.4 ± 6.2a	50.7 ± 6.2a
1.5	48 ± 5.7b	50.8 ± 4.8c	80.9 ± 7.3a	63.3 ± 7.2b	50.5 ± 7.9b	14.6 ± 3a	76.9 ± 7.9a	54.3 ± 6.1a
3.0	67.4 ± 7.5c	74.4 ± 6.5d	86.6 ± 6.7a	68.6 ± 6.4b	73.5 ± 6.4c	69.3 ± 7.9b	88.3 ± 15b	59.8 ± 3.1a
5.0	76 ± 8c	79.9 ± 8.9d	87.6 ± 10a	71.4 ± 8.1b	95 ± 16c	96.2 ± 4.5c	95.7 ± 6.2b	81.6 ± 9.1b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 3.11 Effects of chelate treatments on the translocation of metals from roots to shoots in corn 7 d after the treatment

Chelate application (mmol kg^{-1} soil)	EDTA				EDDS			
	Shoot to root ratio of metal concentration							
	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
0	0.07 ± 0.01a	0.01 ± 0.01a	0.56 ± 0.02a	0.52 ± 0.03a	0.07 ± 0.01a	0.01 ± 0.01a	0.56 ± 0.03a	0.52 ± 0.01a
0.5	0.03 ± 0.01a	0.03 ± 0.01a	0.37 ± 0.04a	0.68 ± 0.06a	0.15 ± 0.02a	0.02 ± 0.01a	0.53 ± 0.04a	0.55 ± 0.06a
1.0	0.05 ± 0.02a	0.07 ± 0.03a	0.38 ± 0.05a	0.50 ± 0.04a	0.21 ± 0.04a	0.02 ± 0.01a	0.70 ± 0.02a	0.87 ± 0.07b
1.5	0.07 ± 0.03a	0.13 ± 0.02b	0.45 ± 0.3a	0.71 ± 0.08a	0.33 ± 0.05b	0.03 ± 0.01a	0.73 ± 0.04a	0.81 ± 0.04b
3.0	0.06 ± 0.02a	0.15 ± 0.01b	0.37 ± 0.5a	0.86 ± 0.06b	0.53 ± 0.02b	0.10 ± 0.02b	0.91 ± 0.01b	0.91 ± 0.06b
5.0	0.08 ± 0.01a	0.19 ± 0.02b	0.39 ± 0.02a	0.82 ± 0.06b	0.99 ± 0.2c	0.18 ± 0.02b	1.12 ± 0.1c	1.07 ± 0.1c
	Metal absorbed by shoot/metal absorbed by entire plant (%)							
	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
0	12.7 ± 1.5b	2.8 ± 0.5a	52.8 ± 6.5a	50.9 ± 5.6a	12.7 ± 2a	2.8 ± 0.3a	52.8 ± 7.3a	50.9 ± 6.0a
0.5	6.3 ± 0.9a	5.6 ± 0.3a	42.5 ± 4.9a	57.5 ± 6.5a	23 ± 3.5b	4.7 ± 0.5a	51.4 ± 6.1a	52.3 ± 4.9a
1.0	8.9 ± 1.2a	12.4 ± 1.1b	42.9 ± 3.1a	49.8 ± 5.9a	29.8 ± 4.2b	3.9 ± 0.2a	58.4 ± 5.7a	63.6 ± 6.6b
1.5	12.6 ± 1.7b	20.5 ± 1.4c	47.8 ± 5.2a	58.7 ± 6.5a	39.9 ± 4.7bc	6.1 ± 0.8a	59.3 ± 6.3a	61.8 ± 7.2b
3.0	10.7 ± 1.2b	23.5 ± 2.6c	42.5 ± 3.8a	63.3 ± 7.1b	51.5 ± 7.5c	16.3 ± 0.5b	64.6 ± 10ab	64.6 ± 8.9b
5.0	14.3 ± 2b	27.9 ± 3.7c	43.5 ± 5.6a	62.2 ± 4.5ab	66.5 ± 7c	26.8 ± 2.5c	69.1 ± 7.9b	68.1 ± 11b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

3.3.5 The Residual Effects of EDTA and EDDS on the Growth and Uptake of Metals by Corn

Usually, phytoremediation of the contaminated soils needs several successive croppings of plants. For an efficient phytoextraction with the application of chelates, the addition of the chelate in the first cropping should not produce or produce less negative effects on the following plants to ensure substantial amount of plant biomass established prior to the applications of chelates. To evaluate the residual and time effects of the application of EDDS and EDTA in soil, a second cropping with corn was conducted six months after the first crops were harvested. The growth of corn in the second cropping was strongly dependent on the chelate treatment in the first cropping experiment (Fig. 3.6). The yields of shoot DM showed a strong negative effect from the amount of the original EDTA application. The biomass of corn at the second harvest decreased significantly as the level of EDTA applied in the first cropping increased due to the toxicity of the residual chelate in soil. Grčman et al. (2001) also found that residual EDTA had a strong inhibitory effect on red clover. The concentration of Cu in the shoots of corn increased as the rate of application of EDTA increased in the first cropping (see Table 3.11). The highest concentration of Cu (200 mg kg^{-1} DW) in the shoots of the second corn cropping appeared in the 5 mmol kg^{-1} of EDTA treatment, which was 6.8-fold that of the control group. For Pb, a significant increase was found only in the treatments with 3 and 5 mmol kg^{-1} of EDTA compared with the control (Table 3.11). These results indicated that the EDTA-treated soil still had a significant ability to depress plant growth and enhance the concentrations of Cu and Pb in the shoots of corn six months

after the chelate treatment to soil.

For the plants grown on the EDDS-treated soils, no significant residual effects of the EDDS were found in the corn shoot DM and the concentrations of the four metals, regardless of the different rates of application (Table 3.11). These results indicated that there were relatively low concentrations of residual EDDS and metal-EDDS complexes in the soil. EDDS and metal-EDDS complexes could have been degraded before the seeds of corn were sown in the second cropping six months later. Vandevivere et al. (2001a) reported that the Ca-, Mg-, Cd-, Fe(III)-, Al-, Pb-, and Cr(III)-EDDS complexes biodegraded readily at an average rate of 0.3 mmol d^{-1}. The calculated half-life of EDDS in sludge-amended soil was 2.5 days (Jaworska et al., 1999). Meers et al. (2005) observed that the half-life of EDDS varied between 3.8 and 7.5 days when the dose that was applied ranged from 0.8 to 4 mmol kg^{-1} soil. However, the minimum observed effective half-life of EDTA was 36 d. When EDTA was applied at the rate of 4 mmol kg^{-1} to soil, no decrease on the mobilization of metals was observed 40 d after the application. Thus, compared with EDTA, EDDS not only has the advantage of being readily biodegradable and less toxic to fish, daphnia, and soil fungi (Jaworska et al., 1999; Grčman et al., 2003), it also poses less risk with respect to the leaching of metals to the groundwater and other surrounding environmental media.

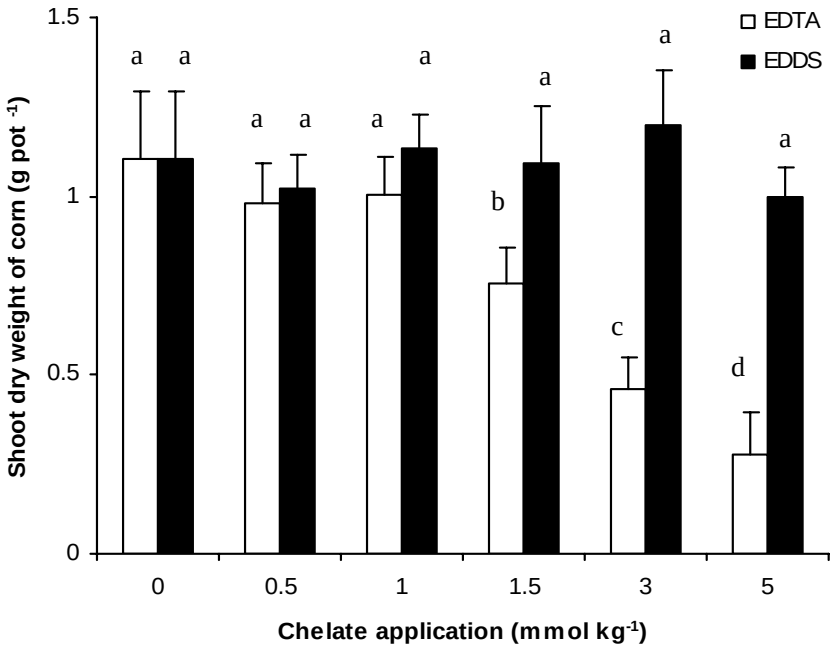


Fig. 3.6 The dry mass yields of corn grown in the second cropping. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 3.12 The concentration of metals in the shoots of corn grown in the second cropping

Chelates (mmol kg^{-1})	Metal concentrations treated by EDTA (mg kg^{-1})				Metal concentrations treated by EDDS (mg kg^{-1})			
	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
0	29.2 ± 1.44 d	4.7 ± 0.55 b	186 ± 35.1 a	10.6 ± 0.65 a	29.2 ± 1.44 a	4.7 ± 0.55 a	186 ± 35.1 a	10.6 ± 0.65 a
0.5	44.3 ± 5.6 d	2.3 ± 0.65 c	203 ± 11.3 a	9.48 ± 1 a	30 ± 1.06 a	1.12 ± 0.1 c	190 ± 19.6 a	10.7 ± 1.85 a
1.0	66.5 ± 8.9 c	3.43 ± 1.09 b	191 ± 25.7 b	7.96 ±1.59 b	32.9 ± 2.19 a	1.13 ± 0.18 c	192 ± 34.7 a	9.05 ± 0.58 a
1.5	76.1 ± 14.2 c	3.94 ± 0.59 b	133 ± 17.1 b	5.85 ± 0.94 b	32.3 ± 5.67 a	1.72 ± 0.57 b	182 ± 18.4 a	8.20 ± 1.35 b
3.0	127 ± 35.1 b	14.8 ± 2.76 a	139 ± 49.2 b	7.29 ± 2.28 b	35.8 ± 4.72 a	1.27 ± 0.26 b	186 ± 31.7 a	7.75 ± 0.79 b
5.0	200 ± 29.5 a	16.8 ± 3 a	133 ± 32.6 b	7.45 ± 1.85 b	39 ± 5 a	0.99 ± 0.49 c	150 ± 19.8 b	7.69 ± 0.59 b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

3.3.6 Effects of EDTA and EDDS on the Growth of Garland Chrysanthemum and Corn Grown in the Cu-Contaminated Soil

The dry mass yields of garland chrysanthemum and corn are shown in Fig. 3.7. The application of 3 mmol kg^{-1} of EDTA and EDDS appeared to be less toxic to the plants grown in the soil added with Cu at the rates of 200 and 400 mg kg^{-1} than to the plants grown in other soils, as shown by the significantly higher dry biomass in the two plant species. On the soil with no addition of Cu, the toxic effects on the plant growth may come from the toxicity of EDTA and EDDS. Vassil et al. (1998) suggested the free EDTA was usually more toxic to plants than EDTA-metal complex. Compared with EDTA, the addition of EDDS was much toxic to plant growth and more significantly depressed the dry mass yields of the two plant species (Fig. 3.7).

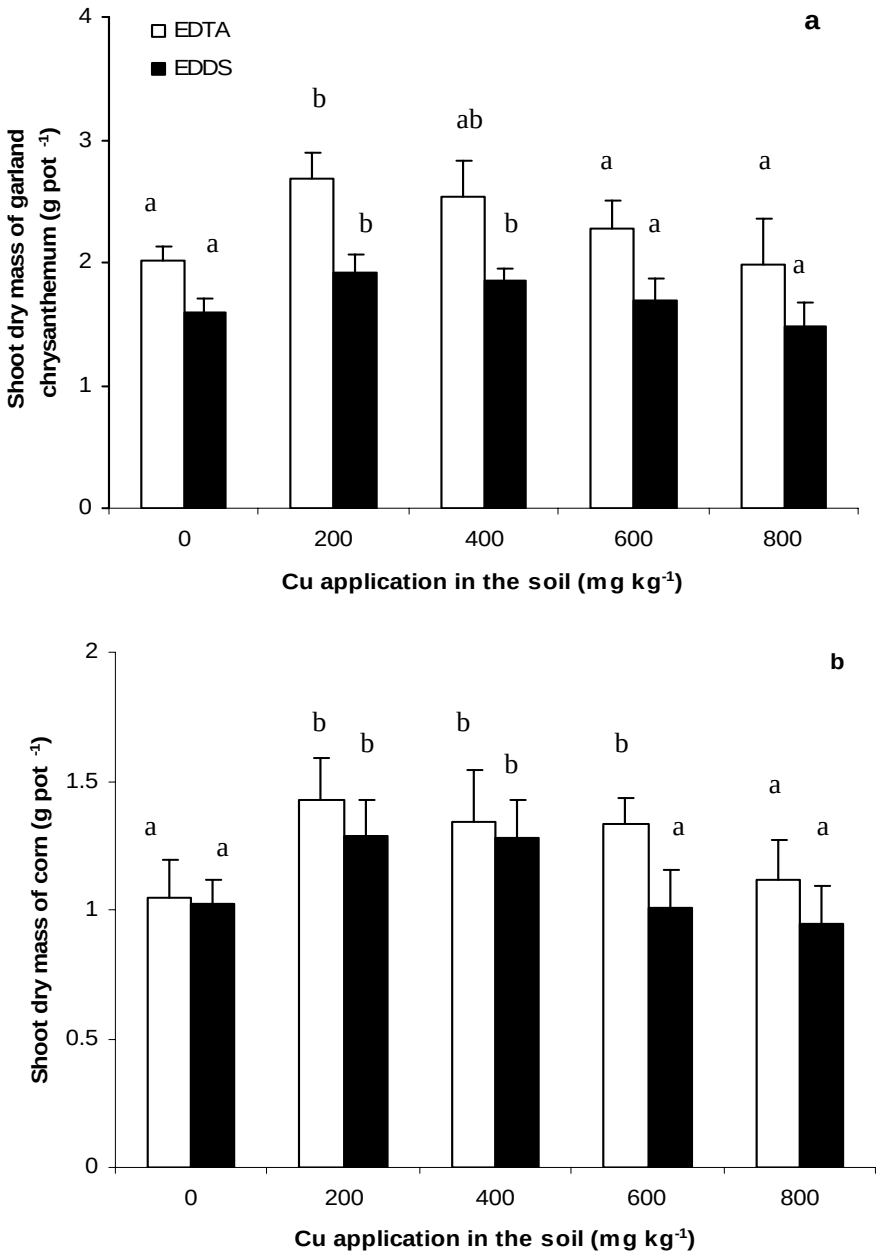


Fig. 3.7 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the dry matter yields of shoots in garland chrysanthemum (a) and corn (b) grown in different Cu-contaminated soil. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

3.3.7 Effects of EDTA and EDDS on Cu Concentration and Phytoextraction by Garland Chrysanthemum and Corn Grown in the Cu-contaminated Soil

The concentrations of Cu in the shoots of garland chrysanthemum and corn increased significantly with the increasing level of Cu added into the soils (Fig. 3.8). Compared with EDTA, EDDS was more effective at increasing the concentration of Cu in the shoots of the two species. Between the two species, a larger increase was observed in garland chrysanthemum than in corn. On Day 7 after the application of 3 mmol kg^{-1} of EDDS, the concentration of Cu in the shoots reached 1480 mg kg^{-1} DW in garland chrysanthemum grown in the soil added with 800 mg kg^{-1} Cu, which was 6.3-fold the amount found in the control group (without the application of Cu). The maximum concentration of Cu in the shoots of corn was 255 mg kg^{-1} DW, representing a 3.5-fold increase over that seen in the control group. The concentrations of Cu in the shoots of the two plant species were comparable to the data in the Fig 3.2 that was achieved in the plants grown in the multi-metal contaminated soils.

The total uptake of Cu in the shoots also increased with the rate of application of Cu to soils (Fig. 3.9). A higher level of Cu phytoextraction was always found in the EDDS treatment, in which with the application of 800 mg kg^{-1} of Cu in the soil, the phytoextraction of Cu was 5.8 and 3.3 times that of the controls for garland chrysanthemum and corn, respectively.

The distribution of metals in the shoots and roots of garland chrysanthemum and corn was also significantly affected by the application of Cu into the soil

(see Tables 3.12 and 3.13). With the application of EDTA and EDDS, the shoot-to-root ratios of the concentration of Cu increased with the increase of Cu concentration in the soil for the two plant species. EDDS was more effective than EDTA in stimulating the translocation of Cu from roots to shoots. As shown in Tables 3.12 and 3.13, when 3 mmol kg^{-1} of EDTA and EDDS were applied to the soil applied with 800 mg kg^{-1} of Cu, the mean percentage of absorbed Cu translocated from roots to shoots increased from 52.2% and 56.2% in the control groups to 96.5% and 98.7% for garland chrysanthemum, respectively, and from 8% and 33.8% to 11.4% and 45% for corn, respectively.

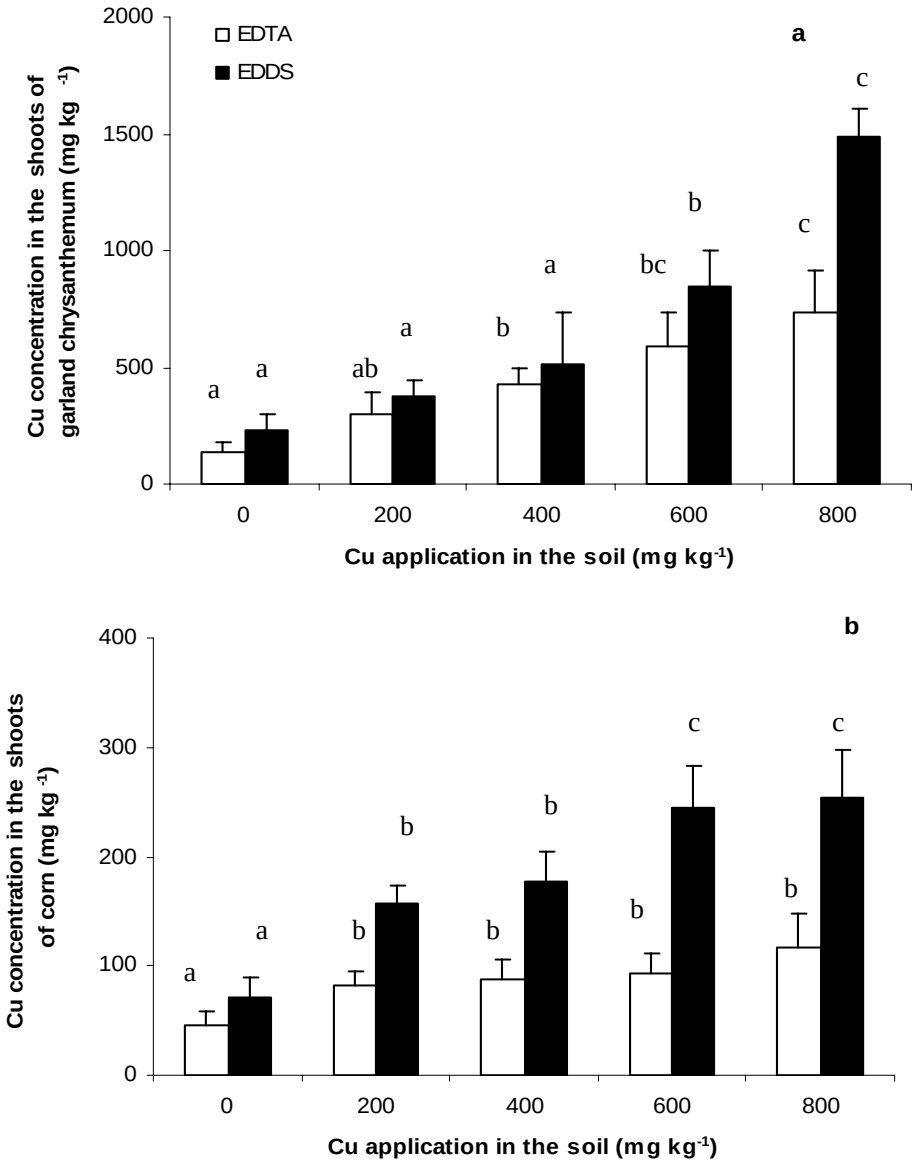


Fig. 3.8 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the concentration of Cu in the shoots of garland chrysanthemum (a) and corn (b) grown in different Cu-contaminated soil. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

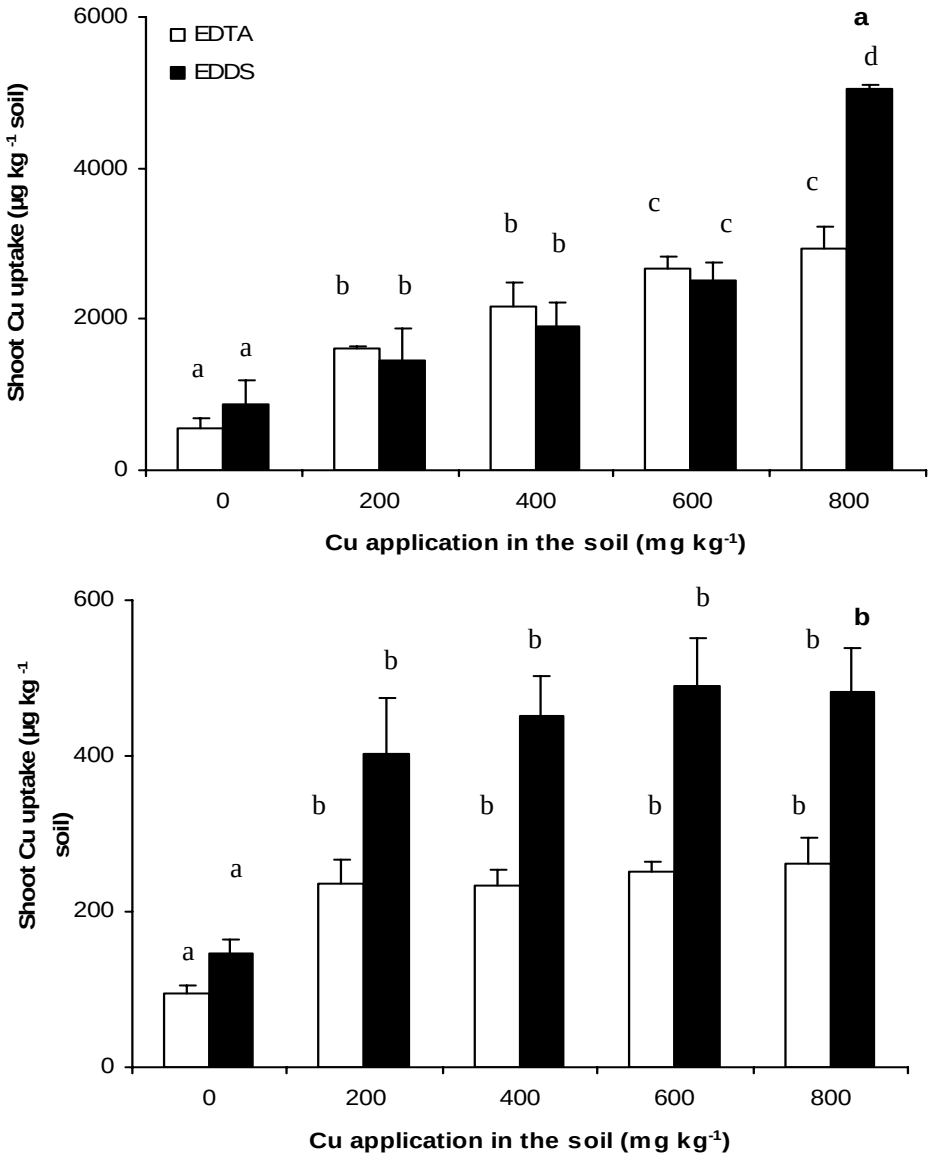


Fig. 3.9 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the phytoextration of Cu in the shoots of garland chrysanthemum (a) and corn (b) grown in different Cu-contaminated soil. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 3.13 Effects of EDTA and EDDS treatments at rate of 3 mmol kg^{-1} on Cu translocation from roots to shoots in garland chrysanthemum 7 d after the treatment

Cu application in soil (mg kg^{-1})	EDTA	EDDS
Shoot to root ratio of metal concentration		
0	0.5 ± 0.02a	0.6 ± 0.01a
200	0.6 ± 0.05a	0.7 ± 0.03a
400	1.4 ± 0.1b	1.5 ± 0.1a
600	2.4 ± 0.3c	3.5 ± 0.3b
800	3.6 ± 0.4d	12.2 ±1.5c
Metal absorbed by shoot/metal absorbed by entire plant (%)		
0	52.2 ± 6.8a	56.2 ± 5.3a
200	57.7 ± 7.8a	59.7± 7a
400	88.5 ± 9b	79.2 ± 8.5b
600	91.7 ± 4.9b	95.1 ± 7.6b
800	96.5 ± 11b	98.7 ± 10b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 3.14 Effects of EDTA and EDDS treatments at rate of 3 mmol kg^{-1} on Cu translocation from roots to shoots in corn 7 d after the treatment

Cu application in soil (mg kg^{-1})	EDTA	EDDS
Shoot to root ratio of metal concentration		
0	0.04 ± 0.01a	0.26 ±0.03a
200	0.05 ± 0.01a	0.27 ±0.04a
400	0.05 ± 0.01a	0.3 ± 0.01a
600	0.07 ± 0.01a	0.31 ± 0.02a
800	0.06 ± 0.01a	0.41 ± 0.04b
Metal absorbed by shoot/metal absorbed by entire plant (%)		
0	8 ± 0.6a	33.8 ± 2.7a
200	8.5 ± 0.7a	35.4 ± 4.6a
400	8.7 ± 0.9a	37.2 ± 4a
600	12.7 ± 1.5a	38 ± 3.2a
800	11.4 ± 1.5a	45 ± 5b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

3.3.8 The Residual Effects of EDTA and EDDS on the Growth and Uptake of Cu by Pea Grown in the Cu-contaminated Soil

Peas were grown in the soil six months after the first crop of corn was harvested in order to study the residual and time effects of the application of EDTA and EDDS to the soil. As shown in Fig. 3.10, in the EDTA treated soil, the peas grown in the soil added with 200 and 400 mg kg^{-1} of Cu produced the higher biomass than those grown in other soils, which was consistent to the trend shown in the first cropping of garland chrysanthemum and corn (Fig. 3.7). For the peas grown in the EDDS treated soil, the growth of peas was dependent on the Cu amounts added into the soil. The yields of shoot DM showed a strong negative effect from the amount of the Cu application into the soil (Fig. 3.10). The biomass of pea decreased as the level of Cu applied in soil increased.

The concentration of Cu in the shoots of peas increased as the rate of application of Cu to the soil increased (see Fig. 3.11a). In comparison with the pea grown in the EDDS treated soil, the plant grown in the EDTA treated soils produced significantly higher concentration of Cu in the shoots. The highest concentration of Cu (1280 mg kg^{-1} DW) in the shoots of peas appeared in the soil (with 800 mg kg^{-1} of Cu addition) treated with EDTA in the first cropping, which was 13.5-fold that of the control group.

Similar to the concentrations of Cu in the shoots, the total uptake of Cu in the pea increased with the increasing level of Cu applied to the soil. Plants grown in EDTA treated soil in the first cropping always produced higher phytoextraction

amounts than those grown in EDDS treated soil. The maximum phytoextraction of Cu was found in EDTA treated soils (with 800 mg kg^{-1} of Cu addition), which was 11-fold that of the control group.

The concentration of water-soluble Cu in soil was also examined immediately after the harvest of peas. In all soils, the concentrations of soluble Cu increased as increasing concentrations of Cu applied to the soil (see Fig. 3.12). Compared with the soil treated with EDDS, a significantly higher concentration of Cu was found in the EDTA treated soils. On the EDTA treated soils with the addition of 800 mg kg^{-1} Cu, the concentration of soluble Cu reached 107 mg kg^{-1}, which was 8.2-fold that of the control group and 3.6–fold that the corresponding soils treated with EDDS. As mentioned above, EDTA and some EDTA-metal complex were very recalcitrant to microbial degradation and they usually could persist in the environment a very long time (Bucheli-Witschel and Egli, 2001), which could explain the significantly higher soluble metals shown in the EDTA treated soils. EDDS is much easily biodegradable in soil. The reported calculated half-life was 2.5 d and no recalcitrant metabolites were found in the degradation profile of EDDS (Jaworska et al., 1999). The EDDS and metal-EDDS complexes probably have been degraded six months later. Therefore, the concentrations of soluble metals was reasonably dependent on the amounts of Cu added to the soil.

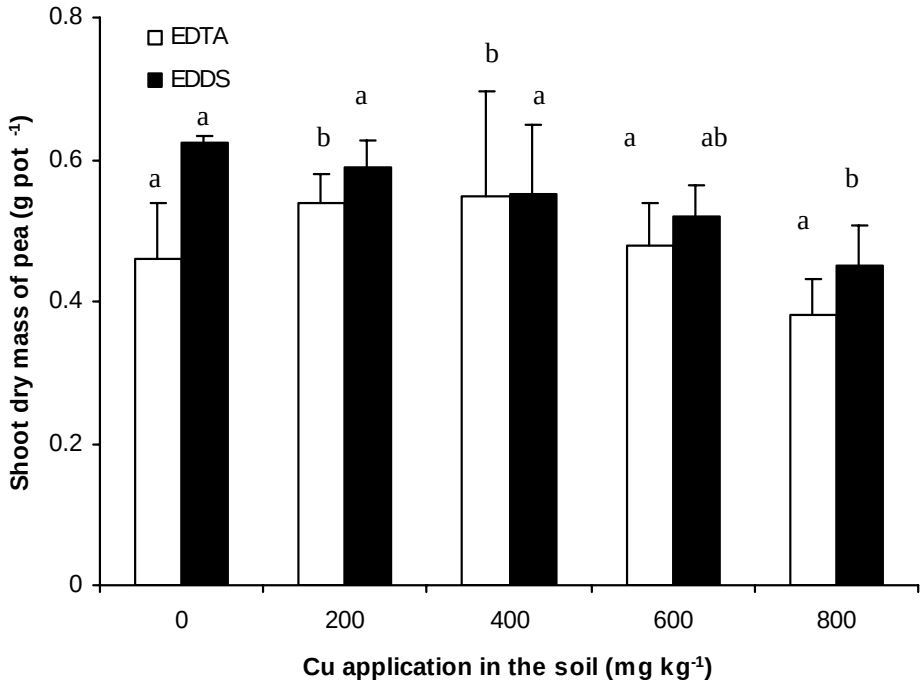


Fig. 3.10 The dry mass yields of pea grown in the second cropping. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

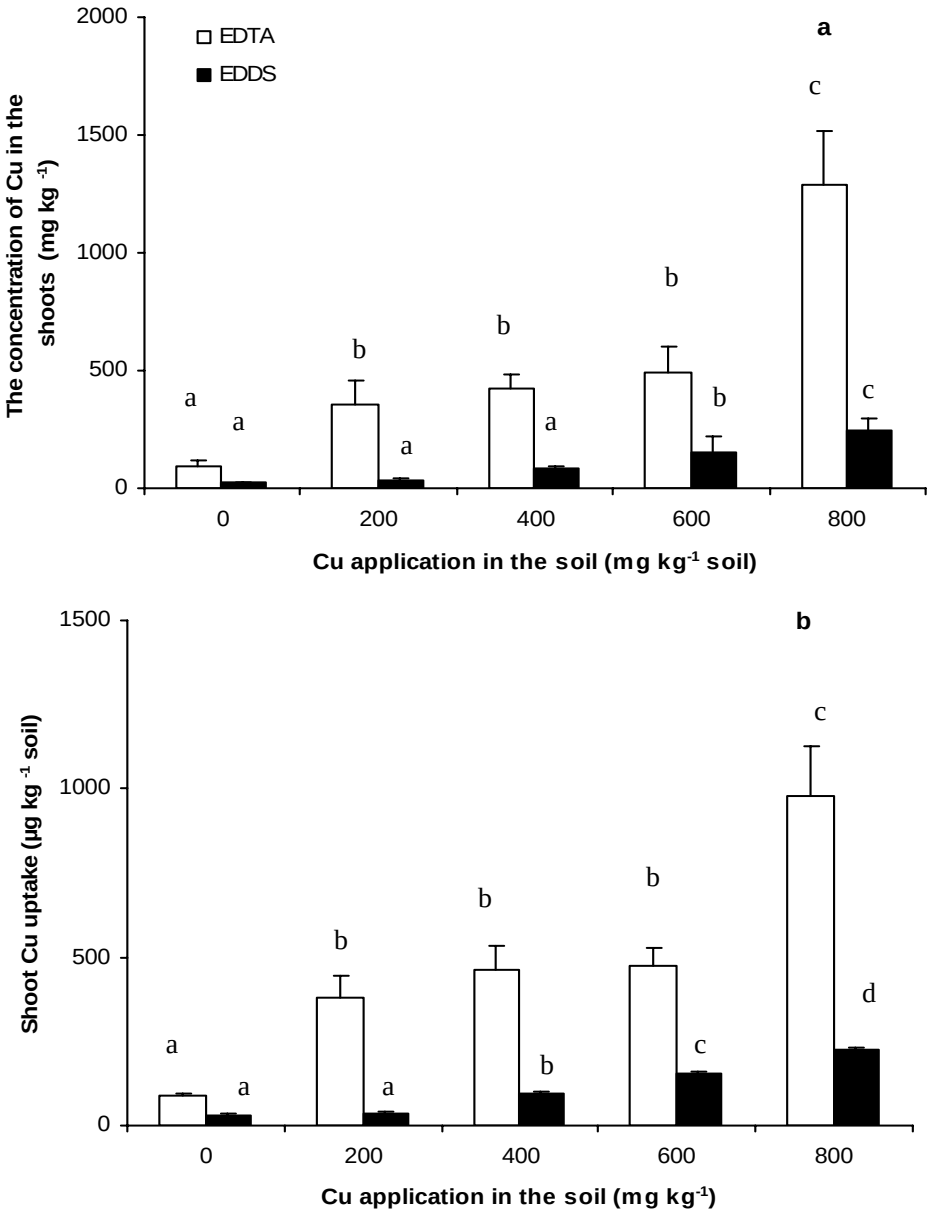


Fig. 3.11 The concentration (a) and the total uptake (b) of Cu in the shoots of pea grown in the second cropping. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

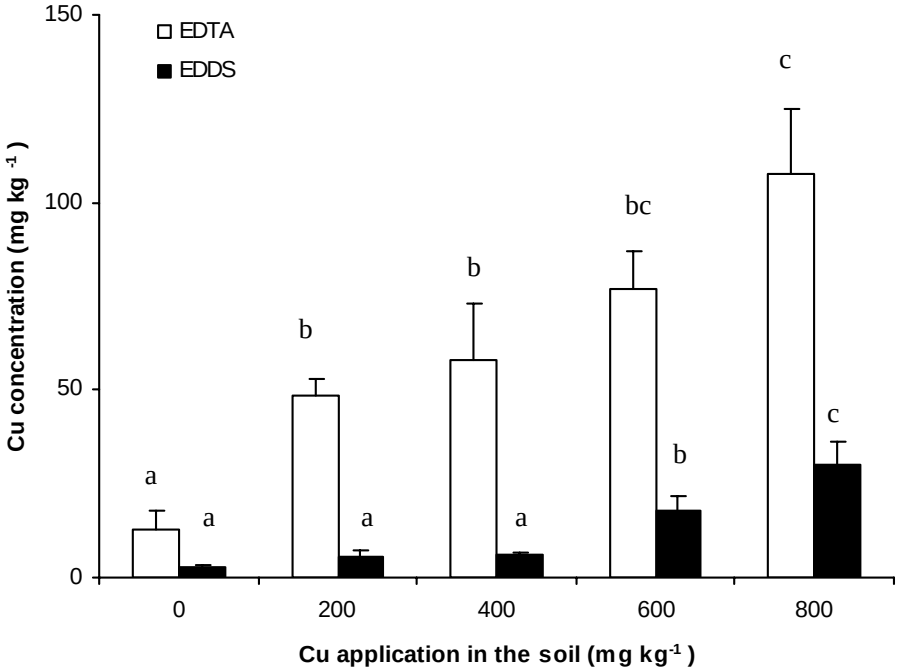


Fig. 3.12 Effects of the application of EDTA and EDDS at the rate of 3 mmol kg^{-1} on the solubilization of Cu in the different Cu-contaminated soil. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Chapter 4 - Selecting Chelates and Optimizing the Strategy of Chelates Application

4.1 Introduction

In the process of chelate-enhanced phytoremediation, besides the plant species, the chelate applied is another important factor governing metal extraction efficiency. EDTA is very effective in mobilizing metals in soils and facilitating metal uptake by plants. However, EDTA and EDTA-heavy metal complexes can be toxic to plants and soil microorganisms and they can be also persistent in the environment due to their low biodegradability (Bucheli-Witschel and Egli, 2001; Grčman et al., 2003). This nature may increase the potential off-site migration of metals, either in surface runoff or by the leaching of metals into groundwater (Nowack, 2002). To minimize the use of EDTA and the potential risk of the migration of solubilized metals into groundwater, further research is still needed.

In recent years, the use of some easily biodegradable chelating agents, such as NTA (nitrilotriacetate) and EDDS (S,S-ethylenediaminedisuccinic acid) has been proposed to enhance the uptake of heavy metals in soil phytoremediation (Kulli et al., 1999; Kayser et al., 2000; Grčman et al., 2003; Kos and Leštan, 2003 a, b; Chiu et al., 2005). However, the accumulation of Cu, Zn and Cd, in maize, Indian mustard and other plants only increased by a factor of 2 to 3, although the solubility of these metals in soil increased by a factor of 9 to 21 (Kayser et al., 2000). Kulli et al. (1999) reported that at the highest NTA

application rate (200 mmol pot^{-1} containing 7.5 kg soil), the concentrations of Cu, Zn and Cd in the aboveground plant biomass were 4- to 24-fold greater than in the control plants. But the total extraction of heavy metals was never more than 2.5 time more than that of the control due to the reduction in yields resulting from the application of NTA. EDDS at an application of 10 mmol kg^{-1} increased the concentrations of Pb, Zn and Cd in cabbage leaves 102-, 4.7- and 3.5-fold, respectively (Grčman et al., 2003). It was concluded that this procedure was far from effective, even at the highest concentrations of heavy metals achieved in the harvestable plant tissues.

The objectives of the present study were to (i) determine the relative effectiveness of EDTA, EDDS, and citric acid in enhancing the accumulation of Cu, Pb, Zn and Cd by monocotyledon of corn (*Zea mays* L.) and dicotyledon of beans (*Phaseolus vulgaris* L.); (ii) investigate whether amendments of biodegradable chelate EDDS or citric acid in combination with EDTA can further enhance the shoot uptake of metals by corn; (iii) compare the solubilzation of heavy metals in soil by various combined treatments of EDTA and EDDS.

4.2 Materials and Methods

4.2.1 Soil Characterization

Soil samples were collected from a disused agricultural field in the Yuen Long area of Hong Kong. The samples were sieved through a 2 mm sieve and air-dried for 3 d. The soils were artificially contaminated with Pb (2500 mg kg^{-1}

of soil) as $Pb_3(OH)_2(CO_3)_2$ (lead hydroxide carbonate) and PbS (a common lead sulfide in mining areas – galena) at a Pb concentration ratio of 1:1; Cu (500 mg kg^{-1} of soil) as $CuCO_3$ (copper carbonate); Zn (1000 mg kg^{-1} of soil) as $ZnCO_3$ (zinc carbonate) and ZnS (zinc sulfide) at a Zn concentration ratio of 1:1; and Cd (15 mg kg^{-1} of soil) with $Cd(NO_3)_2 \cdot 4H_2O$ (cadmium nitrate) (Chen et al., 2004b). The basal fertilizers applied to the soil were 80 mg P kg^{-1} of dry soil, and 100 mg K kg^{-1} of dry soil as KH_2PO_4 (Shen et al., 2002). After adding heavy metals and fertilizers, the soils were equilibrated for 15 d, undergoing five cycles of saturation with de-ionized water and air-drying. Soil parameters, such as electrical conductivity (EC), pH, cation exchangeable capacity (CEC), soil texture, organic matter content, total N and field capacity were measured in the same way as in Chapter 3. The total metal concentrations were determined by ICP-AES (Perkin-Elmer Optima 3300 DV) after strong acid digestion (1:4 concentrated HNO_3 and $HClO_4$ (v/v)) (Li et al., 2001).The selected physical and chemical properties of the soil are presented in Table 4.1.

4.2.2 Comparison of EDTA, EDDS and Citric Acid

Air-dried soils (500 g) were placed in plastic pots. Soil moisture was maintained to near field water capacity by adding DIW on a daily basis. Eight seeds of corn (*Z mays* L.cv. Nongda108) and beans (*P vulgaris* L. white bean) were sown in each pot. After germination, the seedlings were thinned to four plants per pot and grown for two weeks. The plants were allowed to grown 14 d before the application of chelates. The subsets of pots for each species were treated with 50 mL of 50 mM EDTA (as Na_2EDTA salt, from BDH Laboratory Supplies Poole, minimum assay: 99.5%), EDDS (as Na_3EDDS salt, from Fluka Chemie

GmbH) and citric acid (from BDH Laboratory Supplies Poole, minimum assay: 99.7%) in a single application to the surface of the soil at 5.0 mmol chelate per kg soil. Each treatment was replicated four times. All of the experiments were conducted in the greenhouse under natural light. Air temperatures ranged from 18 to 23 ºC. Two plants were harvested by cutting the shoots 0.5 cm above the surface of the soil, removing the roots from the pots 7 and 14 d after the application of chelates. The shoots and roots were washed with tap water and rinsed with DIW, and dried at 70 ºC in a drying oven to a constant weight for dry weight measurements. The dried plant materials were ground using agate mill.

4.2.3 The Combined Application of EDTA, EDDS and Citric Acid

Corn was chosen for the further study. In this part, the combined application of EDTA, EDDS and citric acid was studied. Eight seeds of corn (*Zea mays* L.cv. Nongda108) were sown in each pot. After germinating, the seedlings were thinned to four plants per pot and grown for two weeks. The chelates of EDTA (as Na_2EDTA salt), EDDS (as Na_3EDDS salt) and citric acid were applied as solutions to the surface of the soil as follows: a single application of EDTA, EDDS, and citric acid (5 mmol kg^{-1} soil); a combined application of EDTA and EDDS at a single dose (2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of EDDS); a combined application of EDTA and citric acid (2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of citric acid); and a combined application of EDDS and citric acid (2.5 mmol kg^{-1} of EDDS + 2.5 mmol kg^{-1} of citric acid). A control group without chelate treatment was also used in the experiment. Each treatment was

replicated four times. All experiments were conducted in the greenhouse under natural light. Air temperatures ranged from 18 to 23 ºC. The plants were harvested 7 and 14 d after the application of chelates, as mentioned above.

4.2.4 The Combined Application of EDTA and EDDS at Different Ratios

The moisture of the soil was maintained at near field water capacity by adding DIW on a daily basis. As mentioned above, the seedlings were thinned to four plants per pot and grown for two weeks before the treatment of chelates. EDTA (as Na_2EDTA salt) and EDDS (as Na_3EDDS salt) were applied to the surface of the soil in five different ways: a single application of EDTA and EDDS alone (5 mmol kg^{-1} of soil), an equimolar combined application of EDTA and EDDS (2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of EDDS), and a combined application of EDTA and EDDS at ratios of 1:2 (1.67 mmol kg^{-1} of EDTA + 3.33 mmol kg^{-1} of EDDS) and 2:1 (3.33 mmol kg^{-1} of EDTA + 1.67 mmol kg^{-1} of EDDS). A control group without chelate treatment was also used in the experiment. Each treatment was replicated four times. The plants were harvested 14 d after the application of chelates, as mentioned above.

4.2.5 Extracting Metals with Chelates

4.2.5.1 Extracting Metals with EDTA and EDDS within Different Time

For the soil metal dissolution experiment, 4.0 g of soil (based on dry weight) were placed in a 50-mL polypropylene centrifuge tube. 2 ml 10 mM chelates of

EDTA and EDDS were added to the soil samples, which corresponded to the total amount of chelate added (5 mmol kg^{-1} soil) in the pot experiments. After 0.5 d, 1 d, 2 d, 4 d, 6 d, 8 d, 10 d, 12 d and 14 d, DIW was added to the soil (at a soil-to-water ratio of 1:5) and the suspension was shaken for 30 min. After centrifugation, the supernatant will be filtered through a 0.45 µm paper filter (Whatman [Maidstone, UK] 42), digested with concentrated HNO_3 and analyzed for different metal concentrations by ICP-AES.

4.2.5.2 Extracting Metals with Combined Application of EDTA and EDDS at Different Ratios

For the experiment on the dissolution of metals in soils, 4.0 g of soil (based on dry weight) were placed in a 50-mL polypropylene centrifuge tube. 2 ml of 10 mM chelates of EDTA, EDDS, 1EDTA:1EDDS, 1EDTA:2EDDS, and 2EDTA:1EDDS were added to the soil samples, which corresponded to the total amount of chelates added (5 mmol kg^{-1} of soil) in pot experiments. After 2 d, DIW was added to the soil (at a 1:5 soil-to-water ratio) and the suspension was shaken for 30 min. After centrifugation, the supernatant was filtered through a 0.45 µm filter paper (Whatman No 42), digested with $HNO_{3,}$ and analyzed by ICP-AES for different concentrations of metals.

Soil was extracted with different EDTA and EDDS concentrations at 0.5, 1.0, 2.5, 5.0, 5.5, 6.0 and 7.5 mmol kg^{-1} of soil, a combined application of 5 mmol kg^{-1}g of soil EDTA and 0.5, 1.0, and 2.5 mmol kg^{-1} of soil EDDS. Chelates were added to a 2 ml solution. After 2 d, DIW was added to the soil (at a 1:5

soil-to-water ratio) and the suspension was shaken for 30 min. The filtration and chemical analysis were conducted as mentioned before.

4.2.6 Plant and Soil Analysis

Plant samples of ground shoots (200 mg) and roots (100 mg) and soil samples (250 mg) were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, by volume) and the major and trace elements in the solutions were determined with ICP-AES in the same way used in Chapter 3. The recovery rates were around 90 ± 6% for all of the metals in the plant reference material. The data reported in this part were the mean values based on the four replicated experiment results. Statistical analyses of the experimental data, such as correlation and significant differences, were performed using SPSS® 11.0 statistical software.

Table 4.1 The physicochemical properties of the soils used in the study

	Soils used in the study
pH ($CaCl_2$)	7.27
Electrical conductivity at 25°C ($\mu S\ cm^{-1}$)	282
Sand (%) > 0.05 mm	54.3
Silt (%) 0.05-0.001 mm	31.1
Clay (%) < 0.001 mm	14.6
N_{Total} (%)	0.10
Organic matter (%)	1.67
Cation exchange capacity (cmol kg^{-1})	3.29
Field water capacity (%)	27.4
Background total metal concentration (mg kg^{-1})	
Pb	44.2
Cu	26.7
Zn	131
Cd	0.45

4.3 Results and Discussion

4.3.1 Effects of EDTA, EDDS and Citric Acid on Plant Growth

The dry mass yields of corn and beans are shown in Figure 4.1. When no chelates were added to the soil, all of the plants showed normal development without visual symptoms of metal toxicity. The treatments with 5 mmol kg^{-1} soil EDTA and EDDS significantly affected plant growth (Fig. 4.1). The plants were strongly chlorotic and necrotic at the end of the experiment (14 d after the application of chelates), and root growth was severely impaired. The addition of EDTA appeared to be less toxic to both species of plants compared to EDDS. On the 14^{th} day after the application of EDTA and EDDS, shoot dry matter yields decreased to 60% and 52% of the control plants for corn, and 76% and 61% for beans, respectively. There were no significant effects from the application of citric acid on the dry-yield production of the two plant species.

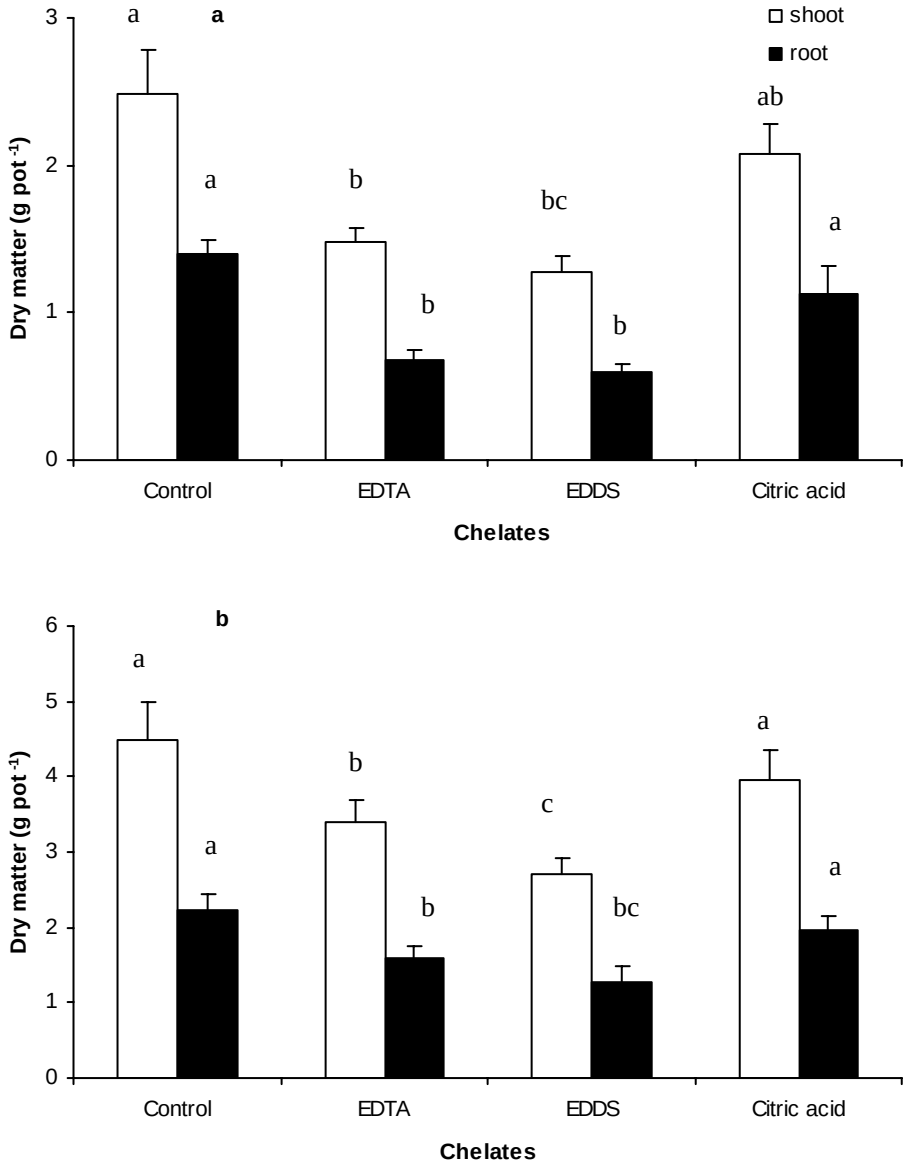


Fig 4.1 Effects of the application of chelates on the dry matter yields of shoots and roots in corn (a) and beans (b). Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

4.3.2 Effects of EDTA, EDDS and Citric Acid on Shoot Metal Concentrations and Phytoextraction

Chemically enhanced phytoextraction has been proposed as an effective approach for removing heavy metals from soils using plants (Huang et al., 1997; Blaylock et al., 1997; Liphadzi et al., 2003). Several chelating agents, such as citric acid, EDTA, CDTA, DTPA, EGTA, EDDHA, EDDS, HEDTA and NTA have been tested for their ability to mobilize and increase the accumulation of heavy metals, particularly Pb. In most cases, the EDTA treatment was superior in terms of solubilizing soil Pb for root uptake and translocation into above-ground biomass due to its strong chemical affinity for Pb (log K_s = 17.88). The results of this study demonstrated that EDTA was most effective in increasing shoot Pb concentrations in corn and beans (Table 4. 2). In the EDTA treatments, the concentrations of Pb in the shoots of corn and beans reached up to 270 and 487 mg kg^{-1} DW, which was 3 and 1.6 times higher than the levels achieved with the EDDS treatments. EDDS has a relatively lower chemical affinity for Pb (log K_s = 12.7) (Tandy et al., 2004). Compared with the control group, the addition of citric acid did not significantly increase the concentration of Pb in the shoots of corn and beans (Table 4.2). Citric acid has a low chemical affinity for Pb (log K_s = 6.5) and is easily biodegradable in soil (Römkens et al., 2002), which led to the lower effectiveness in increasing concentrations of Pb in shoots.

The addition of some chelating agents to the soil dramatically increased the solubility of Cu. These chelating agents included EDTA (Wu et al., 1999;

Lombi et al., 2001), EDDS (log Ks = 18.4, Tandy et al., 2004), EGTA (Römkens et al., 2002), HBED (Wu et al., 1999), and NTA (Kulli et al., 1999; Kayser et al., 2000). However, the chelate-enhanced Cu uptake by plant shoots was generally minimal (Kulli et al., 1999; Wu et al., 1999; Kayser et al., 2000; Lombi et al., 2001; Römkens et al., 2002) except for the result reported by Blaylock et al. (1997). In that study, a 2.5 mmol kg^{-1} EDTA treatment to the soil increased the concentration of Cu to 1000 mg kg^{-1} DW in *B. juncea* shoots. In the present study, the highest concentration of Cu reached 5130 mg kg^{-1} DW in bean shoots on the 14^{th} day after the application of 5 mmol kg^{-1} of EDDS to soil (Fig. 4.3). The effectiveness of EDDS in enhancing the accumulation of Cu was significantly higher than that of EDTA and citric acid (see Figs. 4.2 and 4.3). Compared with the control group, up to 22 and 81-fold increases in the extraction of Cu were found in the shoots of corn and beans on the 14^{th} day after the addition of 5 mmol kg^{-1} EDDS. Similar results were observed in several of our other experiments. The increased uptake of Cu by the application of EDDS in the present study was much higher than that of EDTA, reported previously (Lombi et al., 2001), and of NTA (Kulli et al., 1999; Kayser et al., 2000). The current results showed that the percentage of Cu phytoextracted in one phytoextraction cycle was 0.6-1.0% of the total Cu in the soil by corn and 1.9-5.3% by beans during a 30-day period of plant growth. These values were higher than the data reported by Kos and Leštan (2003b) for Pb extraction with EDDS and EDTA, and comparable with the results of Blaylock et al. (1997). Blaylock et al. (1997) reported that EDTA could enhance Pb uptake in *B. juncea* shoots (plant Pb = 15 000 mg kg^{-1}), and remove 60 kg Pb ha^{-1} in one harvest from soil containing 600 mg kg^{-1} of Pb (assuming 6 000 kg ha^{-1} dry weight per

crop). Based on their study, percentage of Pb extracted in one phytoextraction cycle was calculated to be 4.4% of the total Pb present in the soil (assuming 2250 tons ha^{-1} soils). For efficient soil remediation within a reasonable time span, shoot Pb concentrations exceeding 1% of dry biomass would be required to reduced soil Pb concentrations by 500 mg kg^{-1} over 20-25 years using plants with a high biomass yield (20 000 kg ha^{-1} of dry matter). Unlike Pb, Cu tends to have much higher bioavailability, and it is also more phytotoxic. Copper hyperaccumulators were reported in the literature, but there are still some doubts about their Cu uptake abilities (Baker et al., 2000). In the current study, assuming a constant efficiency of Cu removal, approximately 16 crops of beans with an EDDS application in a concentration of 5 mmol kg^{-1} would be required to reduce the total Cu in the soil from 527 to 100 mg kg^{-1} (P.R. China guidelines for agricultural soil). It appears that chemically enhanced phytoextraction could be an acceptable approach for the remediation of Cu-contaminated soils. The phytoextraction sum of four toxic elements (Cu, Pb, Zn and Cd) reached 8.92 and 40.2 mg metal kg^{-1} soil by corn and beans with the application of EDDS, which were 1.8- and 3.3-fold of the EDTA treatment, respectively (Figs. 4.2 and 4.3). This indicated that EDDS was superior to EDTA in the phytoremediation of contaminated soils with multiple heavy metals. In addition, EDDS has the advantage of being readily biodegradable and less toxic to fish, daphnia and soil fungi (Jaworska et al., 1999; Grčman et al., 2003). The calculated half-life of EDDS in sludge-amended soil was 2.5 days (Jaworska et al., 1999). This implies that residual EDDS in the soil will rapidly be degraded and pose little risk from the leaching of metals to groundwater. The results suggest that EDDS can be regarded as a good chelate candidate for the environmentally safe

phytoextraction of Cu and other metals in soils. Further research is needed to determine the most appropriate plant species and best methods of application before the chelate-assisted phytoextraction technique can be tested in the field.

Most of the increased uptake of Pb after the chelate treatments could be explained as an effect of enhanced Pb solubility (Wu et al., 1999; Kayser et al., 2000). It was reported that the accumulation of Pb in plant shoots correlated with the formation of the Pb-EDTA complex, and that Pb-EDTA was the major form of Pb absorbed and translocated by the plant (Vassil et al., 1998; Epstein et al., 1999). Metal chelate complexes may enter the root through breaks in the endodermis of the root and the Casparian strip, and be rapidly transported to the shoots (Römheld and Marschner, 1981; Bell et al., 1991). Also, it is likely that the physiological barriers in the roots might be destroyed due to the toxic effects of EDDS. Copper may enter the roots of the plant and be transported to the shoots as a Cu-EDDS complex. Kulli et al. (1999) found that a rather abrupt increase in Cu concentrations in the shoots of lettuce and ryegrass when the rate at which NTA, another biodegradable chelate, was applied increased from 70 to 200 mmol pot^{-1} (7.5 kg soil). Plant growth was badly damaged at the 200 mmol pot^{-1} NTA application level. It appears that only at very high Cu concentrations can the breakdown of exclusion mechanisms result in a greatly enhanced Cu uptake (Baker and Brooks, 1989). Wenger et al. (2003) reported that NTA increased the uptake and translocation of Cu into the shoots of tobacco. Neither growth reduction nor any other visible sign of Cu toxicity was found in the presence of 126 µM Cu and 500 µM NTA with a Cu concentration of 190 mg kg^{-1} in the shoots. It was hypothesized that the uptake of Cu-NTA complex by

tobacco occurs via an apoplastic pathway (a passive extracellular transport into the xylem).

The concentration of Zn in shoots was also higher in the plants treated with EDDS than in those treated with EDTA (see Table 4.2). For Cd, EDDS was significantly less effective than EDTA, although enhanced uptake was also observed. Concentration of Cd in the shoots of corn increased 1.5-fold, and in beans 1.5-fold, respectively, in comparison with those in the control group (see Table 4.2). On Day 14 after the application of chelates, the total amount of Zn extracted did not exceed 2.7 times that of the controls, but was always higher than the total extraction of Pb in all treatments of both plant species. Chelates were found to have a less significant stimulating effect on the Cd phytoextraction of these two plants (Fig. 4.3).

In their patent on the induced hyperaccumulation of metals in plant shoots, Ensley et al. (1999) described chemically enhanced phytoextraction as a two-step process. Plants first accumulate metals in their roots. An inducing agent is then applied, which enhances the transfer of the metals to the shoots. This transfer is attributed to a disruption of the plant's metabolism, which regulates the transport of metal to shoots. The results of this study indicate that in the control group, the concentrations of Cu, Pb, Zn and Cd in the roots of the two plant species were significantly higher than those in the shoots. Most of the Cu and Pb absorbed by the plants were concentrated in the roots. The application of EDTA and EDDS significantly increased the shoot-to-root ratios of the concentrations of Cu, Pb, Zn and Cd in both plant species. EDTA was

more effective than EDDS in stimulating the translocation of Pb from roots to shoots. But for the translocation of Cu and Zn, the most effective agent was EDDS. When 5 mmol kg^{-1} of EDTA was applied, the mean percentage of absorbed Pb translocated from the roots to the shoots of both species of plants increased from 3.5% in the control samples to 48% (Table 4.3). The application of EDDS rapidly and dramatically increased Cu concentrations in soil solutions as well as translocate Cu from the roots to the shoots of the two plant species tested. With 5 mmol kg^{-1} of EDDS treatments, up to 46- and 108-fold increases in the shoot-to-root ratios of Cu concentrations were found in corn and beans, respectively (Table 4.3). When 5 mmol kg^{-1} of EDDS was applied, the percentage of absorbed Cu translocated from roots to shoots increased from 8.5% to 83% and from 10.2% to 93% in corn and beans, respectively. Citric acid was less effective in increasing the translocation of metals from the roots to the shoots of the plants.

Table 4.2 Shoot metal concentrations in corn and beans grown in soil with and without the application of chelates (mg kg^{-1})

	Corn				Beans			
	Control	EDTA	EDDS	Citric acid	Control	EDTA	EDDS	Citric acid
				7 d after treatment				
Cu	56.8 ± 2.5a	428 ± 32b	1220 ± 129c	38.1 ± 6.2a	37 ± 3.1a	625 ± 36.4b	2230 ± 89c	50.2 ± 9.7a
Pb	8.9 ± 1.9a	257 ± 65c	67 ± 22b	13.7 ± 1.8a	8.2 ± 1.4a	411 ± 13.3c	293 ± 77b	28.4 ± 13a
Zn	395 ± 51a	778 ± 52b	1200 ± 93c	374 ± 84a	399 ± 36a	857 ± 62.3b	1440 ± 161c	459 ± 100a
Cd	7.8 ± 0.5a	19.8 ± 1.2b	14.9 ± 2.9b	5.4 ± 0.9a	3.7 ± 0.1a	8.8 ± 2.2b	4.7 ± 0.9a	3.2 ± 0.7a
				14 d after treatment				
Cu	45.6 ± 9.2a	560 ± 69b	2060 ± 272c	36.8 ± 1.3a	38.1 ± 1.8a	551 ± 4.5b	5130 ± 349c	51.5 ± 3.4a
Pb	10.1 ± 4.1a	270 ± 19.1c	90.9 ± 7.5b	5.8 ± 2a	7 ± 2.5a	487 ± 23c	298 ± 19b	6.4 ± 0.6a
Zn	580 ± 10.2a	851 ± 174b	1310 ± 148c	401 ± 58a	434 ± 41a	761 ± 74.8b	1950 ± 66c	437 ± 8.9a
Cd	9 ± 2.6a	29 ± 4b	13.7 ± 2.1a	5.2 ± 1.3a	3.1 ± 0.6a	10.1 ± 1.5c	4.6 ± 0.7b	2.8 ± 0.3a

Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

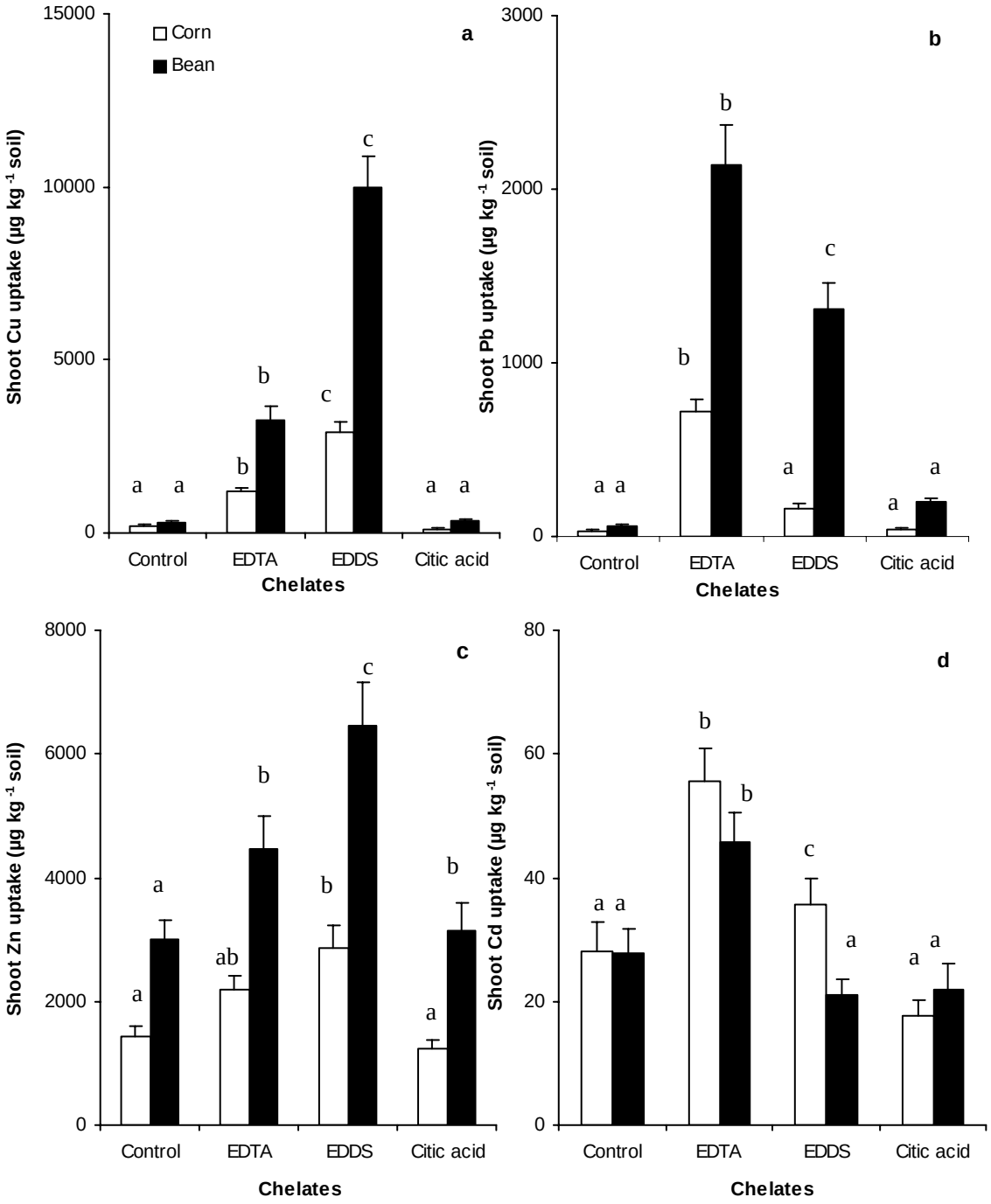


Fig. 4.2 Effects of the application of chelates on the uptake of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn and beans 7 d after the treatment. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

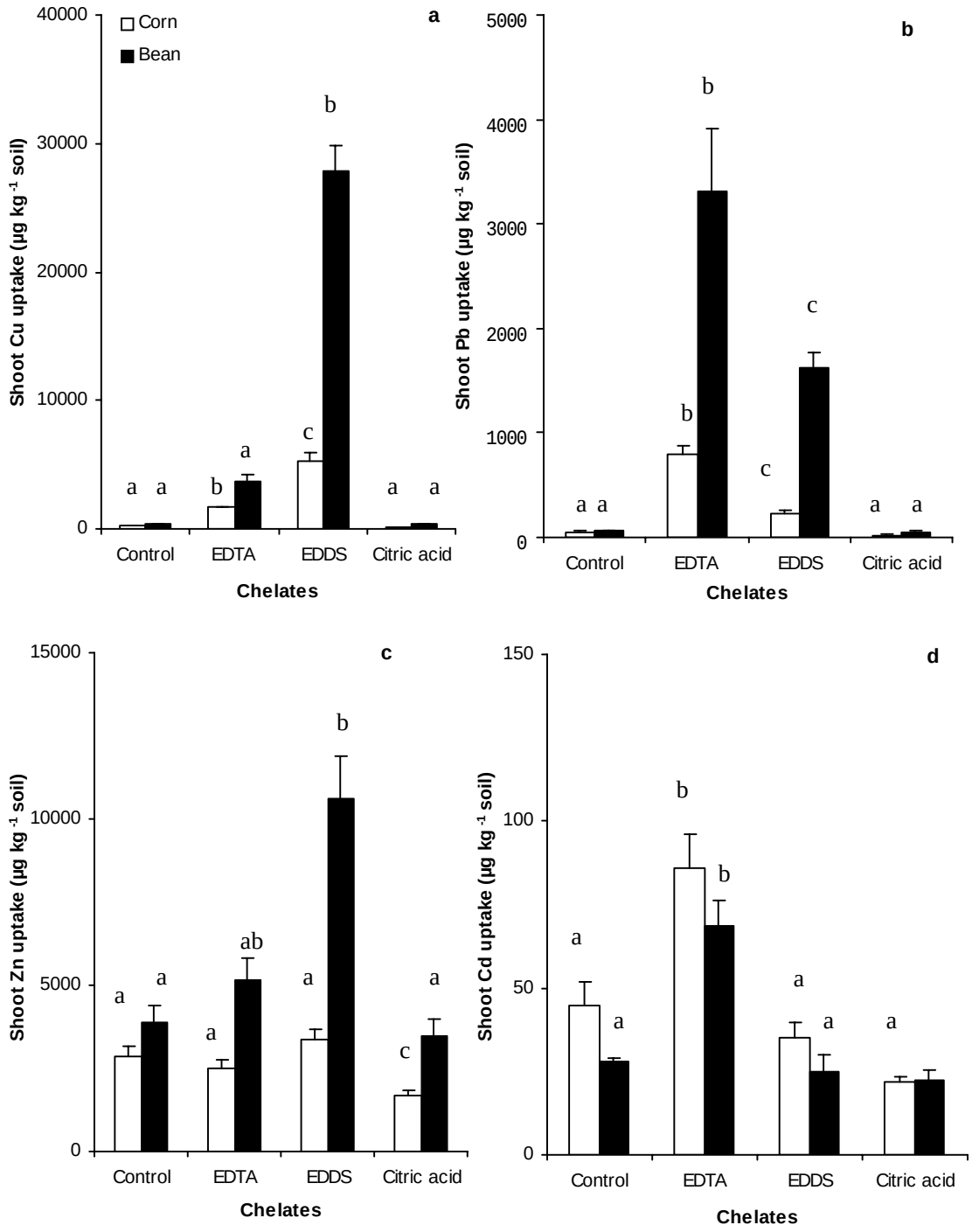


Fig. 4.3 Effects of the application of chelates on the uptake of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn and beans 14 d after the treatment. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 4.3 Effects of chelate treatments on the translocation of metals from the roots to the shoots of corn and beans 14 d after treatment

	Corn				Beans			
	Control	EDTA	EDDS	Citric acid	Control	EDTA	EDDS	Citric acid
				Shoot-to-root ratio of metal concentration				
Cu	0.05 ± 0.01a	0.3 ± 0.02b	2.3 ± 0.05c	0.06 ± 0.01a	0.06 ± 0.01a	0.49 ± 0.02a	6.5 ± 0.3b	0.07 ± 0.01a
Pb	0.03 ± 0.01a	0.41 ± 0.02b	0.08 ± 0.01a	0.03 ± 0.01a	0.007 ± 0.001a	0.45 ± 0.02c	0.12 ± 0.01b	0.008 ± 0.001a
Zn	0.5 ± 0.03a	0.6 ± 0.05a	0.97 ± 0.08b	0.55 ± 0.06a	0.12 ± 0.01a	0.3 ± 0.02b	0.81 ± 0.06c	0.13 ± 0.02a
Cd	0.39 ± 0.03a	0.74 ± 0.08b	0.54 ± 0.06b	0.33 ± 0.02a	0.02 ± 0.01a	0.1 ± 0.01b	0.04 ± 0.01a	0.03 ± 0.01a
				Metal absorbed by shoots/metal absorbed by the entire plant (%)				
Cu	8.5 ± 0.6a	39.8 ± 3.2b	82.8 ± 7.8c	10.6 ± 1.7a	10.2 ± 1.6a	50.9 ± 1.9b	93.2 ± 8.3c	12.3 ± 2.6a
Pb	5.5 ± 0.5a	47.4 ± 5.9c	14.6 ± 2.6b	4.9 ± 3.9a	1.5 ± 0.8a	48.7 ± 2.8c	20.8 ± 3.2b	1.7 ± 0.8a
Zn	46.9 ± 0.3a	56.7 ± 6.1a	67.4 ± 8.6b	50.7 ± 6.1a	19.5 ± 0.9a	39.0 ± 4.8b	63.4 ± 7.8c	20.4 ± 2.9a
Cd	41.2 ± 0.5a	61.8 ± 5.2b	53.3 ± 5.6b	37.7 ± 4.8a	3.3 ± 0.2a	18 ± 2.7c	7.9 ± 0.5b	4.9 ± 0.2a

Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

4.3.3 Effects of the Combined Application of Chelates on the Growth of Corn

The dry mass yields of corn are shown in Fig. 4.4. The treatments with 5 mmol kg^{-1} of EDTA and EDDS significantly depressed the growth of the plants. The addition of EDDS appeared to be more toxic to plants than the application of EDTA, as shown by a significantly lower biomass following the addition of EDDS. Citric acid at 5 mmol kg^{-1} had no significant effects on the dry yields of corn. Combined applications of 2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of citric acid and 2.5 mmol kg^{-1} of EDDS + 2.5 mmol kg^{-1} of citric acid depressed plant growth less than did 5 mmol kg^{-1} of EDTA or EDDS treatment alone, but more than 5 mmol kg^{-1} of citric acid treatment alone, due to the different toxicity of EDTA, EDDS, and citric acid (Fig. 4.4). Plants in the 2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of EDDS treated soil exhibited a slight decrease in biomass compared to those that had received a treatment of 5 mmol kg^{-1} of EDTA alone.

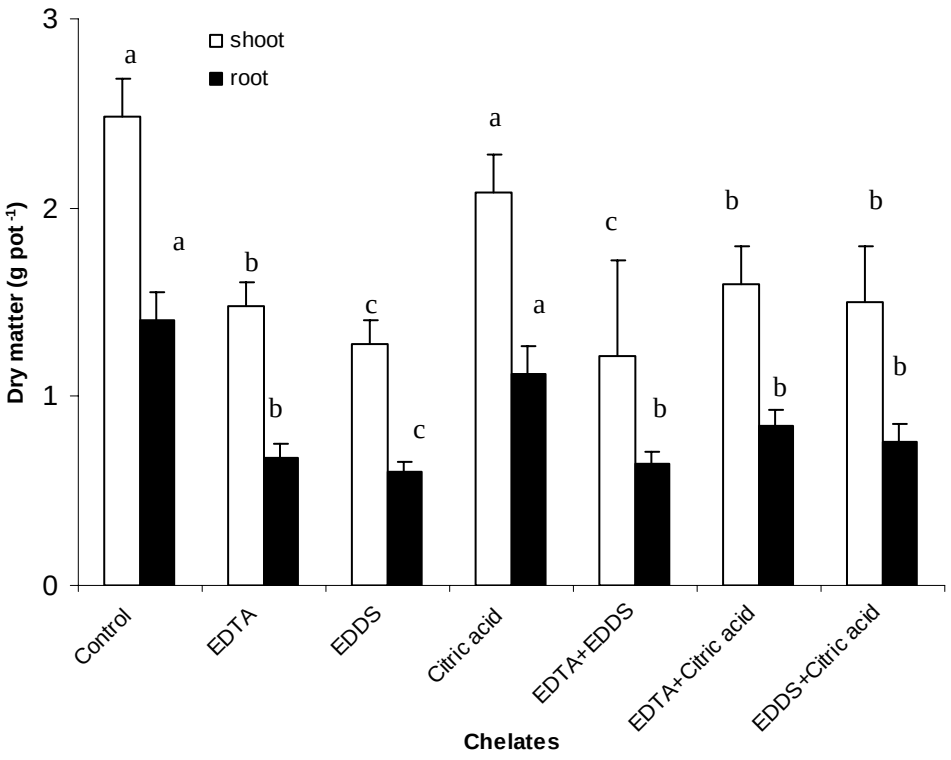


Fig. 4.4 Effects of the application of chelates on the dry matter yields of shoots and roots in corn 14 d after the treatment. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

4.3.4 Effects of the Combined Application of Chelates on Metal Concentrations and Phytoextraction in Corn

Based on the above results that EDTA was more efficient in metal uptake of Pb and Cd, and EDDS was more effective in facilitating the uptake of Cu and Zn (see Table 4.2, Figs. 4.2 and 4.3), in the present experiment, EDTA, EDDS and citric acid were combined together to study if the mixed application could further improve metal accumulation in the tested plants compared with the individual application of the three chelates. Results showed that in comparison with the control group, the addition of citric acid had no significant effect on the concentrations of heavy metals in the shoots of corn. The application of EDTA and EDDS at 5 mmol kg^{-1} to the soil significantly increased the concentrations of Cu, Pb, Zn, and Cd in the shoots of corn (Fig. 4.5). The concentrations of Cu, Pb, Zn, and Cd in the shoots of corn treated with 2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} citric acid were lower than in those that had been treated with EDTA alone, but much higher than those that had been treated with 5 mmol $kg^{-1}$of citric acid alone (Fig. 4.5). Similarly, the combined application of 2.5 mmol kg^{-1} of EDDS + 2.5 mmol kg^{-1} of citric acid produced lower concentrations of Cu, Pb, Zn, and Cd in the shoots than the 5 mmol kg^{-1} of EDDS treatment alone, and but the concentrations of metal in the shoots were higher than with the citric acid treatment alone. However, the combined application of 2.5 mmol kg^{-1} of EDTA and 2.5 mmol kg^{-1} of EDDS produced a significant increase in the concentration of Pb in shoots. The concentration of Pb reached 570 mg kg^{-1} DW, which was two and six times that of the 5 mmol kg^{-1} of EDTA and 5 mmol kg^{-1} of EDDS treatment alone, respectively, on the 14^{th} day after the application of

chelates. The concentration of Cd in corn shoots treated with 2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of EDDS was similar to that in shoots treated with 5 mmol kg^{-1} of EDTA alone ($P < 0.05$), but significantly higher than that with 5 mmol kg^{-1} of EDDS alone. For Cu and Zn, there were no significant differences in concentration in shoots treated with 2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of EDDS and those treated with 5 mmol kg^{-1} of EDDS alone ($P <0.05$), but the concentrations were significantly higher than with those treated with 5 mmol kg^{-1} of EDTA alone.

The total levels of phytoextraction of metals by the shoots of corn in this experiment are shown in Fig. 4.6. For Cu, the maximum phytoextraction was found in the EDDS and EDTA + EDDS treatments, which increased the phytoextraction by up to 24 times that of the control. For Pb, the plants treated with EDTA + EDDS reached maximum levels of phytoextraction of 28 and 1.7 times those of the control and EDTA treatment alone, respectively. For Zn and Cd, the total extracted amounts were less than twice those of the control.

The distribution of metals in the plants was also significantly affected by the application of chelates (see Table 4.4). The combined application of EDTA + EDDS was more efficient at enhancing the translocation of Pb from roots to shoots in comparison with EDTA and EDDS alone. When 2.5 mmol kg^{-1} of EDTA + 2.5 mmol kg^{-1} of EDDS were applied together, the percentage of Pb translocated from roots to shoots increased to 45.3% of the total amount of absorbed metals.

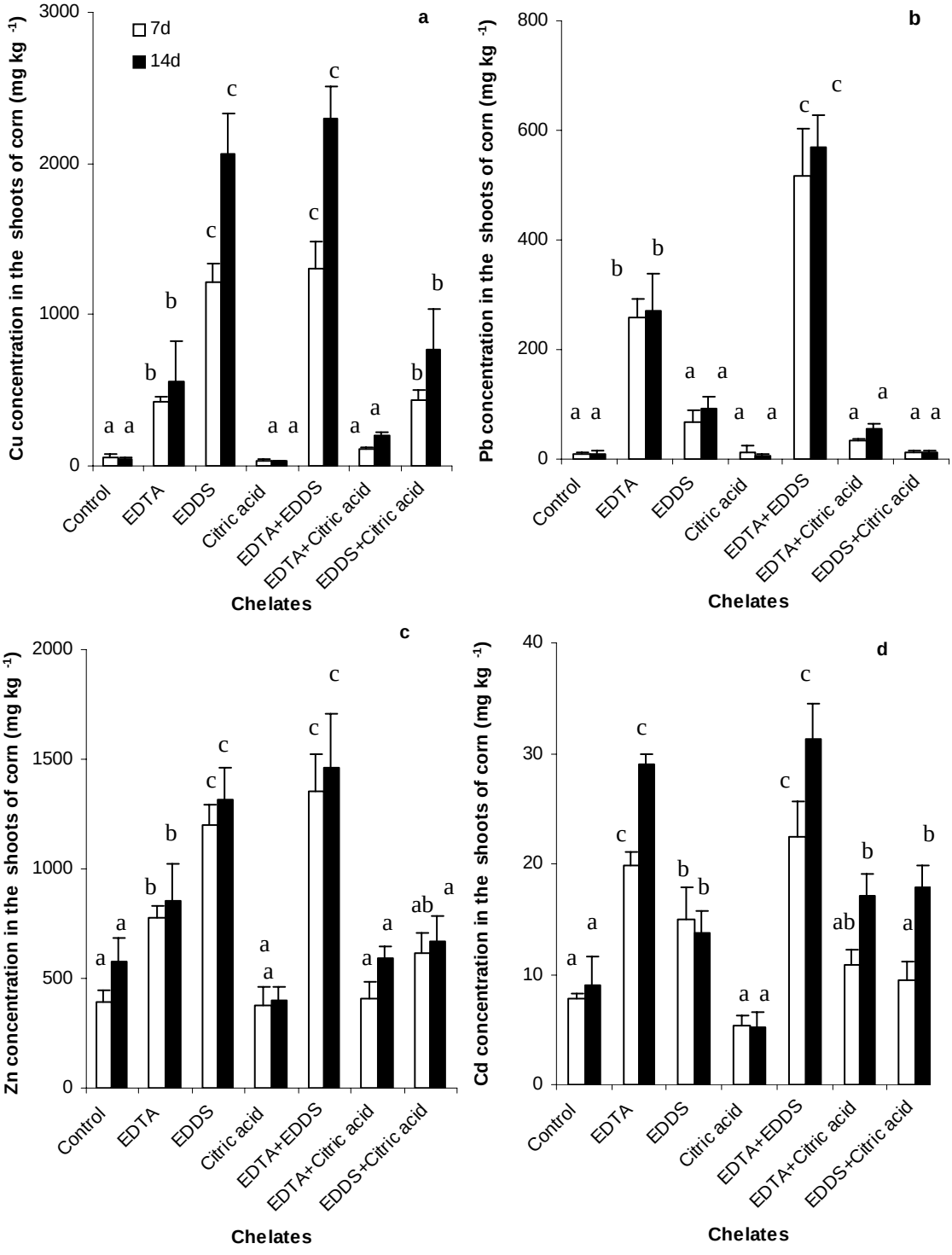


Fig. 4.5 Effects of the application of chelates on the concentrations of Cu (a), Pb (b), Zn (c), and Cd (d) in the shoots of corn. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

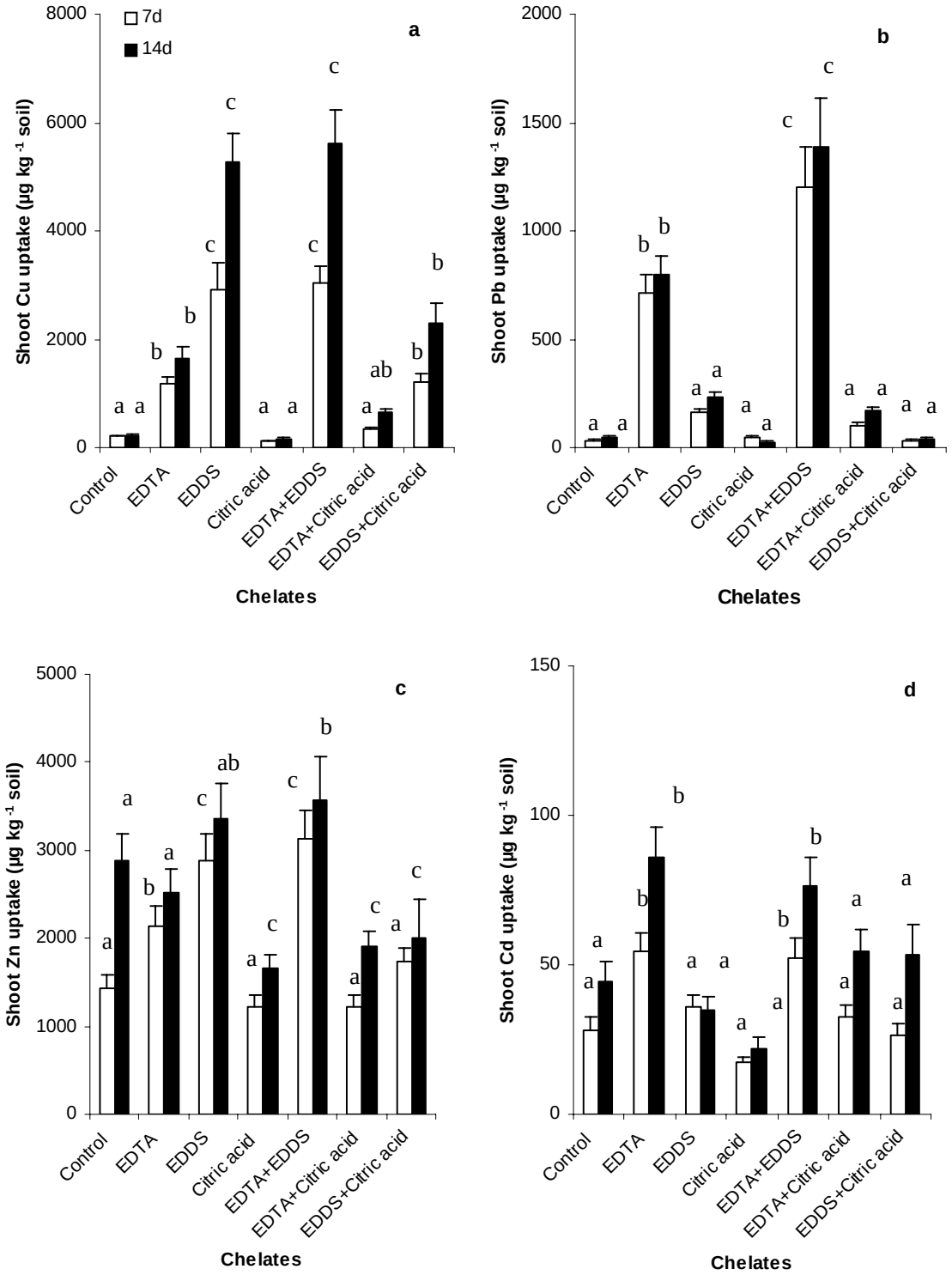


Fig. 4.6 Effects of the application of chelates on the uptake of Cu (a), Pb (b), Zn (c), and Cd (d) in the shoots of corn. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 4.4 Effects of chelate treatments on the translocation of metals from roots to shoots in corn 14 d after treatment

	Control	EDTA	EDDS	Citric acid	EDTA+EDDS	EDTA+Citric acid	EDDS+Citric acid
				Shoot/root quotient for metal concentration			
Cu	0.05 ± 0.01a	0.3 ± 0.02a	2.3 ± 0.05c	0.06 ± 0.01a	1.38 ± 0.09b	0.18 ± 0.01a	0.41 ± 0.05a
Pb	0.03 ± 0.01a	0.41 ± 0.02b	0.08 ± 0.01a	0.03 ± 0.01a	0.65 ± 0.03c	0.09 ± 0.01a	0.02 ± 0.01a
Zn	0.5 ± 0.03a	0.6 ± 0.05a	0.97 ± 0.08b	0.55 ± 0.06a	1.19 ± 0.21b	0.55 ± 0.06a	0.51 ± 0.07a
Cd	0.39 ± 0.03a	0.74 ± 0.08b	0.54 ± 0.06a	0.33 ± 0.02a	1.77 ± 0.1c	0.87 ± 0.06b	0.64 ± 0.04ab
				Metal absorbed by shoots/metal absorbed by the entire plant (%)			
Cu	8.5 ± 0.6a	39.8 ± 3.2b	82.8 ± 7.8c	10.6 ± 1.7a	72.5 ± 9.5c	25.9 ± 3.2b	45 ± 3.5b
Pb	5.5 ± 0.5a	40.4 ± 5.9c	14.6 ± 2.6b	4.9 ± 0.6a	45.3 ±5.6c	14.4 ± 2.1b	3.2 ± 0.5a
Zn	46.9 ± 0.3a	56.7 ± 6.1a	67.4 ± 8.6b	50.7 ± 6.1a	66.3 ± 9.1b	51.4 ± 7.5a	50.1 ± 4.9a
Cd	41.2 ± 0.5a	61.8 ± 5.2b	53.3 ± 5.6a	37.7 ± 4.8a	77.1 ± 8.9b	62.4 ± 9.8b	56 ± 8a

Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

4.3.5 Effects of the Combined Application of EDTA and EDDS at Different Ratios

Compared with the control group, the application of EDTA and EDDS at 5 mmol kg^{-1} to the soil significantly increased the concentrations of Cu, Pb, Zn, and Cd in the shoots of corn (Fig. 4.7). The interesting part of the current study was that a higher efficiency (or a synergy effect) in the phytoextraction of Pb could be achieved by replacing one-third of the EDTA used with an equal amount of EDDS compared with the treatment with 5 mmol kg^{-1} of EDTA alone, although EDDS was less efficient at enhancing the phytoextraction of Pb from the soil than EDTA when EDDS was applied alone (Figs. 4.2 and 4.3). The combined application of 3.33 mmol kg^{-1} of EDTA + 1.67 mmol kg^{-1} of EDDS produced 650 mg kg^{-1} of Pb in the shoots. The value was 2.4 and 5.9 times the concentration of Pb in corn shoots treated with 5 mmol kg^{-1} of EDTA and EDDS alone, respectively. The total phytoextraction of Pb reached 854 μg pot^{-1} (e.g. 1710 μg kg^{-1} soil), which was 2.1 and 6.1 times the total Pb from 5 mmol kg^{-1} of EDTA and EDDS alone, respectively. These results showed that the significantly enhanced phytoextraction of Pb by the combined application of EDTA and EDDS was not attributed to the condensing effects caused by the decrease in dry mass yields (Figs. 4.8 and 4.9). The effect was thought to be related to the decreased potential competition from other ions, and might also be caused by the change of soil pH. In addition, EDDS has the advantage because it is readily biodegradable and is less toxic to fish, daphnia, and soil fungi (Jaworska et al., 1999; Grčman et al., 2003). The calculated half-life of EDDS in sludge-amended soil was 2.5 d (Jaworska et al., 1999). This implies that

residual EDDS in the soil will rapidly be degraded and pose a relatively lower risk with respect to the leaching of metal into groundwater. The results suggest that the combined application of EDTA and EDDS, with less use of EDTA, can be regarded as a good alternative for the phytoextraction of Pb and other metals in soil, reducing the potential of Pb leaching into deep soil, groundwater, and surrounding sites.

For Cu and Zn, there were no significant differences in concentration in shoots treated with 3.33 mmol kg^{-1} of EDTA + 1.67 mmol kg^{-1} of EDDS and those treated with 5 mmol kg^{-1} of EDDS alone ($P < 0.05$), but the concentrations were significantly higher than those treated with 5 mmol kg^{-1} of EDTA alone. The concentration of Cd in corn shoots treated with 3.33 mmol kg^{-1} of EDTA + 1.67 mmol kg^{-1} of EDDS was similar to that in shoots treated with 5 mmol kg^{-1} of EDTA alone ($P < 0.05$), but significantly higher than that with 5 mmol kg^{-1} of EDDS alone. The maximum phytoextraction of Cu was found in the EDDS and 2EDTA:1EDDS treatment, which increased the phytoextraction by up to 42 times that of the control. For Zn and Cd, the total extracted amounts were less than twice those of the control (Fig. 4.9).

The distribution of metals in the plants was also significantly affected by the application of chelates (see Table 4.5). The combined application of EDTA + EDDS was more efficient at enhancing the translocation of Pb from roots to shoots in comparison with EDTA and EDDS alone. When EDTA and EDDS were applied at ratios of 1:1 and 2:1, respectively, the mean percentage of absorbed Pb that was translocated from roots to shoots increased from 5.2% in

the control group to 48% and 57%.

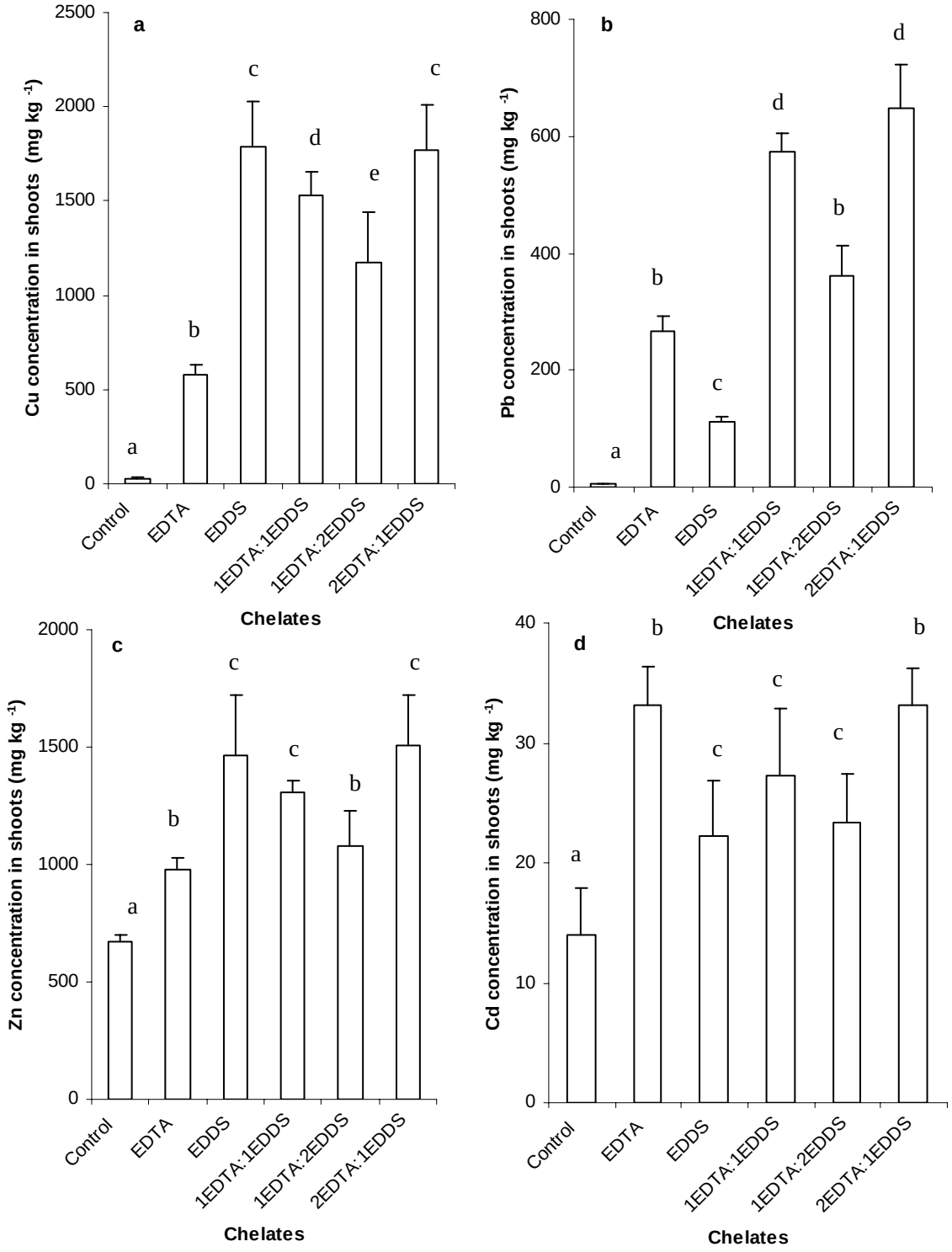


Fig. 4.7 Effects of the combined application of EDTA and EDDS at different ratios on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

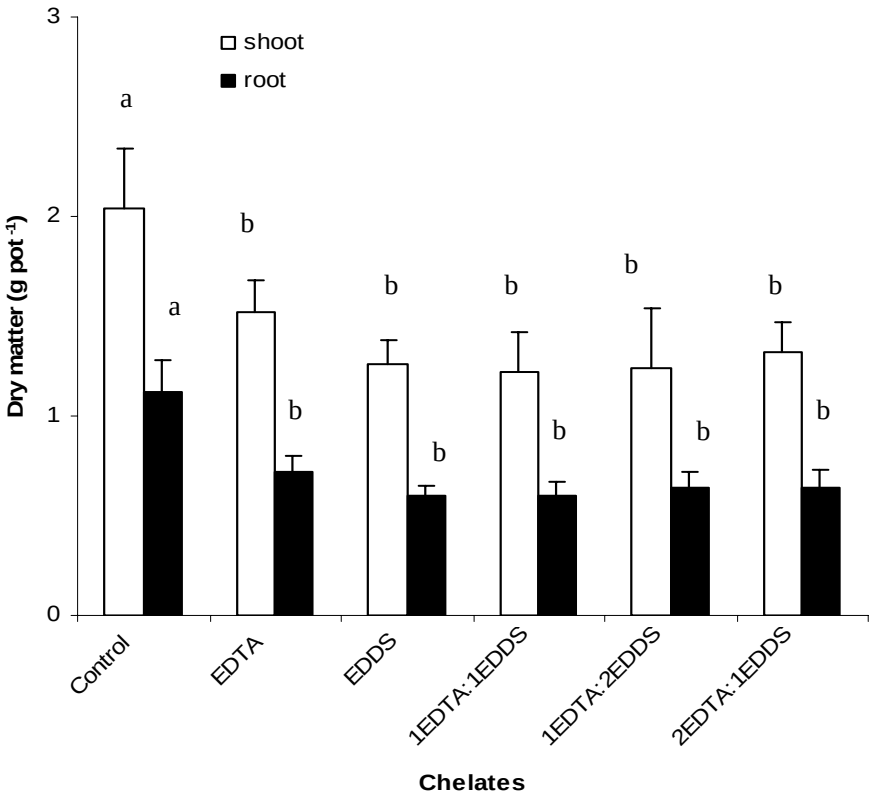


Fig. 4.8 Effects of the combined application of EDTA and EDDS at different ratios on the dry matter yields of shoots and roots in corn. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

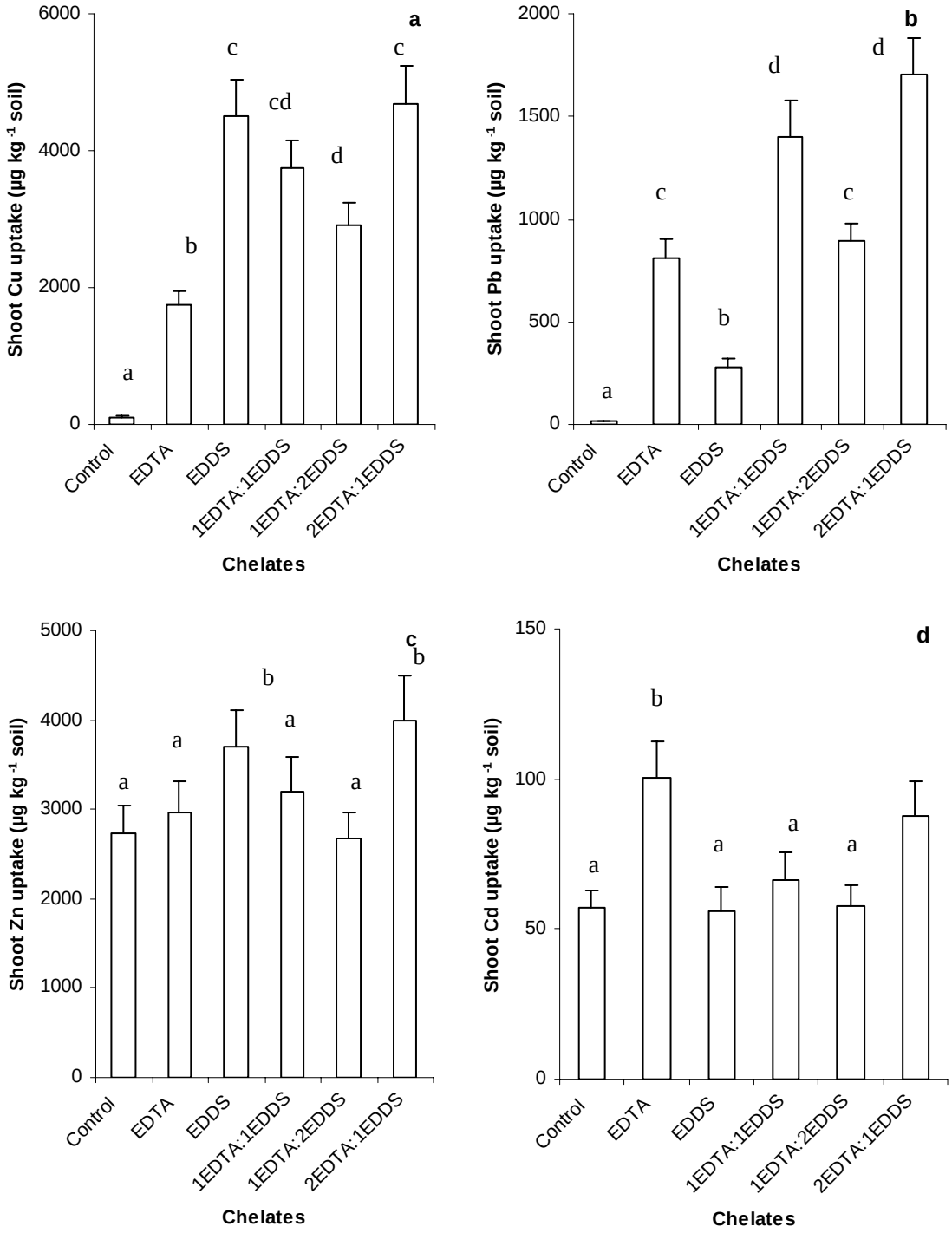


Fig. 4.9 Effects of the combined application of EDTA and EDDS at different ratios on the uptake of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 4.5 Effects of chelate treatments on Pb translocation from roots to shoots in corn 14 d after treatment

Control	EDTA	EDDS	1EDTA:1EDDS	1EDTA:2EDDS	2EDTA:1EDDS
Shoot/root quotient for metal concentration					
0.03±0.01a	0.33±0.04b	0.11±0.03a	0.46±0.05b	0.56±0.07bc	0.64±0.04c
Metal absorbed by shoots/metal absorbed by the entire plant (%)					
5.2±0.8a	40.9±5.1b	18.3±1.1a	48.4±5.6b	52±6.8b	56.9±7.3b

Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

4.3.6 Effects of EDTA and EDDS on the Solubility of Metals in Soil

The concentrations of water-soluble metals in soil were examined to assess the relative efficiency of EDTA and EDDS in enhancing metal solubilization from the soil. In all treatments, the concentrations of soluble Cu, Pb, Zn and Cd increased as increasing concentrations of EDTA and EDDS were applied (see Fig. 4.10). The addition of EDTA at 5 mmol kg^{-1} for 2 d significantly increased the concentrations of soluble Cu, Pb, Zn and Cd in soil, which were 102-, 496-, 5- and 114-fold higher than those in the control soil (see Fig. 4.10). After 2 d, the concentrations of soluble metals remained relatively constant over the period of the experiment, for up to 14 d. However, in the soil treated with EDDS, considerably different rates of solubilization for different metals were shown. EDDS was more effective in solubilizing soil Cu than Zn. Compared with the control, EDDS increased soluble Cu and Zn concentrations by a factor of 192 and 8, respectively. Within the first 8 d, concentrations of soluble Cu tended to increase as the time spent on the EDDS treatment increased, indicating that soil Cu could be solubilized by EDDS at a slower rate than by EDTA. However, the soluble Zn concentration reached a peak 2 d after the addition of EDDS, and then tended to decrease with time. For solubilizing soil Pb and Cd, EDDS was less effective than EDTA.

The effectiveness of chelate-enhanced metal accumulation was consistent with the ability of EDDS to solubilize soil metals (Figs. 4.2 and 4.3). It was suggested that EDDS is a better extractant for Cu and Zn than EDTA at pH values of above 6 with low chelate-metal ratios because it forms only a weak Ca

complex (Tandy et a., 2004). The comparatively low extraction efficiency of EDTA for Cu resulted from competition between the heavy metals and co-extracted Ca. The extraction of Pb at a low chelate-metal ratio seems to depend mainly on the stability constants of the Pb complexes, apart from the competition of Ca in the case of EDTA at high pHs. For Pb and Cd, the lower extraction efficiency of EDDS compared to EDTA may be attributed to the possible rapid biodegradability of EDDS. The biodegradation of chelate-metal complexes strongly depends on the type of metal involved and is not related to the stability constant of the chelate complex (Vandevivere et al., 2001b). For example, the biodegradability of the metal-EDDS complex decreased in the following order: Cd- > Pb- > Zn- > Cu-EDDS. Pb- and Cd-EDDS complexes biodegraded much more readily than Zn- and Cu-EDDS, although Pb- and Zn-EDDS complexes have practically the same stability constant. Cd-EDDS complexes were readily biodegraded as Ca-EDDS, although Ca-EDDS has a much greater stability constant.

Table 4.6 showed the addition of EDTA and EDDS at 5 mmol kg^{-1} for 2 d produced 41 and 1.1 mg kg^{-1} of soluble Pb in soil, respectively, which were 508 and 14 times higher than the levels found in the control soil. When EDTA and EDDS were applied together at ratios of 1:1, 1:2, and 2:1 of total 5 mmol kg^{-1}, the concentration of extracted Pb remained the same as with the 5 mmol kg^{-1} of EDTA treatment alone, although the total amount of EDTA applied was reduced by 50%, 67%, and 33%, respectively. EDDS was more effective than EDTA in solublizing Cu and Zn (see Table 4.6).

Some changes in soil conditions such as pH, total ligand, or superior ion concentrations may affect the chelating power of chelates (Jones and Williams, 2001). It was reported that the addition of free EDTA or the existence of other metal-EDTA complexes could result in a partial remobilization of adsorbed metals from the metal oxides and in the dissolution of minerals (iron and aluminium oxides) and remobilization of adsorbed metal (Nowack and Sigg, 1995; Vandevivere et al., 2001b). Tandy et al. (2004) reported that the extraction of Pb by EDTA seemed to depend mainly on the pH at the low chelate: metal ratio of 1, at which EDTA showed very strong ability to extract Pb up to pH 6. Above this, the efficiency of extraction declined by 50% because of the competition for EDTA from Ca. In the present study, however, Na_2EDTA salt and Na_3EDDS salt were used. A significant decrease in soil pH was not found in the combined treatment of EDTA + EDDS, compared to that of EDTA alone (Figs. 4.11c and 4.12c) ($P < 0.05$). Thus, the mechanism of enhancing the phytoextraction of Pb by the combined application of EDTA + EDDS does not involve a change in the pH of the soil. Wu et al. (1999) reported that there was no simple correlation between the amount of soluble Pb extracted and the binding constant of ligand with Pb. This could be related to the presence of other cations, in particular Ca^{2+}. In order to evaluate the additional effects of the combined application of EDTA and EDDS on the extraction of Pb, different methods of applying EDTA and EDDS to extract Pb and Ca within two days were studied. When the soil was extracted by EDTA or EDDS separately, the concentrations of Pb in the soil solution increased as concentrations of EDTA and EDDS increased (Fig. 4.11a). In extracting Ca, the application of EDTA and EDDS produced different effects. The concentration of Ca in the soil solution

increased as the concentration of EDTA increased, and decreased as the concentration of EDDS applied to the soil increased (Fig. 4.11b). When 5 mmol kg^{-1} of EDTA was applied, the increasing levels of EDDS increased the concentration of soluble Pb and decreased the concentration of soluble Ca in the soil (Figs. 4.12a and 4.12b). Competition between heavy metals and Ca has been shown to be an important factor for the extraction of metals with EDTA (Tandy et al., 2004).

Now, we turn back to the results of enhanced Pb phtoextraction by the replacement of one-third EDTA with equal amount of EDDS compared with the treatment with 5 mmol kg^{-1} of EDTA alone. The increase in the phytoextraction of Pb by corn shoots was more pronounced than the increase of Pb in the soil solution after the partial use of EDTA was replaced with EDDS. The combined application of EDTA + EDDS at the ratio of 2:1 increased the concentration of Pb in shoots and phytoextration by 2.4 and 2.1 times. The increased concentrations of soluble Pb in soil were only 1.2 times higher than the EDTA treatment alone (Figs. 4.7, 4.9 and Table 4.6). The shoot-to-root ratio of Pb concentration was two times higher with the application of EDTA + EDDS (2:1) than with the 5 mmol kg^{-1} of EDTA treatment alone (Table 4.5). The percentage of absorbed Pb translocated from roots to shoots increased from 41 % to 57 %. These results indicated that the major role of EDDS might be to increase the uptake and translocation of Pb from roots to shoots rather than to dissolve soil Pb. Vassil et al. (1998) found that the accumulation of Pb in shoots was correlated with the formation of a Pb-EDTA complex in the hydroponic solution and that Pb-EDTA was the major form of Pb taken up and translocated by the

plant. A similar mechanism held true for plants grown in Pb-contaminated soils amended with EDTA (Epstein et al., 1999). To “induce” the accumulation of high EDTA or Pb-EDTA in shoots, a threshold concentration of EDTA was required in the solution (Vassil et al. 1998). At this threshold concentration, EDTA destroyed the physiological barrier(s) in roots that normally functioned to control the uptake and translocation of metals. It has been suggested that synthetic chelates may induce the uptake and accumulation of metals by chelates by removing stabilizing Zn^{2+} and Ca^{2+} from the plasma membrane, which is thought to play a major role in forming the physiological barrier(s). In the present study, the reduction in the shoot biomass was more significant in the treatment with EDDS than with EDTA (Fig. 4.1). It is hypothesized that the physiological barriers in roots were destroyed due to the toxic effects of EDDS, and soil Pb was solubilized by forming soluble complexes with EDTA when EDTA and EDDS were combined and applied. As mentioned above, plants first accumulate metals in their roots and then, through the application of an inducing agent, the enhanced transfer of the metals to the shoots occurs (Ensley et al., 1999). It has been suggested that metal chelate complexes may enter the roots at breaks in the root endodermis and Casparian strip, and be rapidly transported to the shoots (Römheld and Marschner, 1981; Bell et al., 1991).

For environmental and cost considerations, the addition of chelates to soils should be minimized in the phytoextraction process. Some approaches that could help to minimize the amount of chelate used in soil have been explored (Wu et al., 1999; Shen et al. 2002). A significant increase in the uptake and translocation of Pb has been reported for corn transplanted into soil, then treated

with EDTA, in comparison with plants that were germinated and grown in Pb-contaminated soil to which EDTA was subsequently applied (Wu et al., 1999). Shen et al. (2002) found that the application of EDTA in three separate doses was better at enhancing the accumulation of Pb in cabbage shoots than methods in which EDTA was added in one or two doses. Our current results showed that the combined application of EDTA + EDDS could significantly increase the concentrations and uptake of Cu, Zn, Pb, and Cd in the shoots of corn than the application of single chelate, especially for the phytoextraction of Pb. This method may provide a more efficient approach to the phytoremediation of soils contaminated with multiple heavy metals. For soil contaminated by Cu and Zn, EDDS can be a better chelate because of its high extraction efficiency and better biodegradability (Meers et al., 2005).

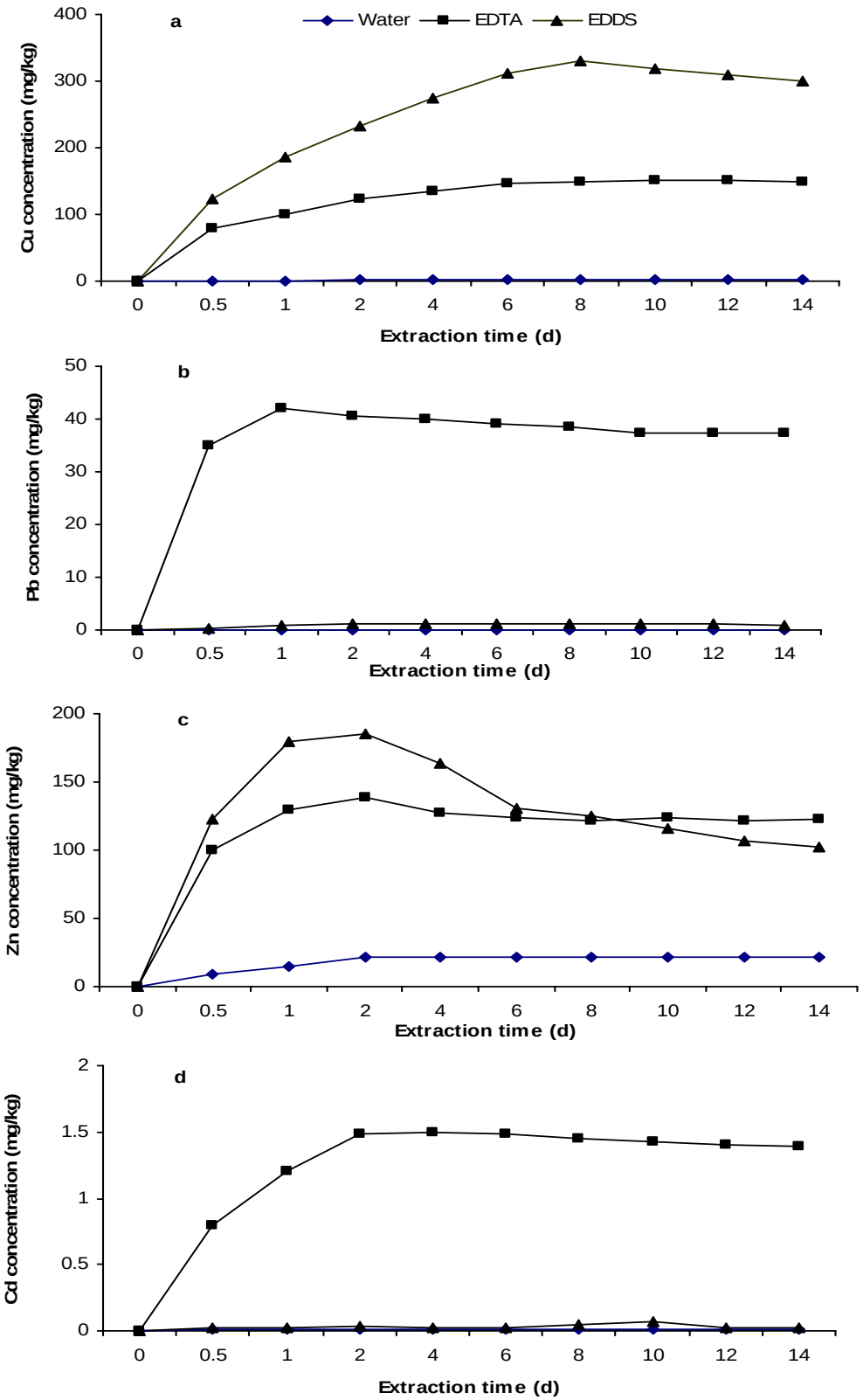


Fig. 4.10 Changes in soluble Cu (a), Pb (b), Zn (c) and Cd (d) concentrations in soil treated with chelates with time. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 4.6 Cu, Pb, Zn, Cd, and Ca concentrations (mg kg^{-1}) in soil solutions extracted with different chelates within two days

	Cu	Pb	Zn	Cd	Ca
Water	1.2 ± 0.2a	0.08 ± 0.002a	21.7 ± 3.2a	0.01 ± 0.001a	410 ± 35a
EDTA	124 ± 14b	40.6 ± 4.1b	139 ± 10b	1.49 ± 0.1b	412 ± 29a
EDDS	231 ± 21c	1.08 ± 0.01a	186 ± 15c	0.03 ± 0.003a	348 ± 41b
1EDTA:1EDDS	218 ± 16c	48.6 ± 3.2b	124 ± 10b	1.72 ± 0.1b	377 ± 32b
1EDTA:2EDDS	236 ± 22c	40.2 ± 3.1b	131 ± 13b	1.41 ± 0.2b	370 ± 31b
2EDTA:1EDDS	176 ± 12b	50.1 ± 4.6b	135 ± 14b	1.82 ± 0.1b	410 ± 45a

Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

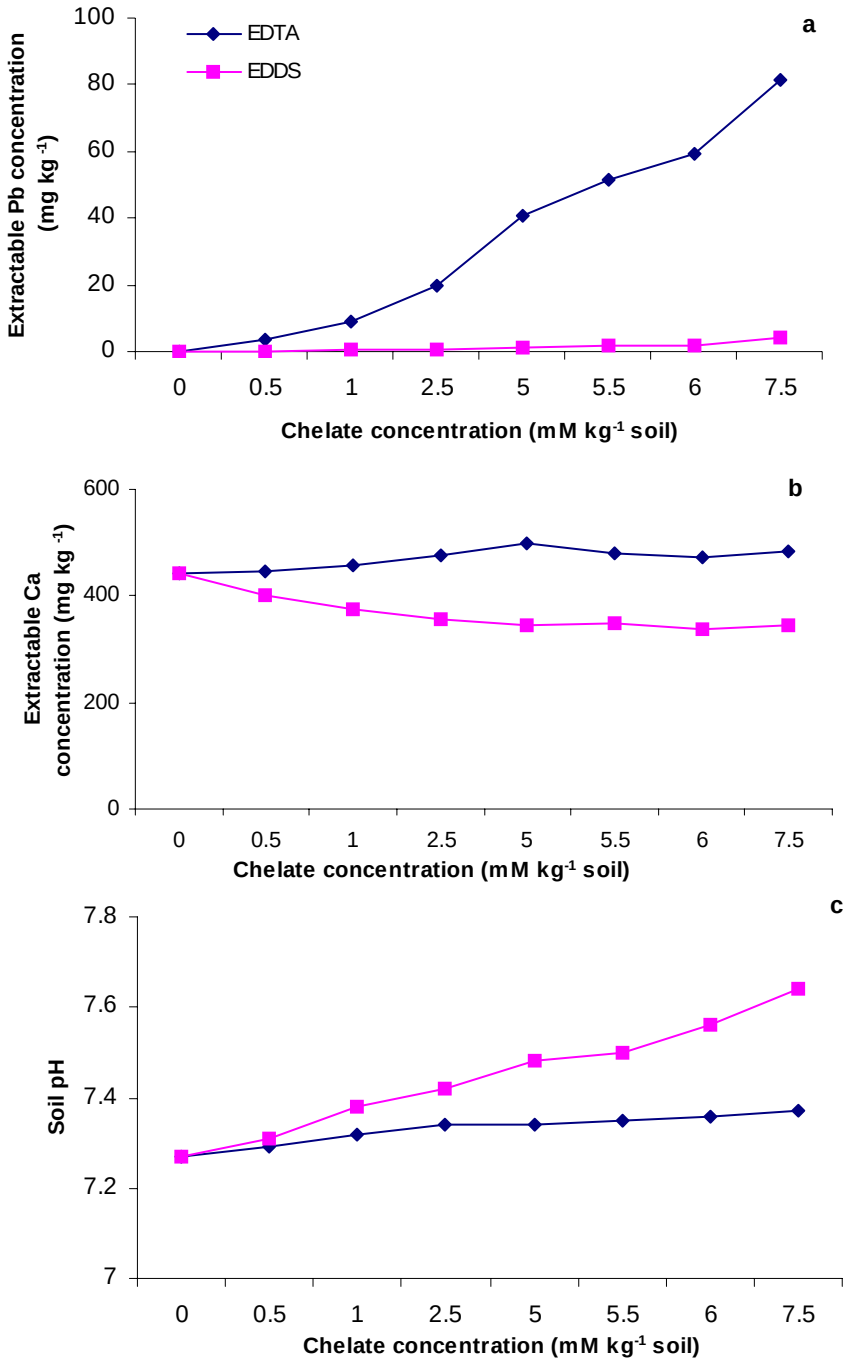


Fig. 4.11 Changes in the concentrations of soluble Pb (a), Ca (b), and pH (c) in soil treated with EDTA and EDDS at different concentrations. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

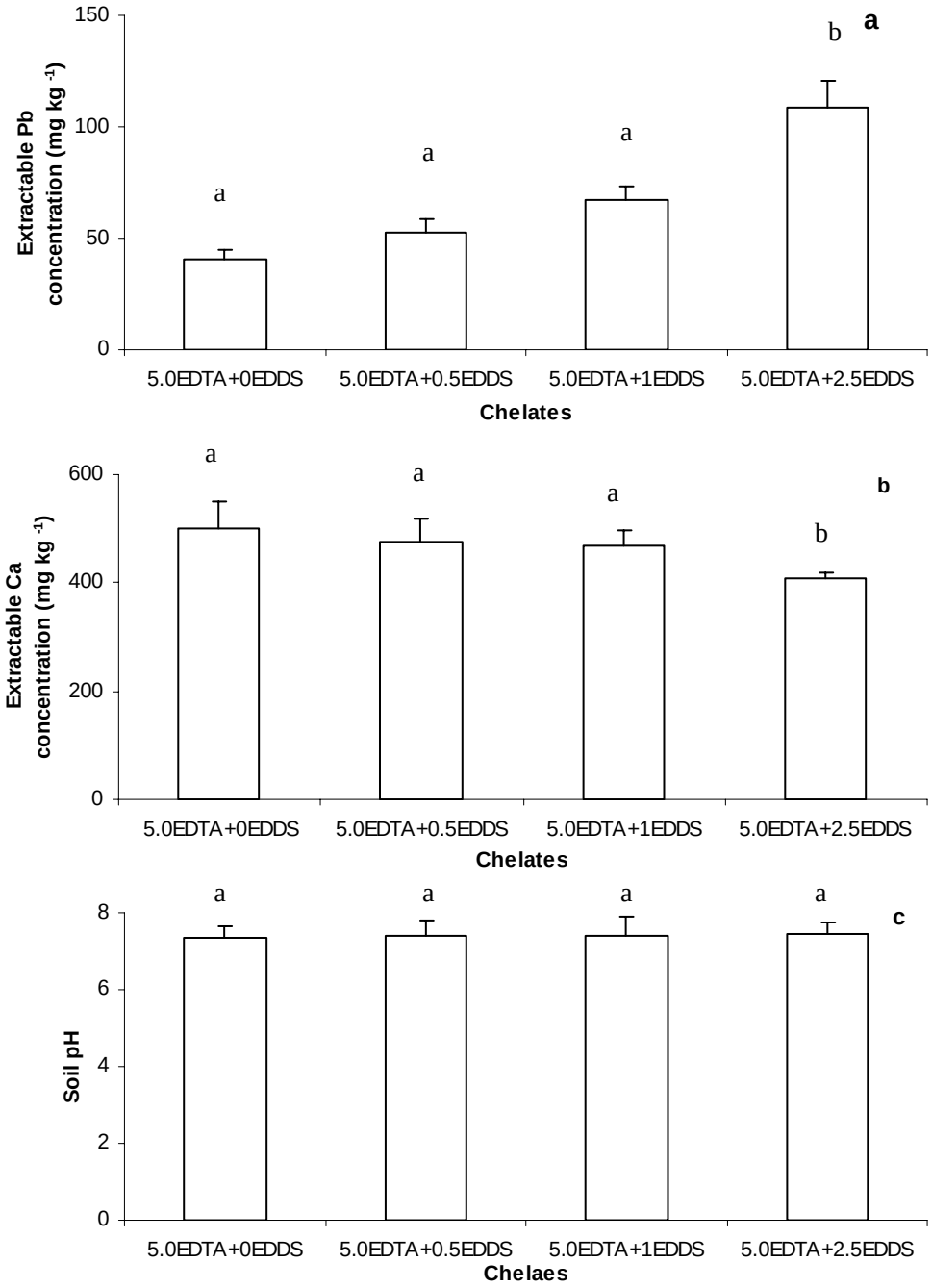


Fig. 4.12 Changes in the soluble concentrations of Pb (a), Ca (b), and pH (c) in soil treated with the combined application of EDTA and EDDS at different ratios. Values are means ± S.D. (n = 4); the different small letters stand for statistical significance at the $p < 0.05$ level.

Chapter 5 Exploring the Mechanism of Chelate-enhanced Phytoextraction and Optimizing the Combination of Plant and Chelate Application

5.1 Introduction

Synthetic chelates have been used to supply plants with micronutrients in both soils and hydroponics. Clarifying the mechanism underlying metal uptake by plants after the application of chelates will be helpful for us to explore some potential methods to improve the efficiency of phytoremdiation. The predominant theory for metal-chelate uptake is the split-uptake mechanism, by which only free metal ions are absorbed by plant roots (Chaney et al., 1972; Marschner et al., 1986; Shen et al., 1998). Fe-EDTA and Zn-EDTA are known to dissociate before plant uptake (Marschner et al., 1986; Sarret et al., 2001). Another theory that has been suggested is that some of the purportedly intact metal chelates are taken up by plants (Wallace, 1983; Bell et al., 1991; Laurie et al., 1991; Salt et al., 1995). Several studies of the accumulation of Pb in plants showed that the metal was absorbed and transferred as a Pb-EDTA complex (Vassil et al., 1998; Epstein et al., 1999). Sarret et al. (2001) reported that both Pb and EDTA could be absorbed by plants, and that some of the Pb present in the leaves of *P. vulgaris* was complexed to EDTA. The complexes of Pb-EDTA can not split through the reduction or oxidation of Pb. Also, it is not likely that Pb-EDTA or EDTA will diffuse across the plasma membrane at any significant rate as they are too large and polar to move through the plasmalemma lipid

bilayer. Bell et al. (1991) suggested that the plant uptake of metal chelate complexes occurs at breaks in the root endodermis and Casparian strip. EDTA could damage the membrane of root cells by chelating the Zn^{2+} and Ca^{2+} cations that stabilize the membrane, thus allowing free equilibration between hydroponic or soil solutions and the xylem sap (Vassil et al., 1998). However, the physiological basis of the uptake of Pb-EDTA complexes, particularly the possibility for these negatively charged large molecules to cross the membrane, is still not well characterized so far. It is also not clear whether some physiological damages to roots would be useful to enhance the uptake of metal-chelates, such as metal-EDTA and metal-EDDS, by plants, which in turn can minimize the application amounts of chelates in the practical operation of chelate-assisted phytoremediation.

The aims of the present study were (i) to assess the influences of EDTA and Pb on the growth of Indian mustard and the accumulation and translocation of Pb from the roots to the shoots; (ii) to examine the effects of different kinds of root damage in Indian mustard on the accumulation of Pb in the shoots and to explore the potential mechanisms underling metal uptake by plants with the application of EDTA; (iii) to investigate whether soil amendments with biodegradable EDDS, in comparison to EDTA, in hot solutions can further enhance the uptake of heavy metals by plants from artificially contaminated soils; (iv) to evaluate the potential leaching of solubilized metals after the application of chelates using soil dissolution experiment; and (v) to further test the mechanisms involved in the chelate-induced metal accumulation after the application of EDTA and EDDS with other plants using hydroponic

experiments.

5.2 Materials and Methods

5.2.1 Plant Culture

Seeds of Indian mustard (*Brassica juncea* L. cv. Baoxinjiecai) (purchased from Nanjing Agricultural University) were sterilized in 0.1% (w / v) $HgCl_2$ for 10 min, and rinsed four times in deionized water before being placed on filter paper for germination. Indian mustard was selected because it has been extensively studied in this field and it showed higher efficiency in Pb uptake with the application of EDTA (Vassil et al., 1998; Epstein et al., 1999). After germination, 15 plants of the same size were selected and transferred to 2-L polyethylene vessels containing a modified 0.2-strength Rorison's nutrient solution (Hewitt, 1966) with the following composition (in μmol L^{-1}): 400 $Ca(NO_3)_2$, 200 $Mg(SO_4)_2$, 50 K_2HPO_4, 300 KCl, 9.2 H_3BO_3, 1.8 $MnSO_4 \cdot 4H_2O$, 0.21 $Na_2MoO_4 \cdot 2H_2O$, 0.31 $CuSO_4 \cdot 5H_2O$, 10 $ZnSO_4 \cdot 7H_2O$, and 10.8 Fe-EDTA at pH 6.0. The nutrient solutions were aerated continuously and renewed every two days. The plants were grown in a glasshouse.

5.2.2 Effects of EDTA on the Accumulation of Pb in Indian Mustard

The 35-day-old seedlings were treated with different concentrations of Pb (0, 50, 100, 250, and 500 μmol L^{-1} of Pb) and EDTA (0, 50, 100, 250, 500, 1000, 2000, and 3000 μmol L^{-1} of EDTA) alone or with 500 μmol L^{-1} of Pb and eight

different concentrations of EDTA in the nutrient solutions, with a final pH adjusted to 6.0 with 1 N HCl and KOH every day. Pb and EDTA were applied in the form of $Pb(NO_3)_2$ and Na_2EDTA solution, respectively. Three replicates were included in each treatment. After 7 d of exposure, the plants were harvested. Shoots and roots were separated, washed with tap water and then with deionized water, and blotted dry with tissue paper. Plant samples were further dried in an oven to a constant weight for dry biomass measurement.

5.2.3 Effects of Pb and EDTA on the Relative Electrolytic Leakage Rate of Root Cells

The 35-day-old seedlings were treated with different concentrations of Pb alone or with 500 μmol L^{-1} of Pb at different concentrations of EDTA. After 7 days of exposure, the roots were cut to small sections (of about 2-3 cm in length) and rinsed with deionized water three times. The relative electrolytic leakage rate of root cells was measured by electrical conductivity (Zhu et al., 1990; Zhou and Leul, 1998). The root samples (0.5 g) were placed in a test tube containing 15 ml of deionized water and the root tissue was immersed and vibrated at room temperature for 2 h. The conductivity of the solution was measured using a conductivity meter (DDS - 11A). After boiling the samples for 10 min, the conductivity was measured again when the solution had cooled to room temperature. The relative electrical conductivity (REC) was calculated as follows: $REC = C_1 / C_2 \times 100$, where C_1 and C_2 were the electrolyte conductivities measured before and after boiling, respectively.

5.2.4 Effects of Root Damage on the Accumulation of Pb in Indian Mustard

For the 35-day-old seedlings, seven different pretreatments were used to assess the effects of damaged roots on the accumulation of Pb in the shoots. The pretreatments to the roots included: pretreatment with an MC solution (methanol : trichloromethane = 2 : 1,v / v) for 1 d, 0.1 mol L^{-1} of HCl for 1 d, 65 ºC of hot water for 1 h, 250 µmol L^{-1} of EDTA for 1 and 3 d, and 3000 µmol L^{-1} of EDTA for 1 and 3 d. After the treatment, all of plants were exposed to 500 µmol L^{-1} of Pb + 3000 µmol L^{-1} of EDTA for 2 days. The plants that had been directly grown in the 500 µmol L^{-1} of Pb + 3000 µmol L^{-1} of EDTA solution for 2 days without any pretreatment were used as the control group. In the meantime, some plants without any pretreatment were exposed to 500 µmol L^{-1} of Pb + 3000 µmol L^{-1} of EDTA + 100 µmol L^{-1} of DNP (2, 4-dinitrophenol) for 2 days to assess the effect of the metabolic inhibitor on the uptake of Pb-EDTA by plants. At the end of these experiments, the shoots and roots were harvested and washed for further analysis.

5.2.5 Hot EDTA and EDDS Treatments with Pot Experiment

Soil used in this part are the same as used in Chapter 3 for the study of garland chrysanthemum (dicotyledon) and corn (monocotyledon) (see Table 3.1, soils used in 3.2.2). The two plants were used here to further test if the root damage theory would be applicable to the two kinds of plants in metal uptake with the application of EDTA and EDDS. The soils were artificially contaminated with Cu (400 mg kg^{-1} of soil) as $CuCO_3$ (copper carbonate); Pb (500 mg kg^{-1} of soil)

as $Pb_3(OH)_2(CO_3)_2$ (lead hydroxide carbonate) and PbS (lead sulfide – galena, a common lead mineral in mining areas) at a Pb concentration ratio of 1:1; Zn (500 mg kg^{-1} of soil) as $ZnCO_3$ (zinc carbonate) and ZnS (zinc sulfide) at a Zn concentration ratio of 1:1; and Cd (15 mg kg^{-1} of soil) with $Cd(NO_3)_2 \cdot 4H_2O$ (cadmium nitrate). Air-dried soils (500 g) were placed in plastic pots (12 cm i.d. x 12 cm height). Soil moisture was maintained to near field water capacity by adding deionized water (DIW) on a daily basis. Seeds of garland chrysanthemum (*Chrysanthemum coronarium* L.) and beans (*P vulgaris* L. white bean) were sown directly in the soils. In order to acquiring uniform seedlings, beans were sown 14 d after the sowing of the garland chrysanthemum seeds. After germination, the seedlings were thinned to four plants per pot. On the 35^{th} day after the sowing of garland chrysanthemum, EDTA (from BDH Laboratory Supplies Poole, minimum assay: 99.5%) and EDDS (from Fluka Chemie GmbH) were applied to the surface of the soils in two different ways (heated and not heated) at rates of 0 (control), 0.5, 1.0, 1.5, 3.0, and 5.0 mmol kg^{-1} soil as 100 ml Na_2EDTA and Na_3EDDS solutions. To make up the different amounts of chelate treatments, EDTA and EDDS were diluted from 50 mM Na_2EDTA (pH 4.8) and Na_3EDDS (pH 10.1) salt solutions. The hot chelate solution treatments were conducted by adding boiled solution to soil in the pots which resulted in the final temperature of the soils was about 40 ºC at the 2/3 depth of the pot. Three replicates were conducted for each treatment. All of the experiments were operated in the glasshouse under natural light. Air temperatures ranged from 16 to 21 ºC. All the plants were harvested by cutting the shoots 0.5 cm above the surface of the soil, removing the roots from the pots 7 d after the application of chelates. The shoots and roots were washed with tap

water and rinsed with DIW, and dried at 70 ºC in a drying oven to a constant weight for dry weight measurements. The dried plant materials were ground using agate mill.

5.2.6 Hot Citric Acid and NTA Treatment

Beans were chosen for a further study on the effects of hot citric acid and NTA on metal uptake. The soil used in this study was the same as mentioned above. After germination, the seedlings of beans (*P vulgaris* L. white bean) were thinned to four plants per pot. On the 21^{th} day after the sowing, citric acid and NTA were applied to the surface of the soils in two different ways (hot solution and normal solution) at rates of 0 (control), 0.5, 1.0, 1.5, 3.0, and 5.0 mmol kg^{-1} soil as 100 ml citric acid and NTA-Na_3 solutions. The hot treatments were conducted in the same way as the above experiment. All the plants were harvested 7 d after the application of chelates.

5.2.7 Metal Leaching Study

After harvesting the plants, soils in pots were brought to 2/3 field capacity. On Day 0, 7, 14 and 21 (i. e. on Day 7, 14, 21 and 28 after the application of chelates), the soil in every pot was mixed thoroughly and 4.0 g of soil (based on dry weight) were placed in a 50-mL polypropylene centrifuge tube. DIW was added to the soil (at a soil-to-water ratio of 1:5) and the suspension was shaken for 30 min. After centrifugation, the supernatant will be filtered through a 0.45 µm paper filter (Whatman [Maidstone, UK] 42), digested with concentrated

HNO_3 and analyzed for metal concentrations by ICP-AES (Perkin Elmer 3000DV).

5.2.8 Root Pretreatment with Hot Water in Hydroponic Experiment

Seeds of beans (*P vulgaris* L. white bean) were sterilized in 0.1% (w / v) $HgCl_2$ for 10 min, and rinsed four times in deionized water before being placed on filter paper for germination. After germination, plants of the same size were selected and transferred to 2-L polyethylene vessels containing a modified 0.2-strength Rorison's nutrient solution (Hewitt, 1966) with the same composition as mentioned above (see 5.2.1) (in μmol L^{-1}): 400 $Ca(NO_3)_2$, 200 $Mg(SO_4)_2$, 50 K_2HPO_4, 300 KCl, 9.2 H_3BO_3, 1.8 $MnSO_4 \cdot 4H_2O$, 0.21 $Na_2MoO_4 \cdot 2H_2O$, 0.31 $CuSO_4 \cdot 5H_2O$, 10 $ZnSO_4 \cdot 7H_2O$, and 10.8 Fe-EDTA at pH 6.0. Nutrient solutions were aerated continuously and renewed every two days. The plants were grown in a glasshouse with the temperature ranged from 17 ºC to 22 ºC.

After 7 d of the transplanting, different pretreatments were conducted to assess the effects of root damage by hot water on the accumulation of Pb in shoots. Nine pretreatments were included: the roots were exposed in hot water at 30 ºC, 40 ºC, 50 ºC, 60 ºC and 80 ºC for 15 min. For the pretreatment at 40 ºC, the roots were exposed in the hot water for 15, 30, 45 and 60 min. The plants without hot pretreatment (room temperature was about 25 ºC) were used as the control. After pretreatments, 15 plants from every treatment were used to measure the relative electrolytic leakage rate of root cells by electrical

conductivity (Zhu et al., 1990; Zhou and Leul, 1998). Half of the rest 30 plants from every treatment were treated with 500 μmol L^{-1} of Pb + 500 μmol L^{-1} of EDTA and another half was treated with 500 μmol L^{-1} of Pb + 500 μmol L^{-1} of EDDS for 2 d, respectively (pH = 6.0). Pb, EDTA and EDDS were applied in the forms of $Pb(NO_3)_2$, Na_2EDTA, and Na_3EDDS solutions, respectively. Every treatment was replicated three times. At the end of these experiments, the shoots and roots were harvested for further chemical analysis. The effects of root damage on the accumulations of Cu, Zn and Cd were studied in the same way, in which Cu, Zn Cd were applied in forms of $CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ and $CdNO_3 \cdot 4H_2O$ solutions, respectively.

5.2.9 Plant and Soil Analysis

The extraction and measurement of the EDTA in the shoots of Indian mustard was conducted using a modified method reported by Vassil et al. (1998) and Epstein et al. (1999). In this study, ground oven-dried material (0.1 g) was extracted with 2 ml of 50% (v/v) ethanol, heated at 80 ºC for 15 min, and centrifuged at 3000 g for 20 min at room temperature. The extraction was conducted three times, the supernatant solutions from each extraction were combined and the solid phase was discarded. The solutions of 50% ethanol plant extracts were centrifuged at 10,000 g for 10 min, and were then filtered through a 0.22-μm nylon membrane filter. The filtered sample (100 μl) was combined with 400 μl of 6.5 mmol L^{-1} ferric chloride in a 7.1 mol L^{-1} acetic acid solution and with HPLC-grade water to a total volume of 1 ml. The mobile phase consisted of a 0.03 mol L^{-1} (pH 4.0) acetate buffer with 0.008 M of

tetrabutyl ammonium hydroxide as the counter ion. The samples were filtered through a 0.45 µm nylon membrane filter and 20 µl was run isocratically at 1 ml / min. EDTA was detected at 254 nm using a SPD - 6UV detector.

Subsamples of ground shoot samples (200 mg) and root samples (100 mg) of Indian mustard were digested in a mixture of concentrated HNO_3 and $HClO_4$ (87 : 13, by volume), and the total concentration of Pb was determined using an atomic absorption spectrophotometer (AAS) (TAS-986). Every sample was replicated three times. Certified standard reference material (SRM 1515, apple leaves) from the National Institute of Standards and Technology, USA, was used in the digestion and analysis. Three reagent blanks were also used where appropriate to ensure accuracy and precision in the analysis. The recovery rates were around 90 ± 10% for Pb in the plant reference material.

Plant samples of garland chrysanthemum and beans and soil samples were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, by volume). The major and trace elements in the solutions were determined with ICP-AES the same way as used in Chapters 3 and 4. The recovery rates were around 94 ± 10% for all of the metals in the plant reference material. Statistical analyses of the experimental data, such as of the correlation and significant differences, were performed using SPSS® 11.0 statistical software. A probability level of 0.05 was considered to be statistically significant for the data set.

5.3 Results and Discussion

5.3.1 Growth of Indian Mustard

The treatment with 50 μmol L^{-1} of Pb for 2 days had no significant effect on the net elongation of the roots (see Table 5.1). From Days 2 to 5 after the 50 μmol L^{-1} Pb treatment, however, the roots decreased in elongation by 39% compared to the roots of the control group. At concentrations of 100 μmol L^{-1} of Pb or higher, the decrease in the elongation of the roots was more pronounced 2 days after the exposure to Pb. The dry matter (DM) yields of both roots and shoots significantly decreased with the increasing concentration of Pb in solution (Table 5.1). The shoot/root quotient for DM increased with increased Pb concentrations in solution, indicating that the growth of the roots was more sensitive than that of the shoots to the Pb treatment.

The EDTA treatment had less significant effect on root elongation than Pb at the same level, although higher EDTA concentrations in solution tended to decrease the elongation of the roots (Table 5.2). Root DM was not affected ($P < 0.05$), while shoot DM substantially decreased when 0 - 250 μmol L^{-1} of EDTA was added to the solution. The shoot/root quotient for DM tended to decrease as the EDTA concentrations in solution increased.

In the presence of 500 μmol L^{-1} of Pb, however, increasing the EDTA concentration from 0 to 500 μmol L^{-1} resulted in progressive increases in root elongation and in shoot and root DM (Table 5.3). However, at higher concentrations of EDTA (> 500 μmol L^{-1}), root elongation and shoot and root DM decreased significantly. The maximum values for root elongation and for

the DM of shoots and roots were obtained in the treatment with 500 μmol L^{-1} of Pb and EDTA.

High concentrations of Pb or EDTA alone in solutions were toxic to plants (Tables 5.1 and 5. 2). It has been demonstrated in the present (Table 5.3) and in previous studies (Denduluri, 1994; Hernandez-Allica et al., 2003; Piechalak et al., 2003) that EDTA reduces the physiological damage of Pb on plants. At the treatment with equimolar Pb and EDTA (500 μmol L^{-1}), Indian mustard plants showed the most root elongation and shoot and root DM without any apparent phytotoxicity. This result is consistent with observations of Vassil et al. (1998), Piechalak et al. (2003) and Hernandez-Allica et al. (2003). The alleviation of toxicity was likely related to the strong affinity of Pb to EDTA and to the limitation of free Pb^{2+} in solution. EDTA can limit the phytotoxicity of Pb by decreasing the concentration of free Pb^{2+} in the solution on one hand. On the other hand, EDTA increases the concentration of the Pb-EDTA complex, which is the major form of Pb uptake by plants. Moreover, the phytotoxic effects of Pb-EDTA complexes were also found in many studies (Vassil et al., 1998; Geebelen et al., 2002; Hernandez-Allica et al., 2003; Piechalak et al., 2003).

Table 5.1 Effects of various Pb treatments on the root elongation and dry matter weights of Indian mustard

Pb treatment	Root elongation (cm)		Dry matter weights (g plant^{-1})			Shoot / root
(μmol L^{-1})	Day 0-2	Day 2-5	Shoot	Root	Total	Ratio of DW
0	0.95 a	1.31 a	0.19 a	0.031 a	0.221 a	6.1 b
50	0.94 a	0.8 b	0.176 a	0.021 b	0.197 a	9.3 a
100	0.6 b	0.62 b	0.168 a	0.02 b	0.188 a	8.4 a
250	0.3 c	0.37 c	0.136 b	0.018 b	0.154 b	7.6 a
500	0.1 c	0.02 d	0.145 b	0.015 c	0.16 b	9.7 a

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 5.2 Effects of different EDTA treatments on the root elongation and dry matter weights of Indian mustard

EDTA treatment	Root elongation (cm)		Dry matter weights (g plant^{-1})			Shoot / root
(μmol L^{-1})	Day 0-2	Day 2-5	Shoot	Root	Total	Ratio of DW
0	1.06 a	1.28 a	0.185 a	0.034 a	0.219 a	5.4 a
50	1.01 a	1.2 a	0.137 b	0.032 a	0.169 b	4.3 b
100	0.97 a	0.98 b	0.121 b	0.028 a	0.149 b	4.3 b
250	0.84 a	0.85 b	0.111 b	0.029 a	0.14 b	3.8 b
500	0.8 a	0.75 b	0.103 c	0.026 b	0.129 b	4 b
1000	0.75 b	0.76 b	0.09 c	0.024 b	0.114 c	3.8 b
2000	0.6 b	0.49 c	0.08 c	0.023 b	0.103 c	3.5 b
3000	0.57 b	0.29 c	0.075 c	0.022 b	0.097 c	3.4 b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 5.3 Effects of different EDTA treatments on the net elongation of the roots and dry matter weights of Indian mustard exposed to 500 μmol L^{-1} of Pb

EDTA treatment	Root elongation (cm)		Dry matter weights (g $plant^{-1}$)			Shoot / root
(μmol L^{-1})	Day 0-2	Day 2-5	Shoot	Root	Total	Ratio of DW
0	0.1 c	0.02 d	0.145 b	0.018 b	0.163 b	8.1 a
50	0.1 c	0.05 d	0.207 a	0.02 b	0.227 a	10.4 a
100	0.2 c	0.15 c	0.197 a	0.025 a	0.222 a	7.9 a
250	0.3 b	0.21 c	0.2 a	0.026 a	0.226 a	7.7 a
500	0.99 a	1.17 a	0.25 a	0.033 a	0.283 a	7.6 a
1000	0.85 a	1.02 a	0.23 a	0.031 a	0.261 a	7.4 a
2000	0.59 b	0.6 b	0.129 b	0.026 a	0.155 b	5 b
3000	0.4 b	0.21 c	0.125 b	0.02 b	0.147 b	6.3 b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

5.3.2 Effects of EDTA on the Concentrations of Pb in Indian Mustard

The results of the present study demonstrated that EDTA effectively increased the uptake and translocation of Pb from the roots to the shoots of Indian mustard (Figs. 5.1 and 5.2). In general, the concentration of Pb in shoots increased with the concentration of EDTA applied to the soil (Blaylock et al., 1997; Shen et al., 2002; Grčman et al., 2003; Kos and Leštan, 2003a). In the presence of 500 μmol L^{-1} of Pb, the concentrations of Pb in shoots increased rapidly as the concentration of EDTA in the solution increased from 0 to 250 μmol L^{-1}, reaching a maximum Pb concentration of 1143 mg kg^{-1} in DW at 250 μmol L^{-1} of EDTA (Fig. 5.1a), which was 10 times higher than that of the control group (500 μmol L^{-1} Pb without EDTA). The maximum concentration of Pb and the efficiency of EDTA-enhancing Pb accumulation were comparable to the results obtained by Jarvis and Leung (2001, 2002) for *Chamaecytisus proliferus* and *Pinus radiata*.

However, the treatments with EDTA at 500 and 1000 μmol L^{-1} led to decreased concentrations of Pb in the shoots compared with the treatment involving 250 μmol L^{-1} of EDTA. Hernadez-Allica et al. (2003) also found the highest concentration of Pb of 192 mg kg^{-1} in the leaves of cardoon plants treated with 500 μmol L^{-1} of EDTA + 1000 μmol L^{-1} of Pb. If it is true that Pb-EDTA follows the mass flow of water into plant at breaks in the root, transpiration and root structure will be critical factors in the accumulation of Pb-EDTA in shoots. In addition to diffusion, a driving force may be required for the translocation of Pb-EDTA across the aqueous-root interface and in xylem from roots to shoots

(Blaylock et al., 1997; Vassil et al., 1998). Crist et al. (2004) showed that the excised roots of Indian mustard did not take up Pb-EDTA because of the deficiency of transpiration. In the presence of 500 μmol L^{-1} of Pb, the decrease in Pb concentrations in the shoots of plants exposed to 500 - 2000 μmol L^{-1} of EDTA (Fig. 5.1a) might be attributed to the fact that there was relatively less damage to plant roots by EDTA (Tables 5.2 and 5.3) and to the decrease in transpiration. Vassil et al. (1998) reported that the presence of free protonated EDTA in solution led to a reduction in the rate of water lost from shoots. In a pot experiment, the transpiration rate was not affected by the application of 5 mmol kg^{-1} of EDTA, but decreased significantly with the application of 10 mmol kg^{-1} of EDTA (Epstein et al., 1999).

The treatments at > 2000 μmol L^{-1} EDTA tended to increase the concentration of Pb in shoots, and the Pb concentration reached 1115 mg kg^{-1} in the 3000 μmol L^{-1} of EDTA treatment, which was speculated to damage the membranes of the roots, causing an unusually high uptake of Pb-EDTA (Fig. 5.1a). It has been suggested that a threshold concentration of EDTA is required to induce the accumulation of the metals in shoots (Blaylock et al., 1997; Vassil et al., 1998). In the pot experiments reported in the literature, the amounts of chelating agents applied to effectively enhance the accumulation of Pb in plants varied between 3 and 10 mmol kg^{-1} of soil (Blaylock et al., 1997; Shen et al., 2002; Grčman et al., 2003). These chelates were usually applied to the surface of the soil as a solution when the vegetation was well-established. The concentrations of EDTA in the root zone would reach at least 4 to 13 mmol L^{-1} (assuming a soil water content of 75%). Under the condition of such a high concentration of

EDTA, the plant roots would suffer from some physiological damage which would lead to a breakdown of the root exclusion mechanism and to the indiscriminate uptake of solutes, including Pb-EDTA, by plants. The concentration of Pb in roots decreased significantly as the concentration of EDTA increased (Fig. 5.1b).

When no EDTA was added to the solution, most of the Pb taken up was concentrated in the roots, accounting for more than 98% of total plant Pb. The EDTA treatment increased the ratio of the Pb concentrations between the shoots and roots (Fig. 5.2a). The percentage of Pb accumulated in the shoots of the total Pb taken up by plants increased from 1.2% in the control to 91% in the 3000 μmol L^{-1} of EDTA treatment (Fig. 5.2b). This result indicated that the EDTA treatment facilitated the uptake and translocation of Pb from the roots to shoots of Indian mustard.

The increased uptake of Pb induced by the application of EDTA can be explained by the effect of enhancing the solubility of Pb and the uptake of the Pb-EDTA complex by plants. In the present study, the concentration of EDTA in shoots also increased significantly as the concentration of Pb in shoots increased (Fig. 5.3). There was a significantly positive correlation between the concentrations of Pb and EDTA in the shoots ($R^2 = 0.69$, $n = 24$) ($P < 0.05$). This result was in agreement with the observations of Vassil et al. (1998) and Epstein et al. (1999), who demonstrated that Pb-EDTA was the major form of Pb taken up and translocated by plants. The transport of solution from the external parts of the roots to the central root xylem, where the material is carried

to the shoots, is thought to occur via two major pathways: symplastic and apoplastic (Tanton and Crowdy, 1972). In the symplastic pathway, the solutions cross many cell membranes along the path. In the apoplastic pathway, the presence of the Casparian strip at the root endodermis disrupts the apoplastic water flow and directs it across to cell plasma membranes at least twice, where the selective transport as well as passive permeation of the solution occurs. Tanton and Crowdy (1972) showed that the uptake of Pb-EDTA by plants was restricted to the region of between 3 and 140 mm from the tip of the root, where suberization of the cell walls had not yet occurred. Also, the apoplastic uptake of the intact Pb-EDTA complex could occur at breaks in the Casparian strip. Along the primary root, the process was initiated by the budding of lateral roots (Peterson and Edgington, 1975; Sanderson, 1983). Thus, in the chemically enhanced phytoextraction process, the uptake of metal would be strongly dependent on the concentration of the metal-chelate complex and on the breakdown of the root exclusion mechanism.

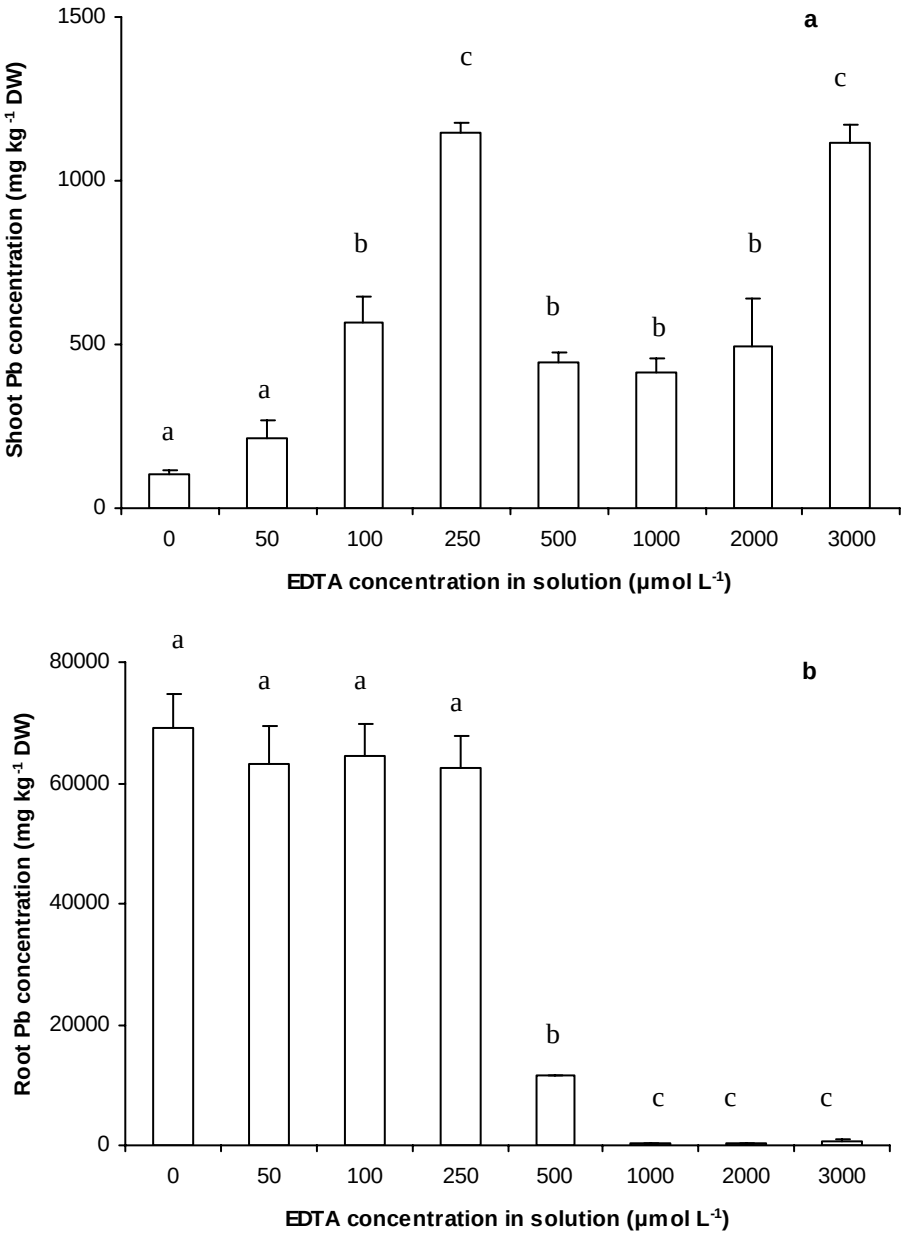


Fig. 5.1 Effects of various EDTA treatments on the concentrations of Pb in the shoots (a) and roots (b) of Indian mustard exposed to 500 µmol L^{-1} of Pb in solution for 7 d. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

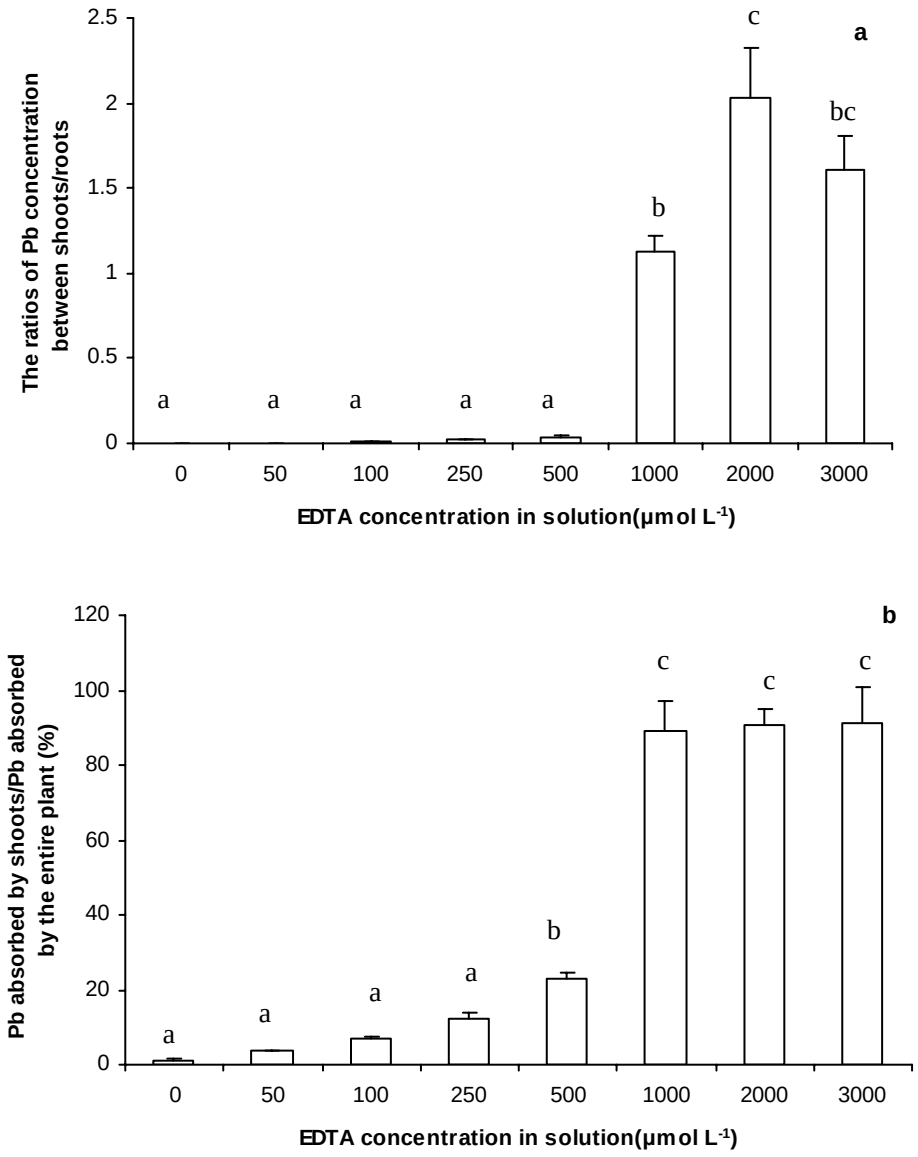


Fig. 5.2 Effects of different EDTA treatments on the ratios of Pb concentration between shoots/roots (a) and the percentages of the Pb absorbed by shoots (b) exposed to 500 μmol L^{-1} of Pb in solution for 7 days. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

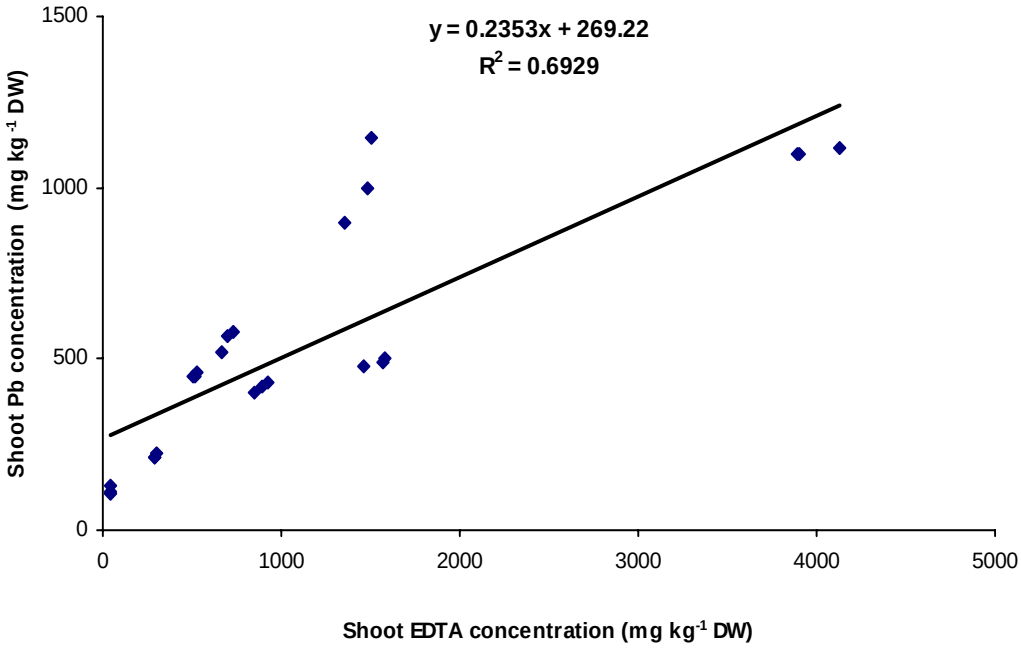


Fig. 5.3 Relationship between the concentrations of EDTA and Pb in the shoots of Indian mustard exposed to 500 µmol L^{-1} of Pb and various concentrations of EDTA in solution for 7 days. Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

5.3.3 Relative Electrolyte Leakage Rate of Root Cells

Lead is effective at displacing various cationic metals from roots (Vassil et al., 1998); thus, it might play an important role in changing the physiological barrier to the solute movement in roots. This was supported by the fact that the Pb treatments increased the relative electrolytic leakage rate of mustard roots (Fig. 5.4a). Furthermore, the concentration of Pb in shoots was significantly correlated with the relative electrolytic leakage rate of root cells (Fig. 5.4b). Kumar et al. (1995) also found that the Pb concentration in the shoots of Indian mustard increased significantly only when the concentration of Pb in the solution reached a value of 100 mg L^{-1} (about 480 μmol L^{-1}). Therefore, it can be hypothesized that high concentrations of free Pb^{2+} may physiologically damage the roots of plants, leading to the rapid equilibration of Pb-EDTA between the external solution and the sap of the xylem. After entering the xylem, Pb-EDTA would be translocated from the roots to shoots with the transpiration stream and lead to a high concentration of Pb in shoots.

In the presence of 500 μmol L^{-1} of Pb, the addition of 100 or 250 μmol L^{-1} of EDTA had no significant effect on the relative electrolyte leakage rate of root cells in comparison with the control group without the EDTA treatment (Fig. 5.5a). The lowest rate was recorded in the treatment with 500 μmol L^{-1} of Pb and EDTA. At EDTA concentrations of greater than 500 μmol L^{-1}, it dramatically increased as the EDTA concentration increased. No significant relationship was found between the concentration of Pb in shoots and the relative electrolytic leakage rate of root cells in the combined Pb and EDTA

treatment (Fig. 5.5b).

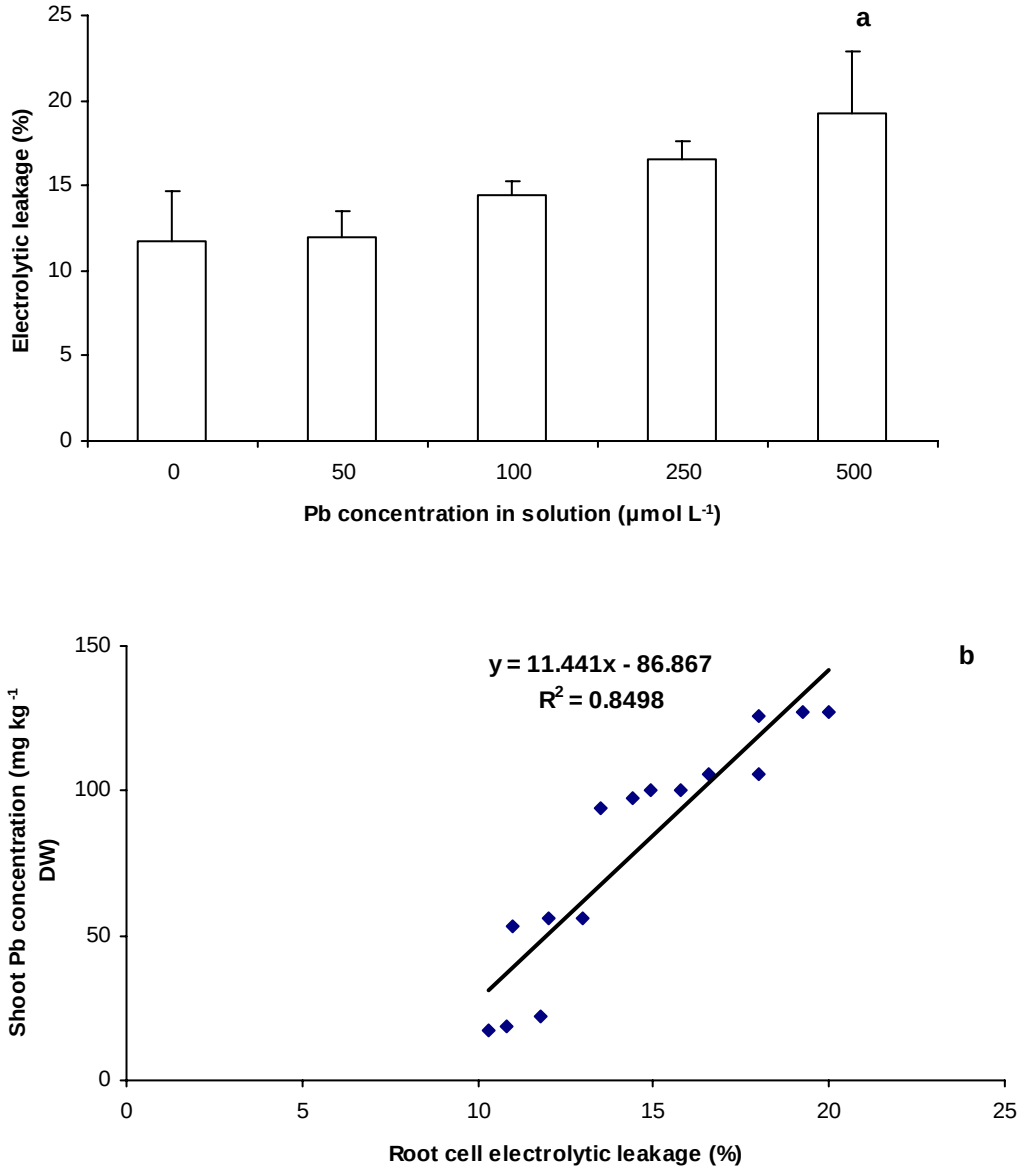


Fig. 5.4 Effects of various Pb treatments on the relative electrolyte leakage rates of root cells (a) and the correlation between the relative electrolyte leakage rate and the concentration of Pb in the shoots of Indian mustard (b). Values are means ± S.D. (n = 3).

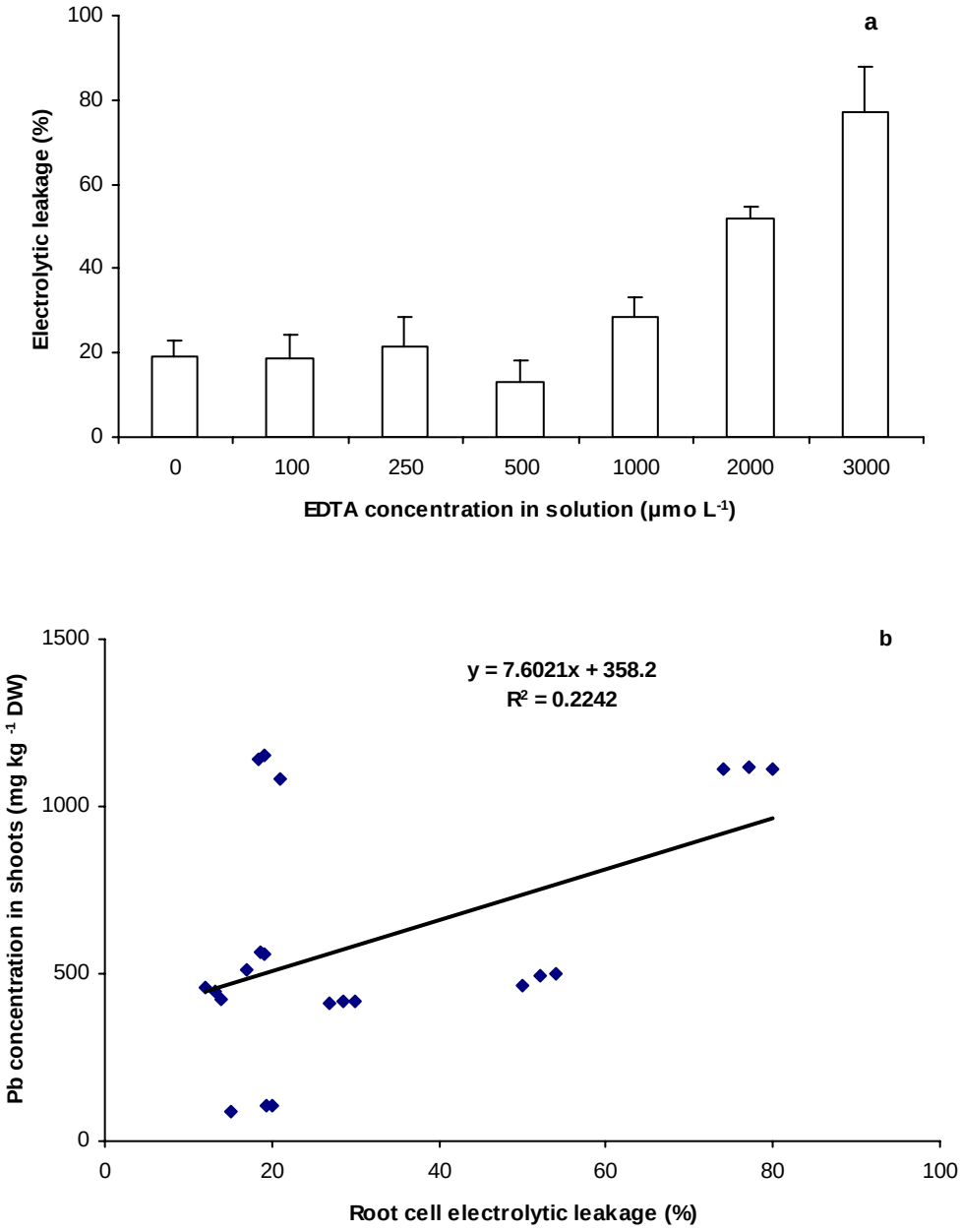


Fig. 5.5 Effects of different EDTA treatments on the relative electrolyte leakage rates of root cells (a) and the correlation between the relative electrolyte leakage rate and the concentration of Pb in the shoots (b) of Indian mustard exposed to 500 µmol L^{-1} of Pb in solution for 7 days. Values are means ± S.D. (n = 3).

5.3.4 Effects of Root Damage Pretreatments on the Accumulation of Pb in Shoots

It is thought that EDTA can destroy the physiological barrier(s) of plant roots that normally function to control the uptake and translocation of solutes, which would lead to the rapid equilibration of the hydroponic or soil solution with the sap of the xylem (Vassil et al., 1998). In the present study, roots of Indian mustard were pretreated with different solutions for 1 or 3 days before they were exposed in solutions containing 500 μmol L^{-1} of Pb + 250 μmol L^{-1} of EDTA or 500 μmol L^{-1} of Pb + 3000 μmol L^{-1} of EDTA. Results showed that the pretreatment of Indian mustard roots with 250 or 3000 μmol L^{-1} of EDTA for 1 d had very little effect on the concentration of Pb in the shoots (Table 5.4). Even when the pretreatment was prolonged for another 2 days, the concentration of Pb in the shoots increased only 2 time more than that in the plants that had not received any pretreatment. In addition, more significant stimulation of the accumulation of Pb in shoots by the pretreatments with the MC solution, 0.1 M HCl, and hot water (65°C) was observed. The Pb concentrations in shoots increased 4, 7, and 15 times following the pretreatment with MC and HCl for 1 day, and hot water for 1 h, respectively, compared to the plants that had not received any pretreatment. When 100 μmol L^{-1} of DNP (dinitrophenol) was added to the solution containing 500 μmol L^{-1} of Pb + 3000 μmol L^{-1} of EDTA, the concentration of Pb in the shoots increased 13 times in comparison with the plants that had not received the treatment (Table 5.4). Therefore, the effect of the EDTA pretreatment on the uptake of Pb in shoots was much smaller than that of other pretreatments with MC, HCl, and hot water

(Table 5.4). The results indicated that the membrane of root cells was not in the condition to cause the rapid equilibriumof hydroponic solution with the sap of the xylem during the one-day 3000 μmol L^{-1} EDTA pretreatment of the roots. Geebelen et al. (2002) showed that there was no reduction in the fresh weight of the roots and leaves or in the length of stems in *P. vulgaris* plants cultivated in the Hoagland's solution containing 50 to 200 μmol L^{-1} of EDTA. EDTA concentrations of up to 500 μmol L^{-1} had no significant effect on the water content of Indian mustard shoots (Vassil et al., 1998).

In the chemically enhanced phytoextraction process, the addition of chelates to soil should be minimized for environmental and cost considerations. The results of the present study showed that some type of physiological damage to roots such as the pretreatments with MC, HCl, and hot water, and the treatment with DNP, dramatically increased the concentrations of Pb in shoots with the EDTA treatment (Table 5.4). Thus, treating plant roots beforehand would help to minimize the dose of EDTA applied in a practical operation. Transplanting seedlings rather than planting seeds resulted in an increased uptake of chelates probably through the breaks of the Casparian strip due to the mechanical damage of roots (Wallace and Hale, 1962). A significant increase in the uptake and translocation of Pb has been reported for corn transplanted into soil, then treated with EDTA, in comparison with the plants that were germinated and grown in Pb-contaminated soil to which EDTA was subsequently applied (Wu et al., 1999). Similarly, some environmental stresses, such as excessive toxic metals, high temperatures, and droughts, also may result in a breakdown of the root exclusion mechanism, sequentially influencing the EDTA-enhanced

accumulation of Pb in plant shoots. This may be one of reasons behind the different Pb phytoextraction efficiencies of the EDTA treatment reported by various researchers even for the same plant species, such as Indian mustard (Blaylock et al., 1997; Epstein et al., 1999; Barocsi et al., 2003; Salido et al., 2003; Walker et al., 2003; Lim et al., 2004) and corn (Huang et al., 1997; Wu et al., 1999; Lombi et al., 2001a; Meers et al., 2004).

Table 5.4 Effects of various pretreatments of Indian mustard roots on the uptake of Pb in 500 μmol L^{-1} of Pb + 250 μmol L^{-1} of EDTA, 500 μmol L^{-1} of Pb + 3000 μmol L^{-1} of EDTA or 500 μmol L^{-1} of Pb + 3000 μmol L^{-1} of EDTA + 100 μmol L^{-1} of DNP solutions for 2 d

Pretreatment	Treatment (μmol L^{-1})	Pb concentration (mg kg^{-1})	
		Shoot	Root
	500 Pb + 250 EDTA	153 ± 13.1 c	20200 ± 157 a
250 EDTA 1 d	500 Pb + 250 EDTA	186 ± 45 c	15200 ± 470 a
250 EDTA 3 d	500 Pb + 250 EDTA	215 ± 48.4 c	13500 ± 896 a
	500 Pb + 3000 EDTA	128 ± 42.7 c	478 ± 23.5 b
3000 EDTA 1 d	500 Pb + 3000 EDTA	189 ± 75.8 c	218 ± 5.2 b
MC 1 d	500 Pb + 3000 EDTA	1820 ± 21.5 a	49 ± 14.3 c
0.1 M HCl 1 d	500 Pb + 3000 EDTA	839 ± 217 b	131 ± 29.2 c
65 ºC hot water 1 h	500 Pb + 3000 EDTA	1880 ± 221 a	50 ± 16.5 c
3000 EDTA 3 d	500 Pb + 3000 EDTA	339 ± 153 c	353 ± 49.4 b
	500 Pb + 3000 EDTA + 100 DNP	1800 ± 372 a	59 ± 7.2 c

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

5.3.5 Plant Growth in Pot Experiment

The application of EDTA and EDDS had a significant effect on the growth of plants and the production of shoot biomass. The dry weights of the shoots of garland chrysanthemum and beans decreased with the increasing level of the chelate applied to the soil (Fig. 5.6). The results also showed that the decrease was more pronounced when EDTA and EDDS were applied as hot solutions to the soil surface than that of the treatment without heating. Compared with those in the treatments with corresponding chelate solutions without heating, shoot dry matter yields decreased 13% and 15% for garland chrysanthemum, and 21% and 24% for beans by the treatments with hot solutions of EDTA and EDDS, respectively, on the 7^{th} day after the application of the chelates.

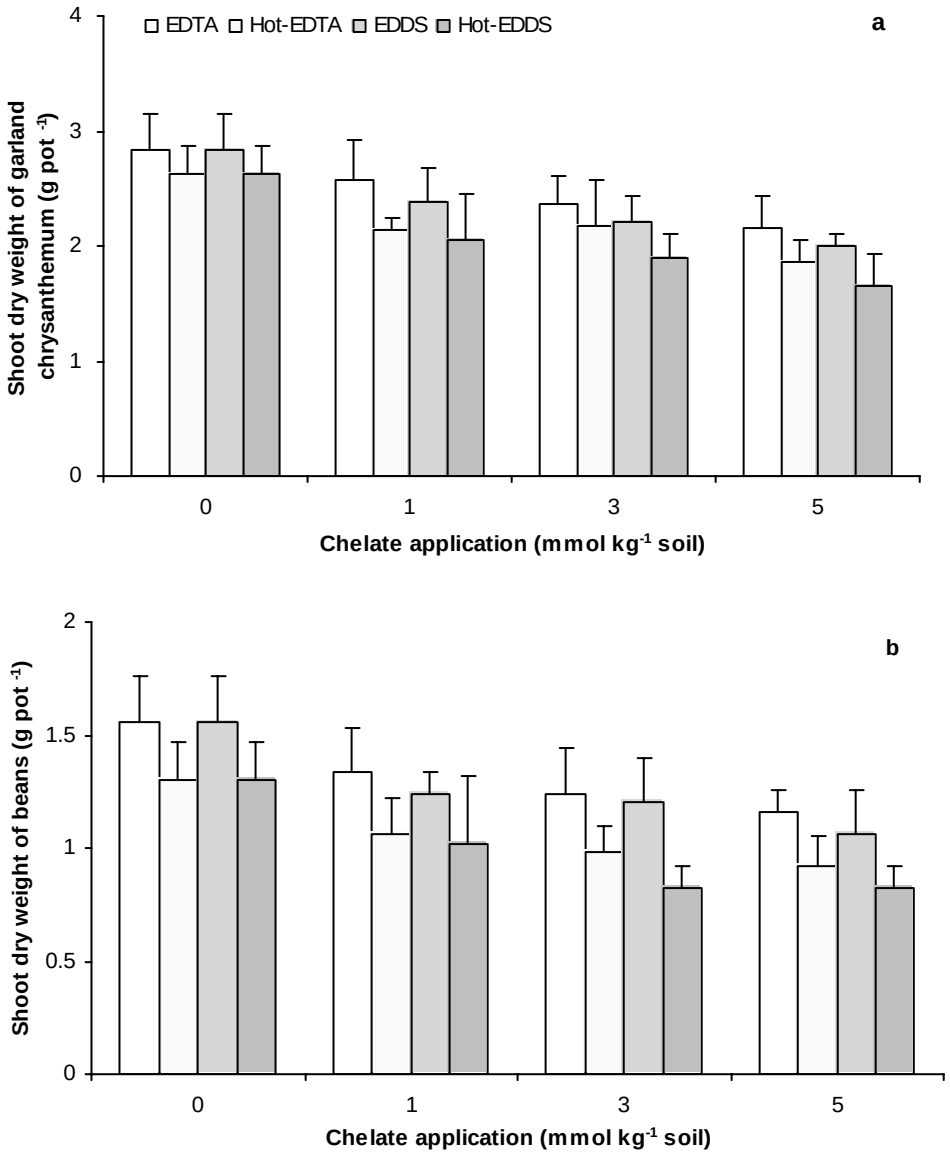


Fig. 5.6 Effects of the application of EDTA and EDDS on the dry matter yields of garland chrysanthemum (a) and beans (b). Values are means ± S.D. (n = 3).

5.3.6 Metal Concentrations and Phytoextraction in Hot EDTA and EDDS Treatments

The chemically enhanced phytoremediation of soils contaminated with heavy metals has been shown to be a potential way of removing heavy metals from soils with high biomass plants (Huang et al., 1997; Liphadzi et al., 2003). In the present study, the results demonstrated that the application of chelates to soils led to a rapid and significant increase in the concentrations of heavy metals in the shoots of garland chrysanthemum and beans. The results also showed that the accumulation of Cu, Pb, Zn and Cd in plant shoots improved dramatically when chelates, including non-degradable EDTA and biodegradable EDDS, were added as hot solutions to the soil (Figs. 5.7 and 5.8). EDDS was more effective at increasing the concentration of Cu in the shoots of the two species than EDTA, but less effective for Pb and Cd. In all treatments, uptake of the metals in the shoots of garland chrysanthemum was higher than in beans.

At the same application dosage, the application of hot chelate solutions produced higher concentrations of Cu, Pb, Zn, and Cd in the shoots of both plant species than the application of chelate solutions without heating (Figs. 5.7 and 5.8). ($P<0.01$) The concentrations of Cu ranged from 3850 to 5850 and 2710 to 3710 mg kg^{-1} in the shoots of garland chrysanthemum treated with hot EDDS and EDTA, respectively, which were 4-21 and 6.8-16 times of those with the normal chelates treatments without heating, and 136-207 and 96-131 times of that in the control group (without the application of chelates), respectively. The highest Pb concentration of 2330 mg kg^{-1} was found in the shoots of

garland chrysanthemum treated with hot EDTA at the rate of 5 mmol kg^{-1}, followed by 2080 mg kg^{-1} in the treatment with hot EDDS of 5 mmol kg^{-1}. The average enhanced effects of hot EDTA and EDDS on the Pb shoot uptake were 10.4 and 6.7 times that in the corresponding chelate treatment without heating, respectively. Chelates were found to have a less significant stimulating effect on uptake of Zn and Cd in these two plants. When the normal EDTA and EDDS were applied at rates of 1-5 mmol kg^{-1}, the concentrations of Zn and Cd in the shoots of both plant species did not exceed 3.2 and 5.9 times those of the controls, respectively. The applications of hot EDTA and EDDS increased the concentration of metals in shoots by 3.8-13.1 and 2.6-11 times for Zn, and by 5.5-67 and 1.4-23 times for Cd, compared with the controls, respectively. The concentrations of Cd were much higher in the shoots of both plant species treated with hot EDTA than in those treated with hot EDDS.

Total metal phytoextraction by the shoots of garland chrysanthemum and beans is shown in Table 5.5. Of the two plant species tested, garland chrysanthemum was better in the phytoextraction of metals than beans. Similar to the effects of chelates on the concentration of metals in the shoots, the maximum phytoextraction of Cu was found in the heated EDDS treatments at the rate of 1 and 3 mmol kg^{-1} soil, which increased 150- and 72-fold in garland chrysanthemum and beans, respectively, compared with the control group (adding normal water). For Pb, the plants treated with 5 mmol kg^{-1} of hot EDTA attained the maximum level of phytoextraction of approximately 270- and 127-fold that in the corresponding control garland chrysanthemum and bean plants, respectively. The total amounts of Zn extracted did not exceed 7.8 times

that of the controls, but were significantly higher in the plants treated with hot EDTA and EDDS than in those treated with chelates without heating. The maximum Cd phytoextraction was observed in the heated EDTA treatment at the rate of 3 mmol kg^{-1} soil, which were 5.5- and 42-fold of the level seen in the control group of garland chrysanthemum and beans, respectively.

In general, for all heavy metals that were studied when chelates were applied as hot solutions at the rate of 1 mmol kg^{-1}, metal concentrations and total phytoextraction by plant shoots exceeded or approximated those in the shoots of plants treated with normal chelates at a rate of 5 mmol kg^{-1}. This result implies that the amount of chelate applied could be greatly decreased for a given effectiveness of chelates in enhancing the phytoextraction of heavy metals from contaminated soils.

The *in situ* application of chelates may pose the potential risk of causing ground water pollution through uncontrolled metal solubilization and migration (Nowack, 2002; Römkens et al., 2002; Shen et al., 2002; Madrid et al., 2003; Chen et al. 2004a). The concentrations of soluble metals in soil significantly increased with the level of chelate applied to the soil (Table 5.6). A reduction in the amount of chelate applied could result in a marked decrease in the concentrations of water-soluble metals in the soil. Therefore, the application of hot chelate solution could not only help to reduce the cost of the operation, but also alleviate the potential risk of the migration of chelate and heavy metals to ground water and to the surrounding environment.

Previous studies indicated that EDDS was more effective at increasing the concentration of Cu in shoots than EDTA (Chapter 4; Meers et al., 2005). It was suggested that EDDS-assisted phytoextraction could be an acceptable approach for the remediation of Cu-contaminated soils (see Chapter 4). The results of the current study prove that EDDS is superior to EDTA in the extraction of Cu by plant shoots from the contaminated soil. The increased uptake of Cu by the application of hot EDDS was much higher than that of EDTA (Lombi et al., 2001a; Meers et al., 2005), EDDS (Kos and Leštan, 2003a, b; Meers et al., 2005) and NTA (Kulli et al., 1999; Kayser et al., 2000). The percentage of Cu extracted was 3.4-6% of the total Cu in the soil by the shoots of garland chrysanthemum during a 42-d period of plant growth and 1-1.3% by beans for 28 days. These values were higher than the data reported by Kos and Leštan (2003b) and comparable with the results of Blaylock et al. (1997) for the Pb extraction with EDTA.

Of the chelates tested for solubilizing soil Pb and enhancing the accumulation of the metal in plant shoots, EDTA has been found to be the most effective due to its strong chemical affinity for Pb (log K_s = 17.88) (Huang et al., 1997; Tandy et al., 2004; Shen et al., 2002; Chapter 4). In the present study, the concentrations of Pb in the shoots of garland chrysanthemums and beans reached 2080 and 1320 mg kg^{-1} on the 7^{th} day after the addition of 5 mmol kg^{-1} of hot EDDS solutions to the soil (Figs. 5.7 and 5.8), respectively, which represented a 365- and 176-fold increase compared with that in the corresponding controls (without the application of chelates); and increased 7.2- and 11.5-fold compared with that in the plants treated with 5 mmol kg^{-1} of

normal EDTA. For the extraction of Pb in the shoots of garland chrysanthemum and beans, increases of up to 216- and 93-fold were also found with 5 mmol kg^{-1} of hot EDDS compared with those in the control (Table 5.5). The increased uptake of Pb was much higher by the application of hot EDDS than that of normal EDTA at the same rates of application, as reported previously (Grčman et al., 2003; Chapter 4). This indicated that hot EDDS solutions might also be effective in the phytoremediation of Pb-contaminated soils. In the pot experiments described in the literature, the concentrations of Pb in plant shoots was generally lower than 2000 mg kg^{-1} DW after the application of EDTA (Wu et al., 1999; Bricker et al., 2001; Grčman et al., 2001; Lombi et al., 2001a; Barocsi et al., 2003; Grčman et al., 2003; Kos and Lestan, 2003a; Kos et al., 2003; Walker et al., 2003; Wenzel et al. 2003; Chen et al., 2004a; Lim et al., 2004; Meers et al., 2004), except for the results in a few experiments (Blaylock et al., 1997; Huang et al., 1997; Epstein et al., 1999; Shen et al., 2002). Blaylock et al. (1997) reported that the concentrations of Pb in the shoots of Indian mustard increased from less than 100 to 15 000 mg kg^{-1} when the plants were grown in soil containing 600 mg kg^{-1} of Pb amended with 10 mmol kg^{-1} of EDTA. Huang et al. (1997) measured more than 10 000 mg kg^{-1} of Pb in the shoots of corn grown in soil containing 2 500 mg kg^{-1} of Pb with the addition of 5.5 mmol kg^{-1} of EDTA. The different Pb phytoextraction efficiencies of the EDTA treatment might be attributed to different experimental conditions, for example, soil properties, plant status, and methods of applying chelate.

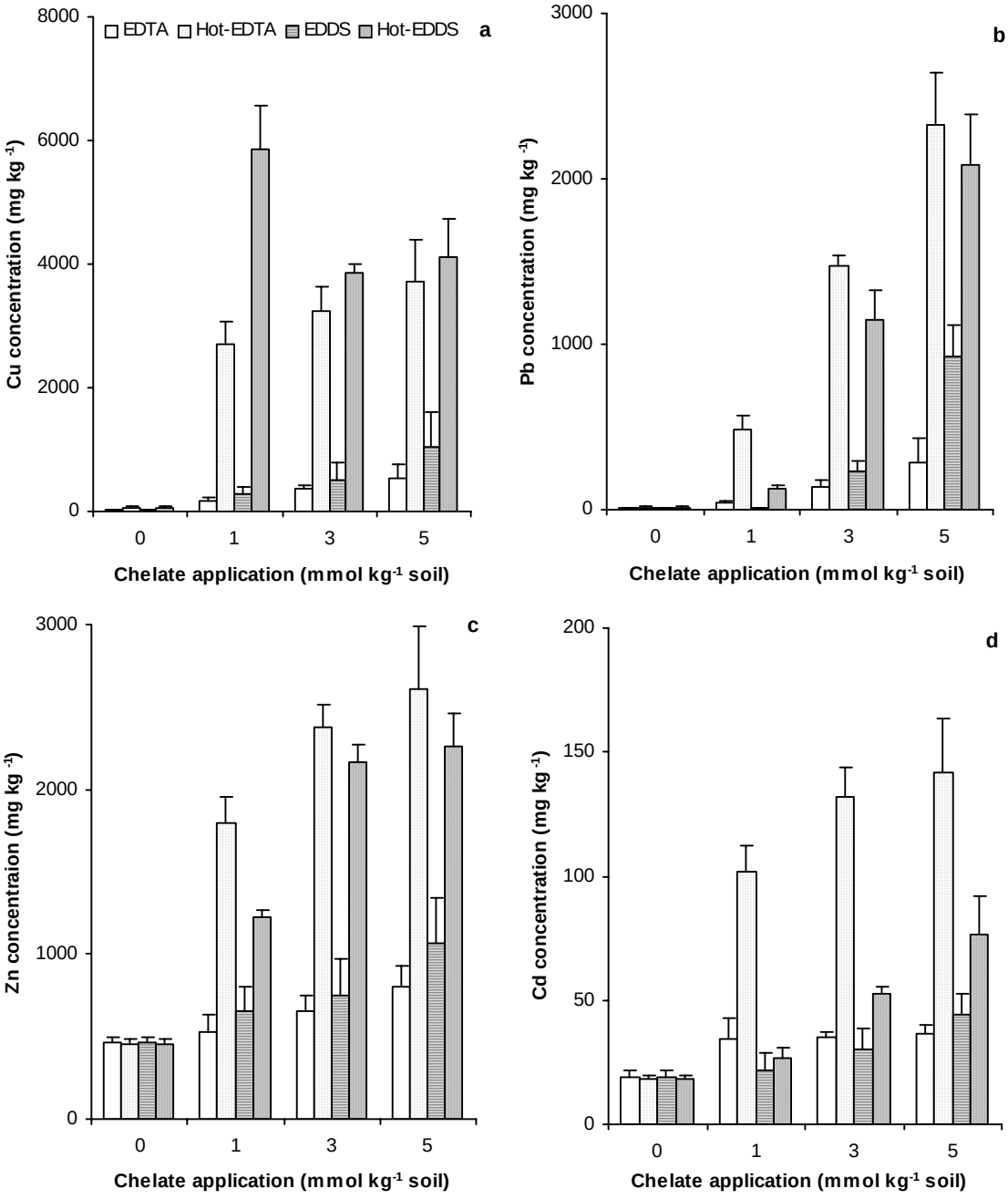


Fig. 5.7 Effects of the application of EDTA and EDDS on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of garland chrysanthemum. Values are means ± S.D. (n = 3).

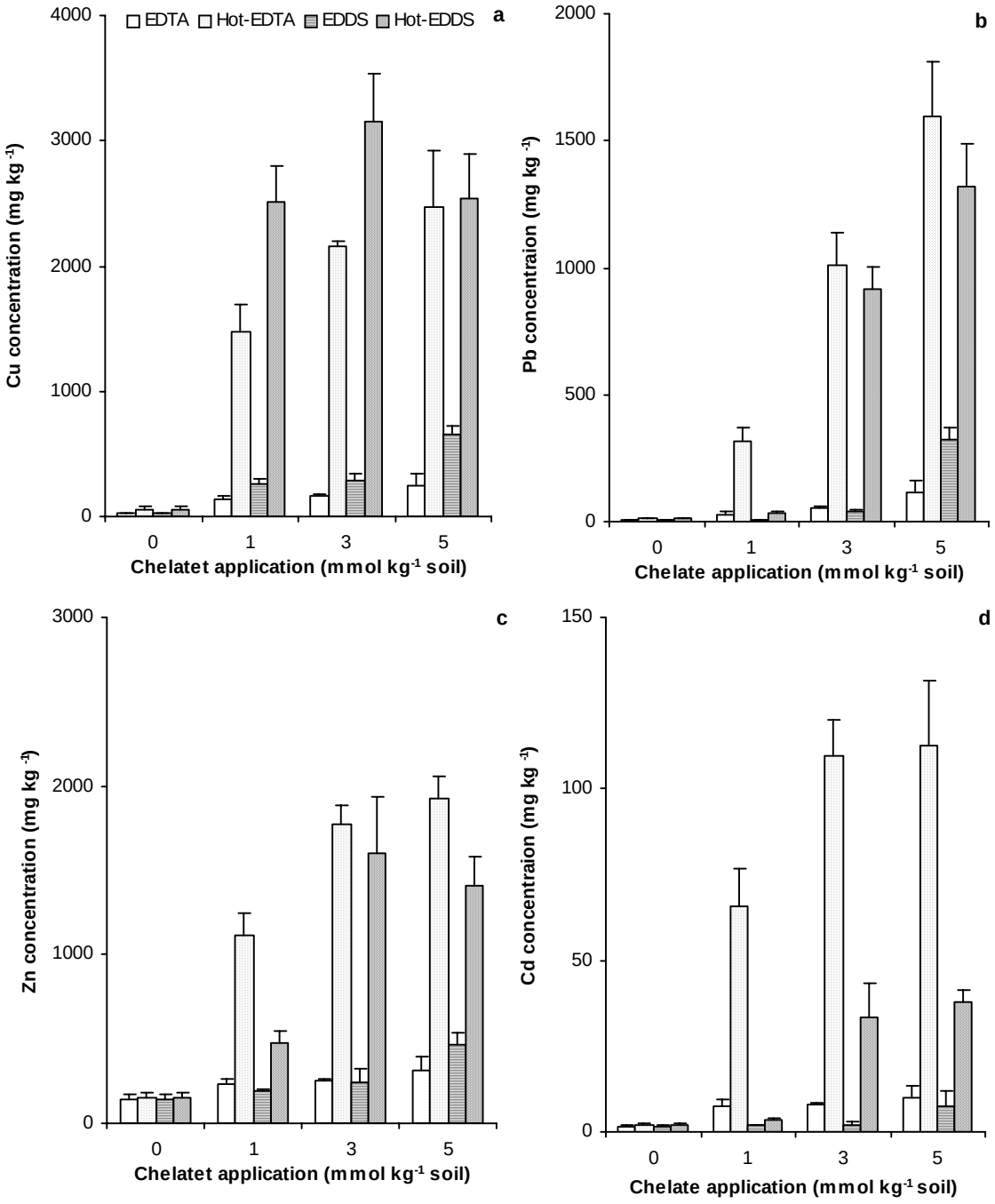


Fig. 5.8 Effects of the application of EDTA and EDDS on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of beans. Values are means ± S.D. (n=3).

Table 5.5 Total phytoextraction (μg kg^{-1} soil) of Cu, Pb, Zn and Cd in the shoots of garland chrysanthemum and beans 7 d after the application of EDTA and EDDS at different concentrations (mmol kg^{-1} soil)

	Garland chrysanthemum				Beans			
Treatments	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
Water	160 ± 19 a	32 ± 6 a	2660 ± 420 a	105 ± 13 a	72 ± 8 a	23 ± 10 a	456 ± 70 a	5.2 ± 2 a
Hot-water	292 ± 48 a	73 ± 9.6 a	2380 ± 300 a	96 ± 12 a	144 ± 18 a	29 ± 6 a	404 ± 42 a	5.4 ± 1.8 a
1mM EDTA	896 ± 138 a	197 ± 22 a	2720 ± 300 a	176 ± 28 a	380 ± 58 a	69 ± 12 a	624 ± 108 a	19 ± 6 a
Hot-1mM EDTA	11600 ± 1340 c	2080 ± 370 b	7660 ± 900 b	436 ± 70 c	3120 ± 500 c	670 ± 80 b	2360 ± 420 b	140 ± 18 c
3mMEDTA	1670 ± 106 b	666 ± 94 a	3080 ± 500 a	166 ± 18 a	402 ± 70 a	126 ± 24 a	634 ± 108 a	19 ± 3.8 a
Hot-3mM EDTA	14200 ± 1820 c	6440 ± 820 d	10400 ± 1360 c	576 ± 80 c	4220 ± 700 c	1970 ± 150 c	3480 ± 420 c	216 ± 34 c
5mM EDTA	2340 ± 380 b	1240 ± 150 b	3460 ± 496 a	160 ± 26 a	568 ± 78 a	266 ± 50 a	740 ± 38 a	23 ± 6 a
Hot-5mM EDTA	13800 ± 1650 c	8660 ± 900 d	9720 ± 1220 c	526 ± 70 c	4540 ± 998 c	2940 ± 420 c	3540 ± 558 c	208 ± 30 c
1mM EDDS	1310 ± 118 a	46.6 ± 7.6 a	3100 ± 720 a	105 ± 12 a	626 ± 94 a	18 ± 4 a	478 ± 42 a	4.6 ± 2 a
Hot-1mM EDDS	24200 ± 1960 d	516 ± 70 a	5040 ± 680 a	108 ± 12 a	5120 ± 780 d	65 ± 8 a	980 ± 70 a	7.1 ± 4 a
3mM EDDS	2260 ± 470 b	1030 ± 160 b	3300 ± 480 a	132 ± 18 a	704 ± 84 a	94 ± 10 a	578 ± 20 a	5.2 ± 0.5 a
Hot-3mM EDDS	14600 ± 1600 c	4360 ± 500 c	8240 ± 1000 b	200 ± 30 a	5180 ± 720 d	1500 ± 136 b	2620 ± 306 b	56 ± 3.8 b
5mM EDDS	4120 ± 620 b	3680 ± 560 c	4260 ± 760 a	176 ± 30 a	1380 ± 156 b	692 ± 90 b	984 ± 58 a	15.6 ± 4 a
Hot-5mM EDDS	13600 ± 1560 c	6920 ± 980 d	7520 ± 970 b	254 ± 42 b	4160 ± 225 c	2160 ± 380 c	2320 ± 348 b	63 ± 5.2 b

Values are means ± S.D. (n=3); the different small letters stand for statistical significance at the $p < 0.05$ level.

5.3.7 Metal Leaching Study after the Treatment with EDTA and EDDS

In order to examine the potential metal leaching in the pots, the soil solution was extracted within 28 days after the application of chelates. For the same metal, the concentrations of water-soluble metals in soil were mainly dependent on the chelate type and application rate (Table 5.6 and Fig. 5.9). No significant differences were observed in the concentrations of soluble metals in the soils between the treatments with hot chelate solutions and with normal chelate solutions at the same application dosage (Table 5.6). The concentrations of soluble Cu were higher in the soil treated with EDDS than that with EDTA. But EDTA was more effective in solubilizing soil Pb and Cd than EDDS. In all treatments, the concentrations of water-soluble metals increased with the increasing levels of EDTA and EDDS applied to the soils and decreased with the prolonging of time (Fig. 5.9). This decrease was more pronounced in the soil treated with EDDS than in the soil treated with EDTA. For example, the average concentrations of soluble Cu, Pb, Zn and Cd decreased by 97, 44, 81, and 82%, respectively, from the 7^{th} day to 28^{th} day after the application of EDDS. On the 28^{th} after chelate application, no significant difference was found in the concentrations of soluble metals between EDDS treatment and the control (without chelate application). But in the soil treated with EDTA, the concentrations of soluble Cu, Pb, Zn and Cd decreased only by 26, 36, 39, and 40%, respectively, from the 7^{th} day to 28^{th} day after the chelate application, and were still significantly higher than those in the control group. Compared with EDTA, EDDS has a clear advantage because it is readily biodegradable and is less toxic to fish, daphnia, and soil fungi (Jaworska et al., 1999; Grčman et al.,

2003). The calculated half-life of EDDS in sludge-amended soil was 2.5 days (Jaworska et al., 1999). The results from the leaching study showed that, at the end of the experiment of 28 d, after the harvesting of the plants, metal solubility in the soil treated with EDDS was not significantly different from that in the control group. This implied that residual EDDS in the soil had been degraded and that the risk of metal leaching to the surrounding environments was relatively low.

Table 5.6 Effects of the application of EDTA and EDDS at different rate (mmol kg^{-1} soil) on metal solubility (mg kg^{-1} soil) 7 d after the application

Treatments	Cu	Pb	Zn	Cd
Water	2.6 ± 0.1 a	2.22 ± 0.1 a	4.37 ± 0.2 a	0.07 ± 0.01 a
Hot-water	2.9 ± 0.3 a	2.49 ± 0.2 a	4.8 ± 0.3 a	0.08 ± 0.01 a
1mM EDTA	36.7 ± 2.5 b	3.39 ± 0.2 a	18.1 ± 1.2b	1.06 ± 0.2 b
Hot-1mM EDTA	31.6 ± 3 b	3.47 ± 0.3 a	16.6 ± 0.6 b	0.85 ± 0.1 b
3mMEDTA	90.1±5.5 c	14.1 ± 0.9 b	65.6 ± 2.9 b	3.8 ± 0.2 c
Hot-3mM EDTA	88.8 ± 6 c	12.8 ± 1.1 b	57.5 ± 3.7 b	3.2 ± 0.3 c
5mM EDTA	131 ± 9.7 bc	43.9 ± 3.3 c	90 ± 5.9 c	5.72 ± 0.4 c
Hot-5mM EDTA	132 ± 6.5 bc	48 ± 2.5 c	85 ± 7.2 c	5.78 ± 0.2 c
1mM EDDS	87 ± 4.7 c	2.52 ± 0.1 a	8.34 ± 0.5 a	0.04 ± 0.01 a
Hot-1mM EDDS	85 ± 3.5 c	2.56 ± 0.2 a	9.37 ± 0.3 a	0.07 ± 0.01 a
3mM EDDS	176 ± 17 d	3.44 ± 0.3 a	62.3 ± 2.1 b	0.11 ± 0.02 a
Hot-3mM EDDS	169 ± 15 d	2.96 ± 0.2 a	65.2 ± 3.6 b	0.1 ± 0.01 a
5mM EDDS	203 ± 12 d	5.04 ± 0.4 a	97 ± 4 c	0.36 ± 0.03 a
Hot-5mM EDDS	198 ± 18 d	4.1 ± 0.1 a	95 ± 6.8 c	0.22 ± 0.04 a

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

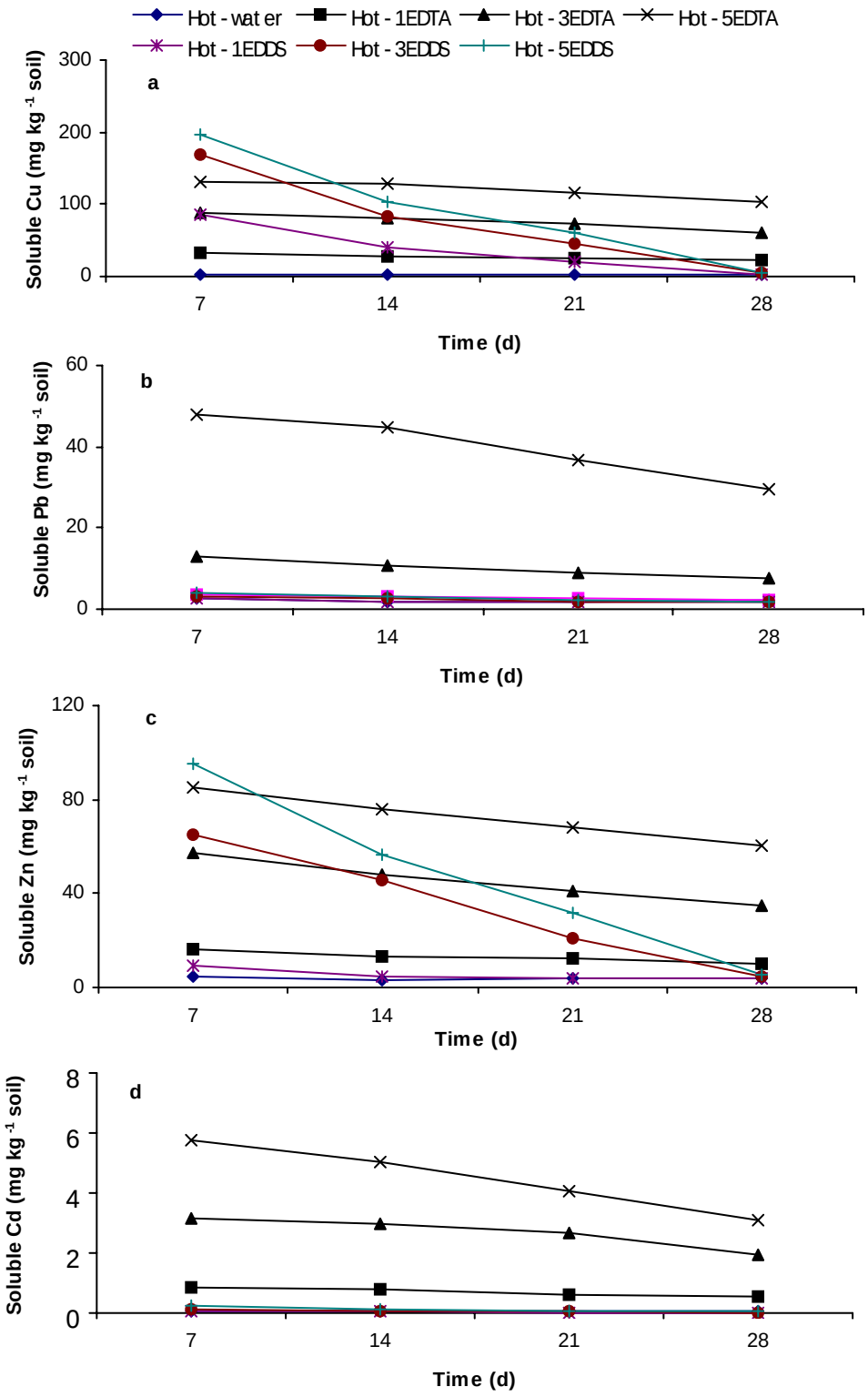


Fig. 5.9 Effects of the application of hot EDTA and EDDS at different concentrations on the solubility of Cu (a), Pb (b), Zn (c) and Cd (d). Values are means ± S.D. (n = 3).

5.3.8 Metal Accumulation in Treatments of Hot Citric Acid and NTA

As compared to the control group, the application of citric acid solutions without heating, even at the rate of 5 mmol kg^{-1} soil, did not produce any significant effect on metal concentrations in the shoots of beans (Table 5.7). When citric acid was added as hot solution at rates of 1, 3 and 5 mmol kg^{-1} to the soil, the concentrations of Cu, Pb, Zn and Cd significantly increased. On average, the concentrations of Cu, Pb, Zn and Cd in the shoots of beans treated with hot citric acid increased 5.9, 3.5, 1.4 and 2.1 times, respectively, of those in the treatments with normal citric acid solutions. Similarly, the application of hot NTA solution to the soil significantly increased the concentrations of Cu, Pb, Zn and Cd in the shoots of beans. The averages of 4.9-, 9.1-, 2.7- and 5-fold enhancement were obtained for the concentrations of Cu, Pb, Zn and Cd in the shoots, in comparison with those in the treatments with normal NTA solutions, respectively.

Table 5.7 Effects of the application of citric acid (CA) and NTA at different concentration (mmol kg^{-1} soil) on the shoot dry weight (g pot^{-1}) and concentration (mg kg^{-1}) of Cu, Pb, Zn and Cd in the shoots of beans

Treatment	Biomass	Cu	Pb	Zn	Cd
Water	1.72 ± 0.02 c	17.5 ± 2 a	2.14 ± 0.2 a	121 ± 14 a	1.65 ± 0.2 a
Hot-water	1.4 ± 0.01 b	33.2 ± 3.2 a	4 ± 0.4 a	142 ± 11 a	2.66 ± 0.4 a
1mM CA	1.79 ± 0.04 c	18.9 ± 2.8 a	2.31 ± 0.1 a	139 ±1 6 a	1.72 ± 0.08 a
Hot-1mM CA	1.51 ± 0.02 b	59.6 ± 8.4 a	7.81 ± 1.1 a	152 ± 4 a	2.55 ± 0.2 a
3mM CA	1.70 ± 0.05 c	17.7 ± 3.3 a	1.71 ± 0.1 a	125 ± 7.8 a	1.5 ± 0.14 a
Hot-3mM CA	1.46 ± 0.02 b	128 ± 35 b	6.08 ± 0.8 a	183 ± 6.3 a	3.19 ± 0.2 a
5mM CA	1.71 ± 0.03 c	16.9 ± 0.1 a	2.11 ± 0.4a	123 ± 15 a	1.55 ± 0.14 a
Hot-5mM CA	1.39 ± 0.04 b	122 ± 44 b	7.65 ± 0.5 a	195 ± 38 a	3.96 ± 1.7 a
1mM NTA	1.64 ± 0.05 c	116 ± 4.6 b	4.97 ± 0.5 a	211 ± 7.8 a	4.32 ± 0.3 a
Hot-1mM NTA	1.35 ± 0.01 b	539 ± 123 c	21.8 ± 2.5 b	493 ± 88 b	10.2 ± 1.9 a
3mM NTA	1.48 ± 0.06 b	172 ± 34 b	8.62 ± 1.1 a	229 ± 20 a	5.11 ± 0.6 a
Hot-3mM NTA	1.25 ± 0.02 a	1250 ± 438 c	151 ± 33 c	1140 ± 336 c	46.8 ± 13 b
5mM NTA	1.41 ± 0.03 b	190 ± 22 b	13.5 ± 3.4 b	275 ± 31 b	6 ± 0.2 a
Hot-5mM NTA	1.21 ± 0.02 a	1110 ± 335 c	113 ± 55 c	1040 ± 151 c	38.9 ± 2.7 b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

5.3.9 Effects of Pretreatment with Hot Water on the Accumulation of Pb in Beans

Roots of beans were pretreated with hot water at different temperature before they were exposed in solutions containing 500 μmol L^{-1} of Pb + 500 μmol L^{-1} of EDTA and 500 μmol L^{-1} of Pb + 500 μmol L^{-1} of EDDS, respectively. Two days after the Pb + EDTA or EDDS exposure, the Pb concentrations in shoots were measured (Fig. 5.10). The results showed that there was a significantly positive correlation between the Pb concentration in shoots and the relative electrolyte leakage rate of root cells (R^2 = 0.91, n = 27 for EDTA treatment; and R^2 = 0.90, n = 27 for EDDS treatment). Similar significantly positive correlation results were also obtained for the metals of Cu, Zn and Cd (see Table 5.8).

Several studies on the accumulation of Pb in plants showed that the metal was absorbed and transferred as a Pb-EDTA complex in the presence of high concentrations of EDTA (Vassil et al., 1998; Epstein et al., 1999). Sarret et al. (2001) reported that both Pb and EDTA could be absorbed by plants, and that some of the Pb present in the leaves of *P. vulgaris* was complexed to EDTA. If the plant uptake of metal chelating complexes occurs at breaks in the endodermis of the root and in the Casparian strip as suggested by Bell et al. (1991), in the chemically enhanced phytoextraction process, the uptake of metal would be strongly dependent on the concentration of the metal-chelant complex in the solution and on the breakdown of the root exclusion mechanism. In the pot experiments, it was assumed that high temperatures caused the breakdown of the root exclusion mechanism, and that the chelate increased the

concentrations of the metal-chelate complex in soil solution, especially when the chelates were applied in hot solutions, which led to the rapid equilibration of metal-chelate between the external solution and the sap of the xylem. After entering the xylem, metal-chelate would be translocated from the roots to shoots by the transpiration stream, leading to high concentrations and the accumulation of metals in shoots. It was found that in the temperature range of 8-48 ºC, each 10 increment resulted in a 6% increase in the metal extracted from soil for Zn, Pb, and Cd (Vandevivere et al., 2001a). Enhanced concentrations of metals in plant tissues with increasing temperature were observed in other experiments (Antoniadis and Alloway, 2000; Fritioff et al., 2005). This hypothesis was also confirmed by the data obtained from the hydroponic experiment. Fig. 5.10 shows a significantly positive correlation between the Pb concentration in the shoots of beans and the relative electrolyte leakage rate of root cells (root damage by hot water). Therefore, the root damage treatment of plants can play an important role in increasing the metal uptake in chemically enhanced phytoextraction. The application of hot EDDS solutions may be a good alternative approach in this direction.

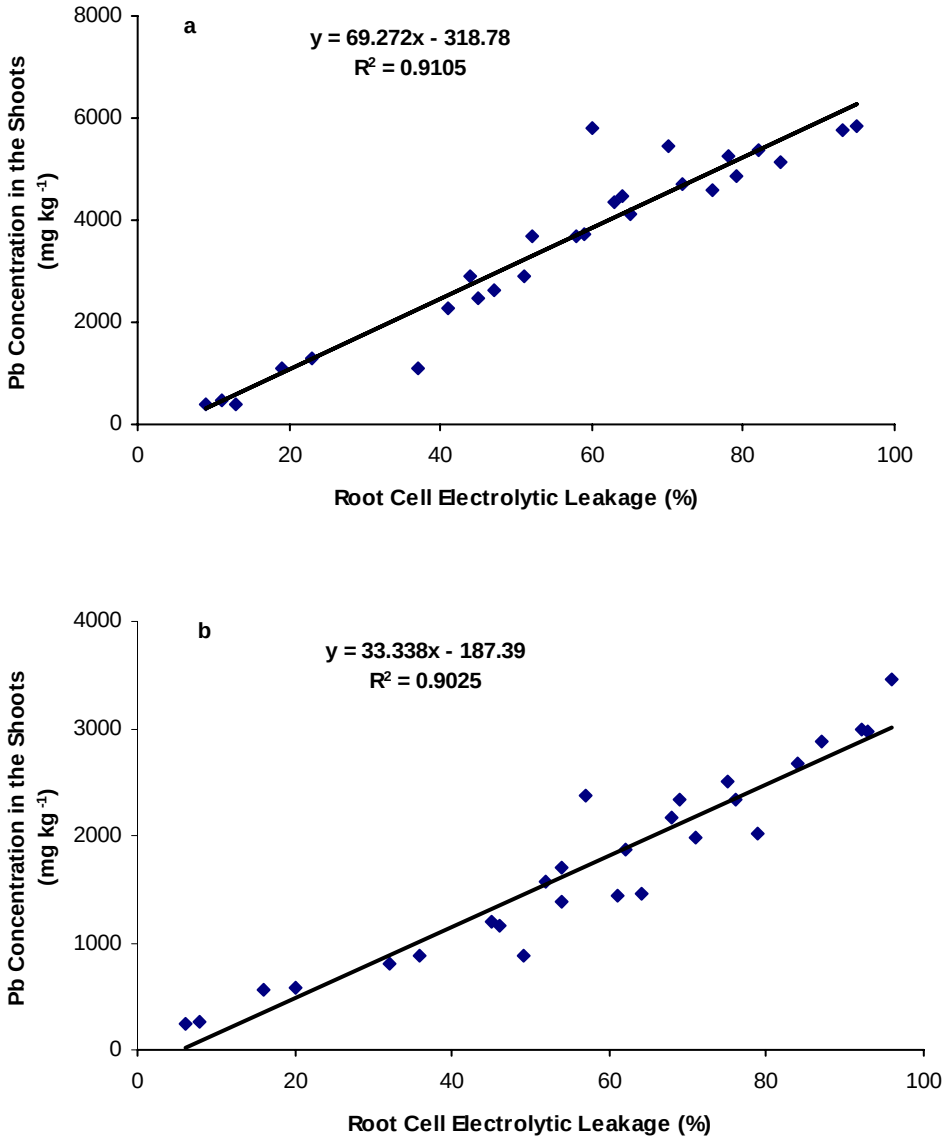


Fig. 5.10 The correlation between the relative electrolyte leakage rate of roots and the concentration of Pb in the shoots of beans. Plants were pretreated with hot water at different temperatures, then exposed in solutions containing 500 µmol L^{-1} of Pb + 500 µmol L^{-1} of EDTA or 500 µmol L^{-1} of Pb + 500 µmol L^{-1} of EDDS for 2 d. The root cell electrolytic leakage was measured immediately after the pretreatment with hot water.

Table 5.8 The correlation between the relative electrolyte leakage rate of roots and the concentrations of Cu, Zn, and Cd in the shoots of beans (R^2 was shown in the Table). Plants were pretreated with hot water at different temperatures, then exposed in solutions containing 500 μmol L^{-1} of Cu, Zn, or Cd + 500 μmol L^{-1} of EDTA or EDDS for 2 d, respectively. The root cell electrolytic leakage was measured immediately after the pretreatment with hot water.

Treatments	R^2
500 μmol L^{-1} of Cu + 500 μmol L^{-1} of EDTA	0.88
500 μmol L^{-1} of Cu + 500 μmol L^{-1} of EDDS	0.95
500 μmol L^{-1} of Zn + 500 μmol L^{-1} of EDTA	0.90
500 μmol L^{-1} of Zn + 500 μmol L^{-1} of EDDS	0.94
500 μmol L^{-1} of Cd + 500 μmol L^{-1} of EDTA	0.86
500 μmol L^{-1} of Cd + 500 μmol L^{-1} of EDDS	0.87

Chapter 6 - Metal Leaching Study After the Application of EDDS

6.1 Introduction

Efficient phytoextraction of metal contaminated soils should satisfy two basic requirements. Firstly, the metals in the soil can be removed to under the regulation standard by harvesting the plants within a reasonable time span. Secondly, the technology should not pose a second contamination threat to the surrounding environment. The widely tested chelate of EDTA and its complexes with metals are usually toxic and poorly photo-, chemo-, and biodegradable in soil environments, which can persist in the soil for several weeks or months after harvest of the phytoextraction crops (Bucheli-Witschel and Egli, 2001; Nowack, 2002; Grčman et al., 2003). The combination of widespread use in fertilizers and slow decomposition in surface environment has led to background concentrations of EDTA in European surface waters in the range of 10 to 50 mg L^{-1} (Kari et al., 1995). The EDTA-enhanced leaching of heavy metals in soils has been extensively documented using batch and column-leaching experiments (Papassiopi et al., 1999; Römkens et al., 2002; Kos and Leštan, 2003a, b; Madrid et al., 2003). In soil columns where cabbage plants were grown, Grěman et al., (2001) observed that 38%, 10%, and 56% of the initial total Pb, Zn, and Cu, respectively, in soil were leached down the soil profiles during the 10 mmol kg^{-1} EDTA treatmens. Kos and Leštan (2003a) found that 2.5-10 mmol kg^{-1} of EDTA caused the leaching of a large portion of

the total Pb initially present in the soil. All the potential risks when using chelates for phytoextraction should therefore be thoroughly evaluated before practical application of this remediation technology in the field.

EDDS (S,S-ethylenediaminedisuccinic acid), as a biodegradable chelate, can strongly complex with transition metals and radionuclides (Jones and Williams, 2001). Mineralization of EDDS in sludge-amended soil was rapid and the process would complete in 28 d. In soil column treated with weekly additions of 10 mmol kg^{-1} EDDS, about 0.8 and 1.5% of initial total Pb and Cd in the soil were leached through the soil profile. However, the same amount of EDTA caused 22.7 and 39.8% of initial total Pb and Cd leaching (Grěman et al., 2003). A biotest with red clover indicated a greater phytotoxic effect of EDTA than EDDS addition to soil. EDDS was also less toxic to soil fungi and caused less stress to soil microorganisms (Grěman et al., 2003). It was reported that the combination of EDDS and permeable barriers might lead to more environmentally safe induced Pb phytoextraction and the *in situ* washing of Pb (Kos and Leštan, 2003a, b).

Reducing metal leaching associated with the application of chelates can be accomplished by decreasing the application dosage and using easily biodegradable chelates. Results from Chapter 5 suggested for a given metal removal efficiency, the reduction of chelate dosage can be reached by the application of hot chelate solutions. In the present study, the similar experimental design was used in pot studies to study the effects of hot EDDS on metal phytoextraction in the development of better chelate-assisted

phytoextraction technologies. Emphasis was given to the assessment of adverse effects arising from EDDS application on the leaching of metals from the root zone.

6.2 Materials and Methods

6.2.1 Soil Preparation

Soil samples used in this part are the same as used in Chapters 3 for the study of garland chrysanthemum and corn and in Chapter 5 (see Table 3.1, soils for 3.2.2). The soils were artificially contaminated with multi-metals: Cu (400 mg kg^{-1} of soil) as $CuCO_3$ (copper carbonate); Pb (500 mg kg^{-1} of soil) as $Pb_3(OH)_2(CO_3)_2$ (lead hydroxide carbonate) and PbS (lead sulfide – galena, a common lead mineral in mining areas) at a Pb concentration ratio of 1:1; Zn (500 mg kg^{-1} of soil) as $ZnCO_3$ (zinc carbonate) and ZnS (zinc sulfide) at a Zn concentration ratio of 1:1; and Cd (15 mg kg^{-1} of soil) with $Cd(NO_3)_2 \cdot 4H_2O$ (cadmium nitrate).

6.2.2 Effects of EDDS Treatment on Plant Growth and Metal Uptake

Air-dried soils (500 g) were placed in plastic pots (12 cm i.d. x 12 cm height). A glass fiber filter GC-50 (D = 1.2 μm) was put in the bottom of the pot to retain the soils. The moisture of the soil moisture was maintained to near field water capacity by adding deionized water (DIW) on a daily basis. Seeds of corn (*Zea mays* L. cv. Nongda No. 108) and beans (*P vulgaris* L. white bean) were sown

directly in the soils. After germination, the seedlings were thinned to four plants per pot. On the 14th day after the sowing plants, EDDS (from Fluka Chemie GmbH) was applied to the surface of the soils in two different ways (heated and not heated) at rates of 0 (control), 1.0, and 5.0 mmol kg^{-1} soil as 100 ml of Na_3EDDS solutions. To make up the different amounts of chelate treatments, EDDS were diluted from 50 mM of Na_3EDDS (pH 10.1) salt solutions. The hot chelate solution treatments were conducted by adding boiled solution to soil in the pots which resulted in the final temperature of the soils was about 40 ºC at the 2/3 depth of the pot. Twelve replicates were conducted for each treatment. All of the experiments were operated in the glasshouse under natural light. Air temperatures ranged from 25 to 33 ºC. All the plants were harvested by cutting the shoots 0.5 cm above the surface of the soil 7 d after the application of chelates. The shoots were washed with tap water and rinsed with DIW, and dried at 70 ºC in a drying oven to a constant weight for dry weight measurements and metal analysis. During the treatment and after harvesting the plants, the moisture of the soil was also maintained at near field water capacity by adding DIW.

6.2.3 Pot Leaching Study with the Addition of Artificial Rainwater

Leaching study was carried out with artificial rainwater on the Day 0, 7, 14, 21, 28 and 35 after the application of EDDS. The composition of the artificial rainwater (mg l^{-1}) used in the present leaching experiment was NO_3^-: 1.94; NH_4^+: 0.49; Na^+: 1.87; Mg^{2+}: 0.25; Ca^{2+}: 0.29; Cl^-: 3.41; SO_4^{2+}: 2.65; pH = 4.40 (Hodson et al., 2001). When carrying out the leaching experiment, two pots

from every treatment were added with 800 ml artificial rainwater (equivalent to about 71 mm of rainwater precipitation) in four equally split applications continuously. One 800 ml plastic beaker was placed under the bottom of the pots to collect the leachate. For the leaching study on the Day 0 of EDDS application, 700 ml of artificial rainwater was added because it was carried out immediately after the addition of 100 ml of EDDS solution.

6.2.4 Chemical and Physical Analysis

The leachate solutions were filtered through Whatman No. 42 filter paper. The volume and pH of the leachate were measured immediately after collection at the bottom of the pots. The dissolved total organic carbon (DOC) was measured with a Shimadzu TOC-5000A analyzer. A sub-sample was digested with concentrated HNO_3, then diluted with 5% HNO_3 and analyzed for heavy metals using ICP-AES.

Metal analysis for the plant and soil samples was carried out as the previous Chapters. The recovery rates were around 90 ± 7% for all of the metals in the plant reference material. The data reported in this paper were the mean values based on the three replicated experiment results. Statistical analyses of the experimental data, such as correlation and significant differences, were performed using SPSS® 11.0 statistical software.

6.3 Results and Discussion

6.3.1 Plant Growth

The dry mass yields of corn and beans are shown in Fig. 6.1. Before the application of EDDS, all of the plants grew very well and no visual symptoms of toxicity were shown. In the control group, the application of 100 ml of hot water severely impaired the growth of the plants. The plant leaves became chlorotic and the dry biomass yields decreased by 19% and 22% for corn and beans, respectively, compared with the plants added with normal water, 7 d after the hot water addition.

Similarly, at the same EDDS application dosage, the decrease in plant dry biomass yields was more pronounced when EDDS was applied as hot solutions to the surface of the soil than was the case without heating. In comparison with the corresponding application of EDDS as the normal solutions, the shoot biomass yields of corn and beans were decreased by 18% and 24%, respectively, when EDDS was added in hot solutions. The dry weights of the two plants decreased with the increasing level of EDDS applied (Fig. 6.1).

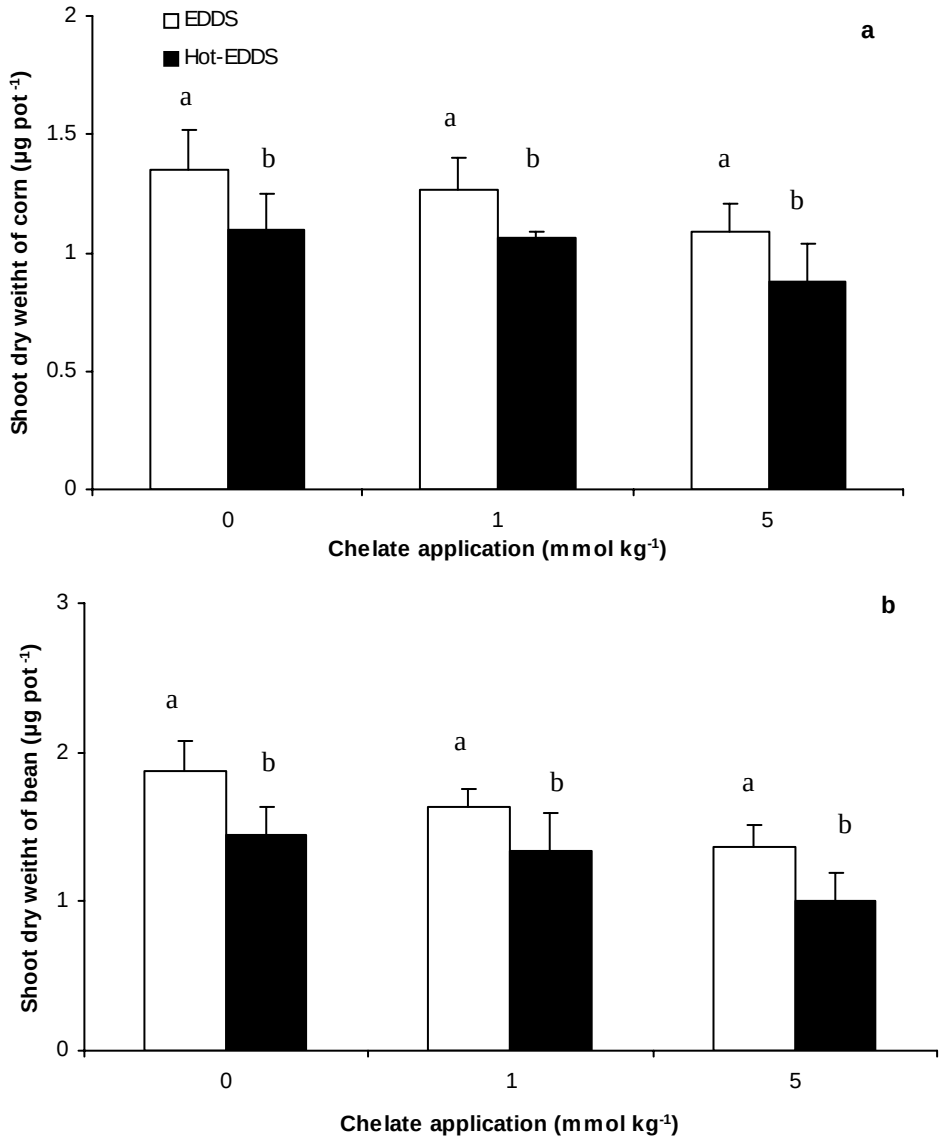


Fig. 6.1 Effects of the application of EDDS on the shoot dry weights of corn (a) and beans (b). Values are means ± S.D. (n = 12); the different small letters stand for statistical significance at the $p < 0.05$ level.

6.3.2 Metal Concentrations and Phytoextraction with the Application of EDDS

Compared with the control group, the addition of EDDS significantly increased the concentrations of Cu, Pb, Zn, and Cd in the shoots of both plant species (Figs. 6.2 and 6.3). In all the treatments, a larger increase of metal concentrations was observed in beans than in corn, which was consistent to the results described in Chapter 4 on chelate application studies.

The metal concentrations in the shoots of corn and beans significantly increased with the increasing level of EDDS applied to the soil (Figs. 6.2 and 6.3). At the same application dosage, the application of hot EDDS solutions produced higher concentrations of Cu, Pb, Zn, and Cd in the shoots of both plant species than the application of EDDS solutions without heating (Figs. 6.2 and 6.3). Compared with the normal EDDS application, the concentrations of Cu, Pb, Zn, and Cd in the shoots were averagely increased to 6.6, 12.3, 2.1 and 1.01 times of the control for corn and 8.1, 11, 3.2, and 12.2 times for beans, respectively, when the EDDS was applied to the soil in hot solutions. The highest concentrations of Cu, Pb, Zn and Cd at 3420, 1980, 1430 and 82 mg kg^{-1} were found in the shoots of beans with the application of hot EDDS at the rate of 5 mmol kg^{-1}, which were 61, 198, 11, and 37-fold of those found in the control group (without the application of EDDS).

Enhanced metal availability to the roots is the most important factor governing the metal uptake by plants. Several studies on the accumulation of Pb in plants

showed that the metal was absorbed and transferred as a Pb-EDTA complex in the presence of high concentrations of EDTA (Vassil et al., 1998; Epstein et al., 1999). The application of EDDS could also greatly increase the water-soluble metal concentrations in soil (see Chapter 4; Meers et al., 2005). In the EDTA-enhanced phytoextraction process, it is thought that EDTA can destroy the physiological barrier(s) in plant roots that normally function to control the uptake and translocation of solutes, by which would lead to the rapid equilibration of the hydroponic or soil solution with the xylem sap (Vassil et al., 1998). Kulli et al. (1999) reported that high NTA application resulted in a breakdown of the exclusion mechanism and high Cu concentrations in test plants. In the present study, it was assumed that high temperatures caused the breakdown of the root exclusion mechanism, and in combination with the increased concentrations of the metal-chelate complex in soil solution, led to the rapid equilibration of metal-chelate between the external soil solution and the sap of the xylem. After entering the xylem, metal-chelate would be translocated from roots to shoots by the transpiration stream, leading to high concentrations and the accumulation of metals in shoots. The majority of Pb and EDTA uptake probably occurred rapidly after the EDTA application, even before a measured transpiration decreased, allowing a high rate of metal uptake followed by the decreased transpiration (Epstein et al., 1999). This was accordance with the significantly higher phytoextraction observed in the plants treated with hot EDDS solutions, although the plant growth and dry biomass was significantly affected, compared with the plants treated with the normal EDDS solutions (see Fig. 6.1). In the present study, the highest phytoextraction of Cu, Pb, Zn, and Cd were found in the shoots of beans, which were 8.94 mg kg^{-1} for Cu obtained

from the treatment of 1 mmol kg^{-1} of hot EDDS, and 4.0, 2.88, and 0.16 mg kg^{-1} for Pb, Zn, and Cd, resectively, achieved in the 5 mmol kg^{-1} of hot EDDS application (See Table 6.1). For corn, the highest phytoextraction of Cu, Pb, Zn, and Cd were 1.92, 0.76, 0.93, and 0.06 mg pot^{-1}, respectively (see Table 6.1).

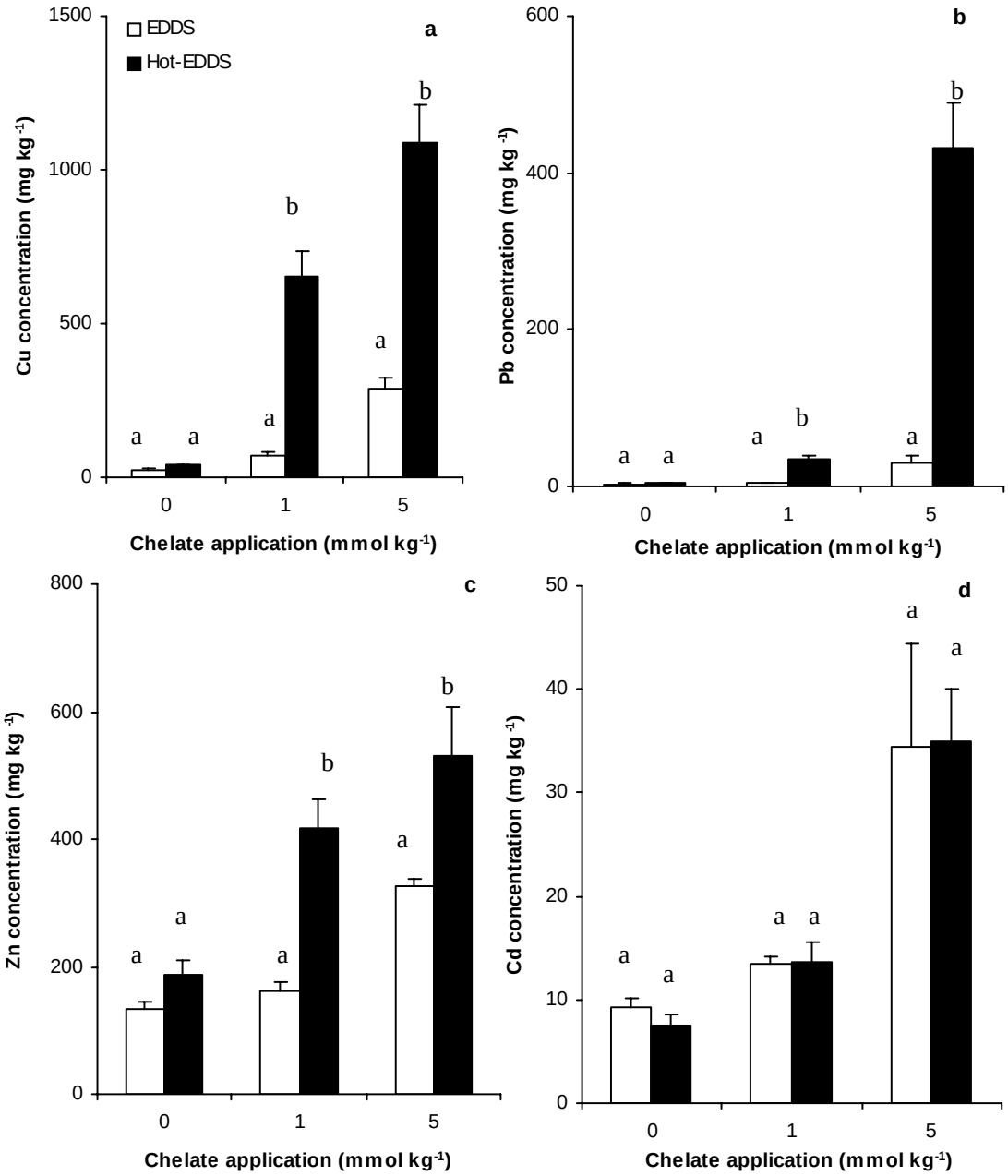


Fig. 6.2 Effects of EDDS application on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of corn. Values are means ± S.D. (n = 12); the different small letters stand for statistical significance at the $p < 0.05$ level.

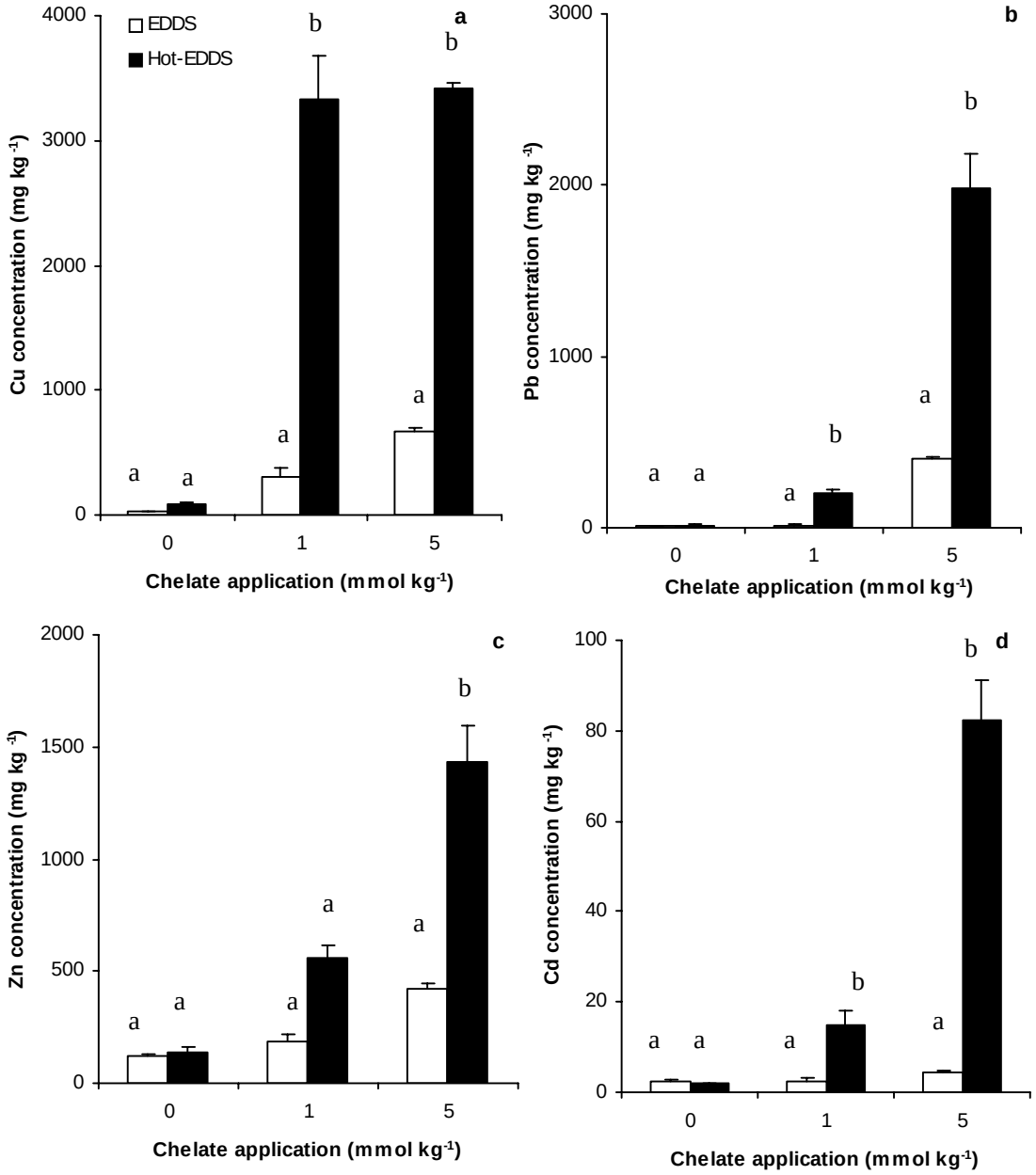


Fig. 6.3 Effects of EDDS application on the concentrations of Cu (a), Pb (b), Zn (c) and Cd (d) in the shoots of beans. Values are means ± S.D. (n = 12); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 6.1 Total phytoextraction (μg kg^{-1} soil) of Cu, Pb, Zn, and Cd in the shoots of corn and beans 7 d after the application of EDDS at different concentrations (mmol kg^{-1} soil)

	Corn				Beans			
Treatments	Cu	Pb	Zn	Cd	Cu	Pb	Zn	Cd
Water	70 ± 5.2 a	8 ± 1.6 a	360 ± 28 a	25 ± 2.2 a	89 ± 1.2 a	20 ± 0.6 a	454 ± 27 a	9 ± 0.9 a
Hot-water	85 ± 8.8 a	9.6 ± 2.2 a	410 ± 50 a	17 ± 1.9 a	254 ± 26 a	43 ± 6 a	394 ± 66 a	5.4 ± 0.8 a
1mM EDDS	176 ± 34 a	9 ± 0.2 a	408 ±36 a	34 ± 1.8 a	992 ± 114 a	38 ± 10 a	600 ± 112 a	7.8 ± 2 a
Hot-1mM EDDS	1390 ± 160 c	73 ± 7 b	884 ± 95 b	29 ± 4.2 a	8940 ± 532 d	532 ± 56 b	1500 ± 150 b	40 ± 8 ab
5mM EDDS	630 ± 75 b	63 ± 5.6 b	712 ± 20 b	75 ± 15 b	1800 ± 74 b	1090 ± 30 b	1140 ± 69 ab	12 ± 0.4 a
Hot-5mM EDDS	1920 ± 210 c	762 ± 100 c	934 ± 134 b	62 ± 8.8 b	6920 ± 700 c	4000 ± 420 c	2880 ± 320 c	166 ± 18 b

The values are means ± S.D. (n = 12); the different small letters stand for statistical significance at the $p < 0.05$ level.

6.3.3 The Leaching of Metals from the Pots

In order to examine the potential leaching of metals in pots and the effects of root growth on the metal leaching, the soil in pots was leached with artificial rainwater within 35 d after the application of EDDS. The volume of the leachate solutions ranged from 600 to 690 ml (equivalent to about 53-61 mm rainfall), and the pH ranged from 6.95 to 7.26 (Tables 6.2 and 6.3). Slightly higher pHs were observed in the treatments with the 5 mmol kg^{-1} of EDDS application on the day of application, which was speculated to be caused by the higher pH of EDDS solutions applied to soil.

The amounts of DOC and metals leached from the pots were also measured. In the leachates from the control group (without the application of EDDS), the amounts of DOC ranged from 21 to 34.9 mg. The average amounts of Cu, Pb, Zn, and Cd in the leachates were 1.05, 0.9, 1.55 and 0.028 mg, respectively, which accounted only 0.53%, 0.36%, 0.62% and 0.37% of the total initial metals in the soil, respectively.

The application of EDDS dramatically enhanced the amounts of DOC in the leachate solutions. The DOC in the leachates increased as increasing levels of EDDS were applied to the soils (Fig. 6.4). The highest DOC amounts reached 1062, 980, and 954 mg in the leachates without plants, and with corn and beans growth, respectively, on just after the application of 5 mmol kg^{-1} EDDS. Thereafter, the amounts of DOC decreased as time went by, and the decrease was more pronounced in the soil with plant growth than the soil without plants.

No significant differences were observed in the amounts of DOC in the leachates between the pots grown with corn and beans, and between the treatments with hot EDDS solutions and those with normal EDDS solutions at the same application dosage (see Table 6.4).

Amounts of Cu, Pb, Zn, and Cd in the leachate solutions closely followed the pattern of DOC. In all treatments, total metal amounts in the leachate solutions increased with the increasing levels of EDDS applied, and decreased with the prolonging of leaching time (Fig. 6.4).

On the day of chelate application, the application of 1 mmol kg^{-1} of EDDS as normal solutions led to an average 70.4, 1.99, 7.83 and 0.072 mg of Cu, Pb, Zn and Cd leached out from the pots, which were 73.3, 2.2, 4.8 and 2.6 times of that leached from the control group (without the EDDS application), respectively. When the application of EDDS was increased to 5 mmol kg^{-1}, the average amounts of Cu, Pb, Zn and Cd in the solutions were increased to 123, 3.52, 81.7 and 0.252 mg, respectively, which account for 61.5%, 1.4%, 40.1% and 3.4% of the initial metals, respectively.

It was reported that some plants with a long, massive and complex root systems, such as vetiver grass could effectively reduce the leaching of heavy metals downwards in an EDTA-assisted phytoextraction process (Chen et al., 2004b). However, in the present study, no significant differences were found in the amounts of metals leached between the pots with and without plants on the day with the application of EDDS, which meant plant growth in the pots had little

effects on the metal leaching under the current experimental conditions. This could be attributed to the shallow pots (only about 12 cm), the less developed root systems and short time span between EDDS application and the leaching study tests. In the present study, the artificially rainwater was added immediately after the application of EDDS and the whole leaching experiment lasted less than 12 h, which did not allow the roots to have enough time to reabsorb the leached metals.

Increasing temperature could lead to a positive effect on the metal extraction (Vandeviere et al., 2001a). It was found in the temperature range of 8 to 48 ºC, each 10 ºC increment resulted in a 6% increase in metal extraction except for Cu (Vandeviere et al., 2001a). In the present leaching study, at the same dosage, the hot EDDS application only resulted in slightly higher amounts of metals in the leachate solutions. An average of 12%, 14%, and 13% enhancement was observed for Pb, Zn and Cd. In contrast to the results of Vandeviere et al. (2001a), the amount of Cu also increased by 10%, which might be due to the different soil properties, such as different soil texture, metal concentrations and associations. The higher metal amounts in the leachate solutions from the pots with hot EDDS application than the normal EDDS application partially explained the higher metal concentrations in the shoots of corn and beans (see Figs. 6.2 and 6.3), although all the enhancements did not reach the statistically significant levels ($P < 0.05$).

In contrast to the results observed on the day of EDDS application, from 7 d to 35 d (the end of the leaching study), at the same EDDS application dosage, the

amounts of metals in the leachate solutions from the pots treated with hot EDDS solutions were slightly lower than that from the pots treated with normal EDDS solutions. The amounts of metals in the leachate solutions from pots with plants were significantly lower than that from the pots without plants (see Fig. 6.4). For example, on Day 7 after the application of 5 mmol kg^{-1} of normal EDDS, the amounts of Cu, Pb, Zn, and Cd in the leachate solution from the pots without plants were 102, 2.69, 66.6 and 0.2 mg, respectively, which were 1.3, 1.3, 1.4 and 1.25 times of that from the pots grown with plants, respectively. For the soils grown by corn and beans, the average amounts of soluble Cu, Pb, Zn, and Cd decreased by 58, 30, 50, and 39%, respectively, from Day 7 to Day 14 after the application of EDDS. But in the soil without the plants, the amounts of soluble Cu, Pb, Zn, and Cd decreased only by 40, 24, 42, and 26%, respectively. The result indicated that the leached metal amounts decreased more quickly in the soil with plant growth than in the soil without plants. Between the pots grown with corn and beans, there were no significant differences found in the amounts of leached metals.

The differences observed between the leached metal amounts from the pots with plants grown and the pots without plants could not be attributed to the metal uptake by plants. Data in the Table 6.1 shows when the EDDS was applied at the same dosage, significantly more metals were phytoextracted by the plants treated with hot EDDS solutions than the normal EDDS application. Therefore, the amounts of metals leached out from the pots treated with hot EDDS could be significantly lower than that leached out from the pots treated with normal EDDS. However, it was not the case (see Table 6.4). Then it is reasonable to

attribute to the biodegradability of EDDS. EDDS is the only commercially available chelate that is naturally present in soil, where it is readily decomposed into degradation products (Witschel and Egli, 1998). The calculated half-life of EDDS in sludge-amended soil was 2.5 days and it would be completely mineralized within 28 d (Jaworska et al., 1999). The less leached metal amounts from the pots with plants may be attributed to the plant root that would facilitate more microbial population growth and thus accelerate the degradation of EDDS. The application of EDDS did not exert toxic effects on the soil microorganisms, capable of chelate biodegradation, even at the higher concentration of 20 mmol kg^{-1} (Kos and Lestan, 2003a). Consistent with the results of Day 7, the results obtained in the following leaching analysis conducted on Day 14, 21, and 28 showed generally significantly lower amounts of Cu, Pb, Zn, and Cd were always found in the leachate solutions from pots with plants when the EDDS was applied at the same dosage. On Day 28, no significant differences were found in the amounts of Cu, Pb, Zn, and Cd in the leachate solutions from the pots grown with plants and the control group (without plant). For the leachate solutions from the pots with no plants, the differences disappeared until 35 days after the application of EDDS (Fig. 6.4).

The main concerns about chemically-enhanced phoextraction are the efficiency of metal removal from soils, the operation of cost and the potential negative effects to the surrounding environment resulted from the application of chemicals. Compared with the traditionally used chelate of EDTA, the application of biodegradable chelate of EDDS makes the chemically-enhanced phytoextraction of heavy metals in contaminated soils more promising because

of the higher biodegradability and less leachability of the chelate. The present study demonstrated that when the EDDS was applied as hot solutions, the accumulation of heavy metals in plant shoots could be greatly enhanced compared with the same EDDS application in normal temperature solutions (Table 6.1). For all heavy metals that were studied when EDDS were applied as hot solutions at the rate of 1 mmol kg^{-1}, metal concentrations and total phytoextraction by plant shoots exceeded or approximated those in the shoots of plants treated with normal EDDS at a rate of 5 mmol kg^{-1} (Figs. 6.2 and 6.3 and Table 6.1). On the other hand, the leaching study showed when the EDDS application was decreased, metals leached out of the pots decreased accordingly (Fig. 6.4). On an average, the leached metal amounts of Cu, Pb, Zn and Cd after the application of EDDS at the rate of 1 mmol kg^{-1} were reduced by 46%, 21%, 57% and 35% compared with that leached from 5 mmol kg^{-1} of EDDS application, respectively. In the present study, for the treatment of 1 mmol of EDDS, the leached metals decreased to the control group levels 14 days after the application of EDDS. Therefore, if there was no rain fall in the first 14 d after the application of EDDS, metals leached to the surrounding environment would be minimal. From this respect, for a given phytoextraction efficiency, hot EDDS application would be very useful in reducing the operation cost and the potential risk of metal leaching to the surrounding environment. Moreover, if some plants with deep root systems, such as vetiver grass, can be introduced to intercrop with the target plants, heavy metals can be efficiently removed by harvesting the shoots of high biomass plants, at the same time, the metal leaching may be greatly reduced by the retention and re-adsorbing in soil due to the deep root systems.

Table 6.2 The leachate solution volume collected under the pots (ml)

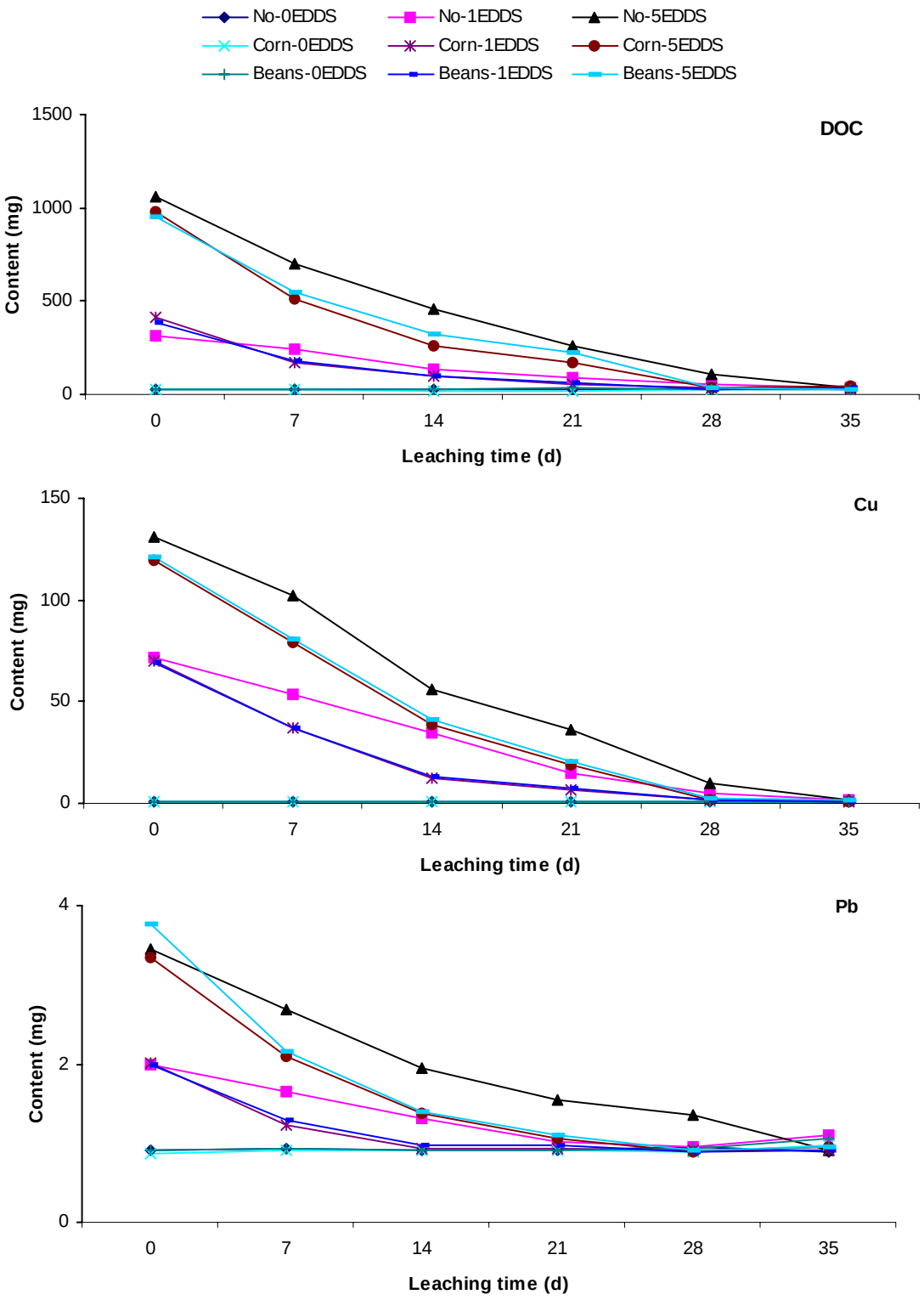
Plants	Treatments	Leachate solution volume (ml)					
		0 d	7 d	14 d	21 d	28 d	35 d
No plant	0 EDDS	650±30	633±14	686±12	634±10	642±7	642±7
	Hot-0 EDDS	678±13	604±13	667±14	642±34	671±34	681±27
	1 EDDS	631±45	618±13	644±18	678±26	687±26	667±43
	Hot-1 EDDS	665±32	624±24	661±10	688±36	656±20	656±30
	5 EDDS	680±35	635±29	677±8	670±41	628±23	688±16
	Hot-5EDDS	617±12	680±35	656±6	626±4	613±28	613±23
Corn	0 EDDS	675±23	660±62	639±15	635±9	633±54	633±20
	Hot-0 EDDS	625±8	653±25	612±25	648±24	669±27	639±23
	1 EDDS	610±18	648±13	625±27	652±29	670±10	660±19
	Hot-1 EDDS	629±20	666±45	663±15	630±16	657±7	677±13
	5 EDDS	608±20	670±23	632±17	667±18	650±3	680±17
	Hot-5EDDS	690±15	686±18	642±20	631±25	647±28	687±10
Beans	0 EDDS	645±30	690±46	679±25	642±28	631±30	671±8
	Hot-0 EDDS	652±32	670±31	652±28	689±34	679±34	639±9
	1 EDDS	675±10	622±20	684±31	637±38	664±30	600±24
	Hot-1 EDDS	682±8	618±50	616±30	678±21	683±54	613±26
	5 EDDS	639±9	622±16	646±9	640±9	613±18	623±17
	Hot-5EDDS	661±27	632±10	662±15	672±52	634±30	664±19

Values are means ± S.D. (n = 2).

Table 6.3 The pH of the leachate solutions

Plants	Treatments	Leachate solution pH					
		0 d	7 d	14 d	21 d	28 d	35 d
No plant	0 EDDS	7.09±0.04	6.99±0.02	6.98±0.02	6.98±0.02	7.12±0.05	7.02±0.02
	Hot-0 EDDS	6.98±0.1	6.98±0.1	6.97±0.04	6.99±0.07	7.08±0.09	7.08±0.01
	1 EDDS	7.16±0.08	7.09±0.09	7.07±0.07	7.09±0.05	7.07±0.02	7.07±0.09
	Hot-1 EDDS	7.09±0.05	7.00±0.13	6.97±0.05	7.05±0.08	7.02±0.03	7.12±0.06
	5 EDDS	7.21±0.06	6.95±0.08	7.00±0.1	7.02±0.04	7.04±0.1	7.04±0.09
	Hot-5EDDS	7.22±0.09	7.07±0.07	7.05±0.03	7.06±0.09	7.08±0.05	7.08±0.1
Corn	0 EDDS	6.95±0.12	6.98±0.06	6.99±0.1	6.99±0.13	6.99±0.13	6.99±0.09
	Hot-0 EDDS	7.03±0.04	7.02±0.05	7.00±0.05	6.99±0.15	7.01±0.15	7.01±0.07
	1 EDDS	7.02±0.13	7.05±0.09	7.08±0.03	7.07±0.15	7.10±0.08	6.99±0.04
	Hot-1 EDDS	7.18±0.1	7.06±0.03	7.00±0.06	7.07±0.02	7.11±0.03	7.01±0.02
	5 EDDS	7.23±0.02	7.09±0.1	7.11±0.04	7.11±0.08	7.01±0.06	7.04±0.03
	Hot-5EDDS	7.21±0.05	7.01±0.09	7.09±0.02	7.09±0.07	7.05±0.04	6.99±0.12
Bean	0 EDDS	6.98±0.1	7.00±0.04	6.97±0.07	6.99±0.07	6.98±0.07	6.98±0.08
	Hot-0 EDDS	7.10±0.08	6.98±0.08	6.99±0.03	6.97±0.04	6.99±0.01	6.99±0.01
	1 EDDS	7.05±0.05	7.09±0.06	7.05±0.08	7.04±0.03	7.00±0.02	7.00±0.01
	Hot-1 EDDS	7.12±0.03	7.01±0.12	7.09±0.09	7.05±0.1	7.06±0.04	7.10±0.04
	5 EDDS	7.26±0.05	7.00±0.09	7.02±0.1	7.01±0.04	7.00±0.09	7.01±0.05
	Hot-5EDDS	7.23±0.1	7.09±0.02	7.01±0.04	7.01±0.02	7.03±0.08	7.09±0.06

Values are means ± S.D. (n = 2).


No-0EDDS
No-1EDDS
No-5EDDS
Corn-0EDDS
Corn-1EDDS
Corn-5EDDS
Beans-0EDDS
Beans-1EDDS
Beans-5EDDS
DOC
Content (mg)
1500
1000
500
0
0
7
14
21
28
35
Leaching time (d)
Cu
Content (mg)
150
100
50
0
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7
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Leaching time (d)

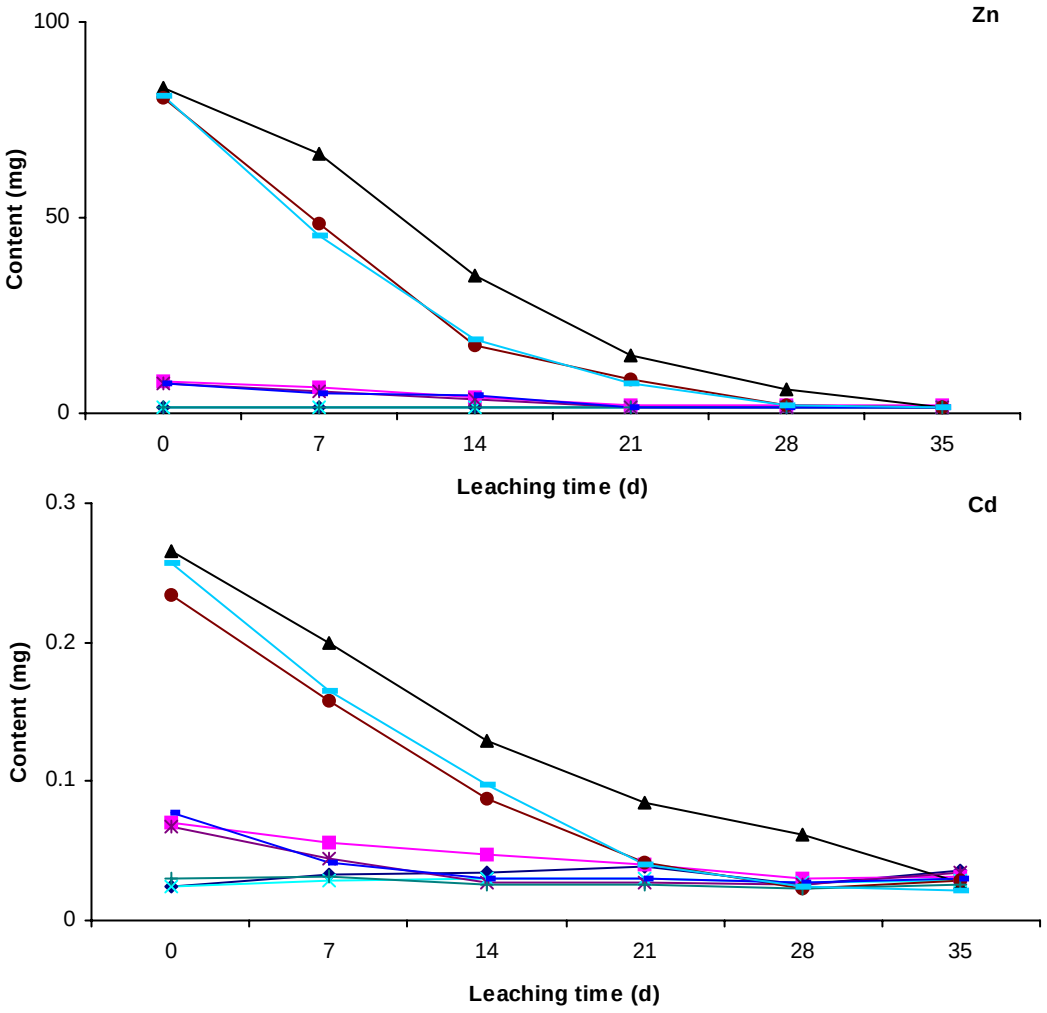

Fig. 6.4 Amounts of DOC, Cu, Pb, Zn, and Cd in the leachates from the soil without plants and the soil grown with corn and beans after the application of normal EDDS during the whole leaching study.

Table 6.4 Total amounts of DOC and metals of in the leachate solutions 7 d after the application of EDDS (mg)

Plants	Treatments	DOC (mg)	Metals (mg)			
			Cu	Pb	Zn	Cd
No plant	0 EDDS	25.4 ± 5.7	1.09 ± 0.3	0.93 ± 0.1	1.5 ± 0.1	0.034 ± 0.001
	Hot-0 EDDS	24.7 ± 3.9	1.13 ± 0.2	0.92 ± 0.3	1.62 ± 0.2	0.027 ± 0.004
	1 EDDS	242 ± 42	53.7 ± 6.8	1.64 ± 0.2	6.57 ± 0.7	0.056 ± 0.01
	Hot-1 EDDS	209 ± 37	50.1 ± 4.8	1.55 ± 0.4	6.61 ± 0.4	0.065 ± 0.008
	5 EDDS	702 ± 106	102 ± 12	2.69 ± 0.2	66.6 ± 7.8	0.2 ± 0.03
	Hot-5EDDS	796 ± 56	95.2 ± 6.8	2.59 ± 0.5	61.5 ± 6.2	0.192 ± 0.02
Corn	0 EDDS	23.1 ± 5	1 ± 0.5	0.91 ± 0.1	1.45 ± 0.2	0.029 ± 0.004
	Hot-0 EDDS	27.3 ± 4.9	1.09 ± 0.1	0.89 ± 0.1	1.24 ± 0.3	0.026 ± 0.004
	1 EDDS	170 ± 25	37.2 ± 4.3	1.22 ± 0.2	5.4 ± 0.4	0.045 ± 0.001
	Hot-1 EDDS	188 ± 31	40 ± 5	1.1 ± 0.3	4.99 ± 0.7	0.04 ± 0.002
	5 EDDS	511 ± 27	79.2 ± 6.4	2.09 ± 0.2	48.6 ± 8	0.158 ± 0.02
	Hot-5EDDS	504 ± 46	81.8 ± 9.7	2.19 ± 0.2	47.4 ± 5.5	0.145 ± 0.02
Bean	0 EDDS	29.2 ± 6	0.89 ± 0.1	0.92 ± 0.1	1.31 ± 0.2	0.032 ± 0.003
	Hot-0 EDDS	34.9 ± 4.8	0.81 ± 0.1	0.87 ± 0.1	1.21 ± 0.1	0.030 ± 0.004
	1 EDDS	182 ± 25	37.4 ± 4.6	1.3 ± 0.2	5.2 ± 0.5	0.042 ± 0.005
	Hot-1 EDDS	166 ± 17	37.4 ± 7	1.4 ± 0.4	5.13 ± 0.8	0.051 ± 0.005
	5 EDDS	544 ± 62	80.9 ± 9	2.17 ± 0.5	45.4 ± 5.1	0.165 ± 0.02
	Hot-5EDDS	601 ± 51	81.6 ± 11	2.32 ± 0.3	41.9 ± 5	0.151 ± 0.01

Values are means ± S.D. (n = 2).

Chapter 7 - Exploring the Naturally Enhanced Phytoremediation: Effects of Root Interaction on Phytoextraction of Metals from Soils

7.1 Introduction

The most common phytoremediation includes the use of hyperaccumulator plants and chemically enhanced phytoextraction to remove metals form contaminated soils. Unfortunately, most hyperaccumulators grow slowly and produce low biomass, which greatly limit the application of this technique in the field (Mulligan et al., 2001; Puschenreiter et al., 2001). The chemically enhanced phytoextraction with the addition of some artificially produced chelates, was suggested as an efficient strategy for the clean-up of the heavy metal-contaminated soils (Blaylock et al., 1997; Huang et al., 1997; Cooper et al., 1999; Wu et al., 1999; Shen et al., 2002). However, the associated leaching of metals poses the potential risk to the ground water and surrounding environment (Nowack, 2002). Hence, it is necessary to explore some more environmental sound and economical technology to solve the problem.

Under nutrient limiting conditions, plants can modify the rhizosphere to enhance acquisition of nutrients (Marschner, 1995). Rhizosphere acidification and release of root exudates are two common mechanisms employed in the process. Root exudates can change the pH condition in the rhizosphere, provide ligands for metal

complexation and facilitate microbial activity, which, in turn, enhance the concentrations of soluble metals in the soil (Tao et al., 2004). Root exudates from different plant species and cultivars may have different capacities in increasing the solubility of metals (Mench and Martin, 1991; Zhao et al., 2001; Ma et al., 2003). Under Fe deficiency conditions which is typical in calcareous soils, graminaceous plants usually increase markedly the release of phytosiderophores to the rhizosphere. Based on the study for an intercropping system, Ma et al. (2003) concluded that the secretion of DMA (2'-deoxymugineic acid), the first product of phytosiderophore synthesis, from the roots of perennial grasses could be partly responsible for the converting of insoluble soil Fe to forms available to fruit trees and for the "re-greening effect" observed in fruit trees grown on calcareous soils after introduction of grasses in the tree rows. Phytosiderophores (PS) are capable of chelating not only Fe, but also Cu, Zn and Mn, thus mobilizing these metals from soils (Treeby et al., 1989). Mench and Martin (1991) observed that the uptake of Cd from soils by the three plant species of *Nicotiana tabacum*, *Nicotiana rustica* and *Zea mays* followed the same order as the extent of Cd extraction by their root exudates. Also, root exudates are confined to the same small portion of the soil where roots are located. Therefore, the adverse result of increased leaching of heavy metals by excess chelates application used in the chemically enhanced phytoremediation can be expected to be negligible in the soil.

For the consideration of cost and potential environmental risk, if intercropping graminaceous plants with other plants could enhance the metal uptake in

harvestable parts of the plants, under some conditions such as Fe deficient soils, it would provide one meaningful alternative for the remediation of low level contaminated soils. In the present study, different growing systems for dicotyledon of pea (*Pisum sativum* L. cv.), such as mixing-cultured soil cultivation with graminaceous plants of corn (*Zea mays* L. cv. Nongda No. 108), barley (*Hordeum vulgare* L.cv. Jian 4) and wheat (*Triticum aestivum* L. cv.) were studied. The metal mobilization capacity of root exudates collected from barley was also studied in the present study.

7.2 Materials and Methods

7.2.1 Mixing-culture Pea with Corn, Barley and Wheat

Soil samples were the same as those used in Chapter 3 for the study of garland chrysanthemum and corn (see Table 3.1, soils for 3.2.2). The only one difference was the pH of the soil used here was adjusted to 8.4 with lime. The samples were sieved to pass through a 2 mm sieve and air-dried for one week. The soils were artificially contaminated with Cu (400 mg kg^{-1} of soil) as $CuCO_3$ (copper carbonate); Pb (500 mg kg^{-1} of soil) as $Pb_3(OH)_2(CO_3)_2$ (lead hydroxide carbonate) and PbS (lead sulfide – galena, a common lead mineral in mining area) at a Pb concentration ratio of 1:1; Zn (500 mg kg^{-1} of soil) as $ZnCO_3$ (zinc carbonate) and ZnS (zinc sulfide) at a Zn concentration ratio of 1:1; and Cd (15 mg kg^{-1} of soil) with $Cd(NO_3)_2 \cdot 4H_2O$ (cadmium nitrate). Basal fertilizers applied to the soil were

80 mg P kg^{-1} of dry soil, and 100 mg K kg^{-1} of dry soil as KH_2PO_4 (Shen et al., 2002). After adding heavy metals, the soils were equilibrated for two months, undergoing seven cycles of saturation with de-ionized water and air-drying processes.

Air-dried soils (500 g) were placed in plastic pots (12 cm i.d. x 12 cm height). The moisture of the soil was maintained at near field water capacity by adding DIW daily. The treatments consisted of mono-culture of corn (*Zea mays* L. cv. Nongda No. 108), barley (*Hordeum vulgare* L.cv. Jian 4), wheat (*Triticum aestivum* L. cv. Yangmai No. 11), pea (*Pisum sativum* L. cv. Qinxuan No. 2) and mixing-culture of pea with corn, barley and wheat, respectively. Seeds of plants were purchased from Nanjing Agricultural University, and directly sown in the soils and the seedlings were thinned to four plants per pot (two plants for one plant species per pot in the intercropping pots). Three replications were used in each treatment. All experiments were conducted in the greenhouse under natural light. Air temperature ranged from 23 to 32 ºC. Plants were harvested 14 d after planting. Shoots and roots were separated, and washed with tap water and rinsed with DIW, and dried at 70 ºC in an oven to a constant weight for dry weight measurements and further analysis.

7.2.2 Collection of Root Exudates

Barley was germinated in the dark on filter paper moistened with DIW. After

germination (4 d), twenty seedlings were transferred to each 2-L polyethylene vessels filled with modified 0.2-strength Rorison nutrient solution with the following composition (in µmol L^{-1}): 400 $Ca(NO_3)_2$, 200 $Mg(SO_4)_2$, 50 K_2HPO_4, 300 KCl, 9.2 H_3BO_3, 1.8 $MnSO_4 \cdot 4H_2O$, 0.21 $Na_2MoO_4 \cdot 2H_2O$, 0.31 $CuSO_4 \cdot 5H_2O$, 10 $ZnSO_4 \cdot 7H_2O$, and 10.8 Fe-EDTA at pH 6.0 (Hewitt, 1966). Two treatments of the control group (full nutrient composition) and Fe deficiency treatment (without the addition of Fe-EDTA) were imposed 10 d after the seedlings were transferred to the nutrient solutions. Each treatment was replicated four times. Before the treatments were imposed, roots were rinsed thoroughly with DIW. The treatments were imposed for 10 d. The nutrient solution was aerated continuously and renewed every two days and the experiment was conducted in the greenhouse under natural light with air temperature ranging from 25 to 34 ºC. The pH of the solution was adjusted to 6.0 with 1 N of HNO_3 and KOH solutions. Before collection of root exudates, the roots were cultured overnight in DIW. Root exudates were collected by bathing the roots in 200 ml autoclaved 0.1 mM $CaCl_2$ solution for 3 h after the onset of the light period during which period the solution was aerated continuously (Cakmak et al., 1996; Zhao et al, 2001). After collection of exudates, the roots of barley were separated from shoots and washed with DIW, and dried in an oven for dry weight measurement.

The exudates solutions were filtered immediately after collection through a sterile 0.45 µm paper filter (Whatman [Maidstone, UK] 42) into an autoclaved glass tube and the filtered solutions (200 ml) were immediately concentrated to 10 ml using a

rotary evaporator at 40ºC. The exudates solutions were immediately added to soils to study the metal mobilization.

7.2.3 Extraction of Metals from Soil

In this part, the soil used was the same as that used in the intercropping experiment. Root exudates were adjusted to pH 5.0 with 1 N HNO_3 and KOH solutions to reach an equal pH of the two different root exudates in order to eliminate the potential effects from pH change on metal extraction. 2 g of soils (passed through a 2-mm sieve) were placed in a 20-mL polypropylene centrifuge tube and shaken with 10 ml root exudates for 30 min. The initial pH of the root exudates collected from full nutrition and Fe deficiency solutions are 5.2 and 4.5, respectively (see Table 7.2). After centrifugation (3500 g, 10 min), the supernatant was filtered through a 0.45 µm paper filter (Whatman [Maidstone, UK] 42), digested with concentrated HNO_3 and analyzed for different metal concentrations by ICP-AES. The soil extracted with 0.1 mM pH 5.0 $CaCl_2$ in the same way was served as the background concentration of water exchangeable metals in the soil.

7.2.4 Metal Accumulation in Pea with the Addition of Root Exudates from Barley

Seeds of pea were directly sown in the same soil and the seedlings were thinned to four plants per pot as mentioned before. Barley root exudates from full nutrient

component treatment and Fe deficiency treatment were collected in the same way as above. After the pea was grown for 10 d, 1000 ml of the root exudates solutions from barley (pH was adjusted to 5.0) were concentrated to 50 ml and added to the soil surface immediately. Pea plants treated with 0.1 mM pH 5.0 $CaCl_2$ solutions in the same way were used as the control. Three replicates were conducted in each treatment. All experiments were operated in the glasshouse under natural light. Air temperature ranged from 23 to 32 ºC. Plants were harvested 7 d after the application of root exudates. Shoots and roots were separated, and washed with tap water and rinsed with DIW, and dried at 70 ºC in oven to a constant weight for dry weight measurements and further analysis.

7.2.5 Plant Analysis

Subsamples of ground shoot samples (200 mg) and root samples (100 mg) were digested in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, by volume) and the major and trace elements in the solutions were determined with ICP-AES (Chen et al., 2004b). A certified standard reference material (SRM 1515, apple leaves) of the National Institute of Standards and Technology, U.S.A., was used in the digestion and analysis as part of the QA/QC protocol. Reagent blank and analytical duplicates were also used where appropriate to ensure accuracy and precision in the analysis. The recovery rates were around 90 ± 6% for all of the metals in the plant reference material.

The concentration of dissolved organic C in root exudates was determined by a Shimadzu TOC-5000A analyzer and the pH was measured using a pH meter.

The data reported here were the mean values based on the three replicated experiment results. Statistical analyses of the experimental data, such as correlation and significant differences, were performed using SPSS® 11.0 statistical software.

7.3 Results and Discussion

7.3.1 Plant Growth and Metal Accumulation in Sole and Intercropping Systems

Iron-deficiency is a common problem for calcareous soils because of the extremely low solubility of soil Fe (Mengel, 1994). According to the mechanisms of iron (Fe) acquisition in higher plants, plants have been grouped into Strategy I plants and Strategy II plants. Strategy I plants include dicotyledons and non-graminaceous monocotyledons, which respond to Fe deficiency by extruding both protons and reducing substances from the roots, and by enhancing ferric reduction activity at the root plasma membrane. Strategy II plants (graminaceous species) usually respond to Fe deficiency by the release of phytosiderophores (PS) and a highly specific uptake system for ferrated PS (Ma and Nomoto, 1996). In the present study, all plants appeared normal and healthy whenever grown in sole and intercropped with other plant species (see Table 7.1). Among the four plant species

used in the present study, pea and corn produced significant higher shoot dry biomass in the same growth period, which reached to 3-fold of the shoot dry matter yields of barley and wheat. There was no significant effect from the cropping system on the dry matter production of the four plant species (Table 7.1) ($P < 0.05$).

Several plant and environmental factors, such as plant species/cultivars, temperature and light intensity can influence the rate of PS release from roots (Cakmak et al., 1998). It was found that the release of PS was correlated positively with $CaCO_3$ contents in soils (Awad, et al., 1999). Under the Fe deficiency condition, rice, sorghum and maize released lower amounts of PS than wheat, barley and oat (Kawai et al., 1988; Römheld and Marschner, 1990). In the present study, when the plants were grown in sole, pea produced the lowest concentrations of metals in the shoots compared with other three plant species (Fig. 7.1), which was consistent to the results of Chen et al. (2004a).

Among the three graminaceous plants used here, barley showed a higher capacity in the accumulation of metals, especially for the accumulation of Zn, which was 1.47- and 1.32-fold of the values in corn and wheat, respectively (see Fig. 7.1). When the three monocotyledon plants were mixing-cultured with pea, the concentrations of metals in the shoots of corn, wheat and barley decreased slightly, in comparison with those planted in sole. However, metal concentrations in the shoots of pea were increased. The concentrations of Cu, Pb, Zn, Cd and Fe in the shoots of pea reached the highest when the pea was mixing-cultured with barley,

which were about 1.4-, 1.3-, 1.2-, 1.2- and 1.1-fold of that in the pea intercropped with corn and wheat (see Fig. 7.1) ($P < 0.05$).

Usually, root exudates can influence nutrient solubility and plant uptake directly by acidification, chelation, precipitation and oxidation-reduction reactions and indirectly through their effects on microbial activity, physical and chemical properties of rhizosphere and root growth patterns (Uren and Reisensuer, 1988). PS are highly efficient in complexing and mobilizing Fe in the rhizosphere. In the present study, when pea was intercropped with corn, barley and wheat, the concentrations of Fe in the shoots were increased by 7.5%, 26% and 17%, respectively, in comparison with the pea grown in sole (Fig. 7.1). Ma et al. (2003) concluded that the secretion of DMA from the roots of perennial grasses may be partially responsible to converting insoluble soil Fe to forms available to fruit trees. The "re-greening effect" observed in fruit trees grown on calcareous soils after introduction of grasses in the tree rows indicated that these grasses were able to improve Fe uptake by tree roots (Tagliavini et al., 2000; Ma et al., 2003).

Table 7.1 The dry matter yields (g plant^{-1}) in the shoots of plants

Cropping system	Plant	Dry weight
Sole	Corn	0.41 ± 0.02
	Barley	0.16 ± 0.02
	Wheat	0.12 ± 0.01
	Pea	0.48 ± 0.04
Corn – pea mixed culture	Corn	0.42 ± 0.01
	Pea	0.45 ± 0.05
Barley – pea mixed culture	Barley	0.18 ± 0.01
	Pea	0.45 ± 0.05
Wheat – pea mixed culture	Wheat	0.14 ± 0.02
	Pea	0.46 ± 0.03

Values are means ± S.D. (n = 3).

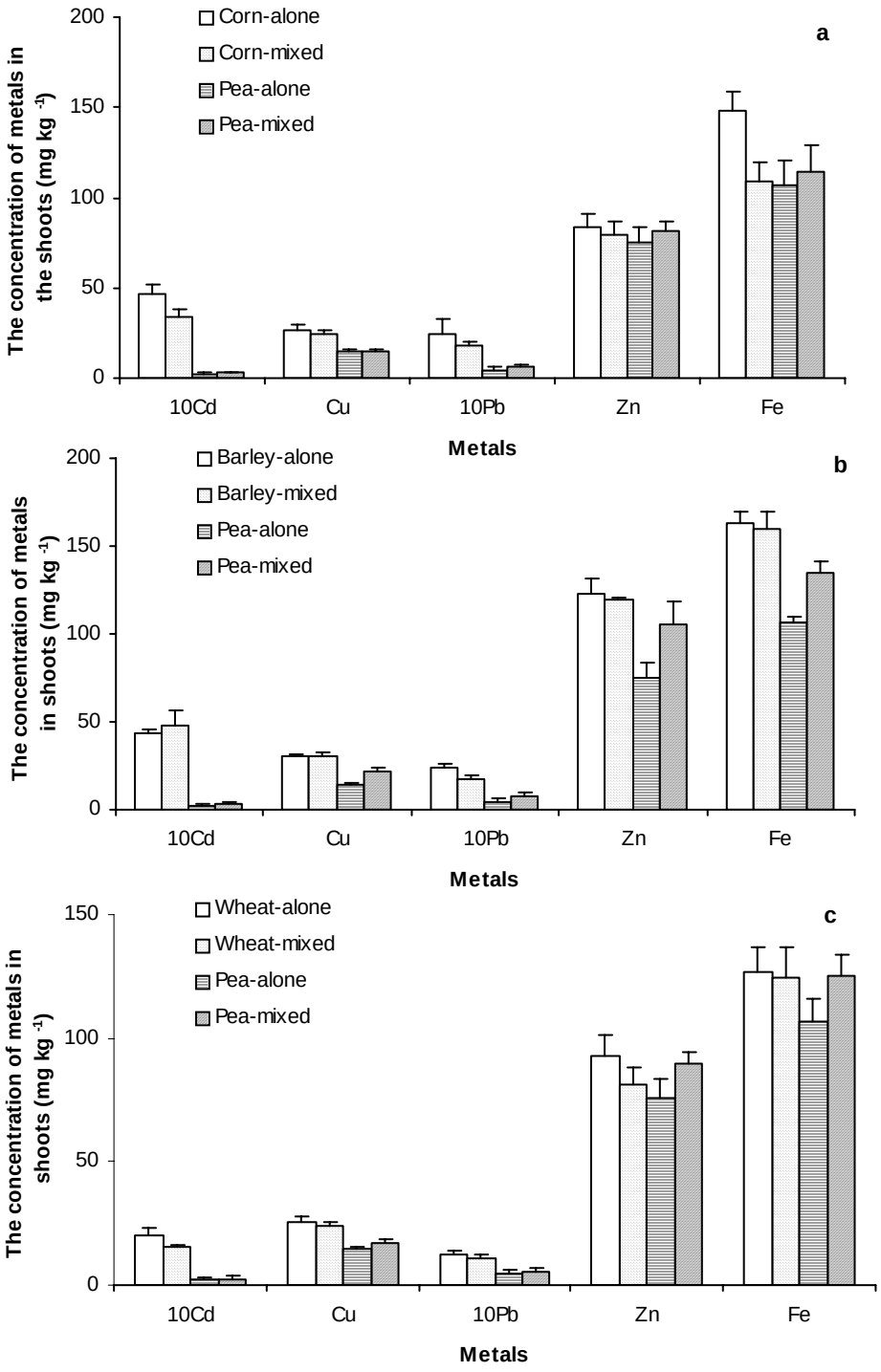

Fig. 7.1 The concentrations of Cd, Cu, Pb, Zn and Fe in the shoots of plants in different cropping system. The data for Cd and Pb shown here are 10 times of the original data. Values are means ± S.D. (n = 3).

7.3.2 Mobilization of Metals from Soil by Barley Root Exudates

The plants of pea and barley were chosen for the following study. Root exudates from barley were collected using the hydroponic experiment. The content of DOC in the root exudates from Fe deficiency treatment was 2.9-fold of that in the control plants (full nutrition cultured). The pH values of the root exudates from the barley were 4.5 for the Fe deficiency treatment and 5.2 for the full-nutrition culture, lower than that of the soil (8.2) (see Table 7.2). In order to eliminate the possible effects from the pH of root exudates on the mobilization of metals in the soil, the pH of root exudates was adjusted to 5.0 before added to the soils. Tao et al. (2004) observed that the effects of pH change on copper fractionation in calcareous soils were relatively insignificant compared with that of root exudate complexation because of the strong buffering capacity of calcareous soils and the acidification of the soil solution was not the reason for the increase in zinc availability (Knight et al., 1997). The effect of root exudates and microbial products could not be easily distinguished. Many components of root exudates can serve as carbon sources for microorganisms and facilitate microbial activity, which in turn, leads to the increased release of organic acids, which can mobilize metals through the formation of metal-organic chelates (Tao et al., 2004). Reduced enzymes released from microorganisms may also play a role in the solubilization of metals (Lombi et al., 1999).

The amounts of Cu, Pb, Zn, Cd and Fe extracted by barley root exudates are shown in Table 7.3. Compared with the control, adding root exudates from the Fe deficiency treatment dramatically enhanced the metal mobilization in soils. On average, the amounts of Cu, Pb, Zn, Cd and Fe in soils extracted by root exudates from Fe deficiency plants were 4.7-, 3.2-, 9.7-, 4.9- and 11.5-fold of that in the control.

Table 7.2 The content of dissolved organic C (mg g^{-1} root DW) and the pH of the root exudates collected from barley in the control (full nutrition solution) and Fe deficiency nutrition solution.

	DOC	pH
Control	6.7 ± 0.3a	5.2 ± 0.4a
Fe deficient nutrition solution	19.5 ± 1.1b	4.5 ± 0.2b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 7.3 The amounts of metals in the soil extracted with the addition of root exudates (mg kg^{-1} root DW of barley)

	Control	Fe deficiency nutrition solution
Cu	23.6 ± 3.5a	110 ± 15b
Pb	8.3 ± 1.2a	26.5 ± 2.1b
Zn	23.3 ± 1.8a	225 ± 30b
Cd	0.09 ± 0.01a	0.44 ± 0.1b
Fe	103 ± 11a	1180 ± 85b

Values are means ± S.D. (n = 3). the different small letters stand for statistical significance at the $p < 0.05$ level .

7.3.3 Metal Concentrations and Phytoextraction by the Shoots of Pea

In a chemically enhanced phytoremediation process, the first step is to produce high plant biomass at contaminated sites and the second step is to induce metals to accumulate in the shoots (Blaylock et al., 1997). Usually, the enhanced solubility of metals in the soil was accomplished by the addition of synthetic chelates. However, the potential leaching of metals associated with the application of chelates limited the application of this technology in field (Nowack, 2002). It is found that plants naturally produce substances of PS, which are capable of chelating not only Fe, but also Cu, Zn and Mn, thus mobilizing these metals from soils (Treeby et al., 1989). It is hypothesized that the release of PS from graminaceous plants mobilize heavy metals by forming corresponding complexes and thus increasing their availability to plants. Table 7.4 showed that comparison with the control group (adding 0.1 mM $CaCl_2$ solutions), the addition of barley root exudates significantly enhanced the metal concentrations in the shoots of pea (Table 7.4). Also, the improved effect was more significant for the root exudates from Fe deficiency treatment than that from the full-cultured plants. The concentrations of Cu, Pb, Zn, Cd and Fe in the shoots of pea treated with barley root exudates from Fe deficiency treatment were 3.8-, 2.3-, 1.6-, 1.7- and 2.6-fold of that applied with root exudates from the full-cultured barley plants, respectively, which was consistent with the higher soluble metal concentrations achieved in the soils extracted with root exudates from the Fe deficiency treatment (see Table 7.3.).

The total metal phytoextraction was presented in Table 7.5. This is expressed on the basis of the amounts of root exudates. In the calculation, the metal phytoextraction for the control group (adding 0.1 $CaCl_2$ mM solutions) was as the base for plants treated with root exudates. Hence, in the table, the control group represents the pea plants treated with the root exudates from full-cultured barley plants. The phyotextraction of Cu, Pb, Zn, Cd and Fe by the shoots of pea was significantly enhanced by adding root exudates of barley from the Fe deficiency treatment, which attained to 3.8-, 2.7-, 8.5-, 2.6- and 8.2-fold of that in the control group. Similarly, Chen et al (2004c) observed that Fe deficiency induced Cu uptake and accumulation in *Commelina communis*. Mench and Martin (1991) reported that the uptake of Cd from soils by the three plant species of *Nicotiana tabacum*, *Nicotiana rustica* and *Zea mays* followed the same order as the extent of Cd extraction by their root exudates. In the shoots of wheat plants precultured for low Fe nutritional status, the concentrations of Zn, Ni, and Cd were between 25 and 200% higher than in the adequate Fe precultured plants (Römheld and Awad, 2000). However, results from Shenker et al (2001) suggested PS release did increase the uptake of Fe, Zn and Mn in barley and wheat, but did not increase the uptake of Cd. Hill and Lion (2002) observed the release of DMA appeared to reduce Cd accumulation in the corn.

Table 7.4 The concentration of metals (mg kg^{-1} DW) in the shoots of pea

	Control	Full nutrition addition	Fe deficiency nutrition addition
Cu	14.5 ± 2.5a	31 ± 2.7a	118 ± 14b
Pb	0.32 ± 0.1a	0.9 ± 0.1a	2.1 ± 0.1b
Zn	70 ± 8.9a	97 ± 8.1a	157 ± 13b
Cd	0.2 ± 0.1a	0.29 ± 0.03a	0.48 ± 0.1b
Fe	102 ± 13a	138 ± 15a	359 ± 39b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level.

Table 7.5 The total phytoextraction of metals (mg kg^{-1} root DW of barley) in the shoots of pea

	Control	Fe deficiency treatment
Cu	25.2 ± 3.1a	94.6 ± 11b
Pb	0.9 ± 0.1a	2.4 ± 0.3b
Zn	37.6 ± 2.9a	319 ± 23b
Cd	0.13 ± 0.02a	0.34 ± 0.1b
Fe	49.4 ± 5.5a	405 ± 24b

Values are means ± S.D. (n = 3); the different small letters stand for statistical significance at the $p < 0.05$ level

Chapter 8 – Conclusions and Recommendations

8.1 Major Findings

Screening plant species more sensitive to the application of chelate is helpful to reduce the chelate dosage for a given phytoextraction efficiency. In the present study, using pot experiment with the addition of EDTA at the rate of 3 mmol kg^{-1}, 18 different plants to be used in the chemically enhanced phytoextraction of Cu, Pb, Zn and Cd was assessed. The major finding on the plant screening and the study of the residual effects of EDTA and EDDS treatments in soils are as following:

1. Compared with the dicotyledon species, the five graminaceous monocotyledon plants showed relatively less response to the application of EDTA, with less chlorosis and smaller reductions of shoot biomass.
2. The application of EDTA at the rate of 3 mmol kg^{-1} dramatically increased the concentrations of Cu, Pb and Zn in the shoots of plants. The enhancement was more pronounced for the dicotyledon plants than for graminaceous monocotyledon plants
3. Of all of the plants that were tested, garland chrysanthemum was the species most sensitive to the application of EDTA, and had the highest concentrations of Cu, Pb, Zn and Cd in its shoots.
4. The highest amounts of Cu and Pb extracted were achieved by garland

chrysanthemum, with 1.42 mg Cu kg^{-1} soil and 0.93 mg Pb kg^{-1} soil, respectively. For Zn, the highest extraction (2.0 mg Zn kg^{-1} soil) was achieved by the greengrocery (*Brassica chinensis* L. cv. Xiaoza No. 56). The application of 3 mmol kg^{-1} of EDTA did not significantly improve the phytoextraction of Cd by all species of plants.

5. The study of the residual effects of various chelates treatments in multiple metals contaminated soils indicated that the EDTA-treated soil still had a significant ability to enhance the concentrations of Cu and Pb in the shoots of corn six months after the chelate treatment. However, the EDDS-treated soil did not have any effect in enhancing the concentrations of metals in the shoots of corn in the second crop test.
6. The results may indicate that EDDS biodegrades more rapidly than EDTA in soil, and has better chance in limiting the potential metal leaching from soil.

The major findings on the comparison of different chelates and optimizing chelate application methods are as follows:

1. EDDS was more effective than EDTA at increasing the concentration of Cu and Zn in the shoots of corn and beans. For Pb and Cd, EDDS was less effective than EDTA. Citric acid showed the lowest improving effects on the metal uptake by the two plants.
2. The application of EDTA and EDDS also significantly increased the shoot-to-root ratios of the concentrations of Cu, Pb, Zn and Cd in both plant

species.

3. The results of metal extraction with chelates showed that EDDS was more efficient at solubilizing Cu and Zn than EDTA, and EDTA was better at solubilizing Pb and Cd than EDDS.

4. When EDTA and EDDS were combined applied at the ratios of 1:1, 1:2, and 2:1 (EDTA:EDDS), 2:1 of EDTA:EDDS was the most efficient ratio for increasing the uptake of Cu, Pb, Zn and Cd by corn, especially for the phytoextraction of Pb.

5. The combined application of 3.33 mmol kg^{-1} soil of EDTA + 1.67 mmol kg^{-1} soil of EDDS (2EDTA:1EDDS) produced 2.4 and 5.9 times the concentration of Pb in the shoots treated with 5 mmol kg^{-1} of EDTA and EDDS alone, respectively. The total phytoextraction of Pb reached 2.1 and 6.1 times the total Pb from 5 mmol kg^{-1} EDTA and EDDS alone, respectively.

6. The mechanism of enhancing the phytoextraction of Pb by the combined application of EDTA + EDDS did not involve a change in the pH of the soil. The increase in the phytoextraction of Pb by the shoots of corn was more pronounced than the increase of Pb in the soil solution with the combined application of EDTA and EDDS. It was thought that the major role of EDDS might be to increase the uptake and translocation of Pb from the roots to the shoots of plants.

7. The combined application of EDTA and EDDS may provide a more efficient

approach to the phytoremediation of soils contaminated with multiple heavy metals. For soil contaminated by Cu and Zn, EDDS can be a better chelate because of its high extraction efficiency and better biodegradability.

The major finding on the exploring of the chelate-enhanced metal uptake by plants and metal leaching study are as follows:

1. A significantly positive correlation was found between the concentrations of Pb and EDTA in the shoots of mustard, indicating that Pb-EDTA was probably the major form of Pb taken up and translocated by the plants.
2. High concentrations of free Pb^{2+} rather than EDTA may physiologically damage the roots of plants and, in turn, lead to the uptake and translocation of the Pb-EDTA complex from roots to shoots in mustard.
3. Some physiological damage to roots such pretreatment with MC solution (methanol: trichloromethane solution), 0.1 mol L^{-1} of HCl, and 65 ºC hot water would be useful to enhance the uptake of metal chelates by plants and to minimize the application of doses of EDTA in the practical operation of chelate-assisted phytoremediation.
4. Using pot experiments, when the biodegradable chelating agent of EDDS was added in a hot solution at 90°C to the soil in which garland chrysanthemum and beans were growing, the uptake of heavy metals by plants was significantly improved compared with the application EDDS at the normal temperature at 25°C.

5. When 1 mmol kg^{-1} of EDDS as a hot solution was applied to soil, the concentrations of Cu, Pb, Zn and Cd and the total phytoextraction by the shoots of the two plant species exceeded or approximated those in the shoots of plants treated with 5 mmol kg^{-1} of normal EDTA solution.
6. The concentrations of metals in the shoots of beans were significantly correlated with the relative electrolyte leakage rate of root cells, indicating that the root damage resulting from the hot solution might play an important role in the process of chelate-enhanced metal uptake.
7. The leaching study showed when the EDDS application was decreased, metals leached out of the pots decreased accordingly. There was no significant difference between the treatment of hot and normal EDDS application when the dosage was at the same rate.
8. On an average, the leached metal amounts of Cu, Pb, Zn and Cd on the application of EDDS at the rate of 1 mmol kg^{-1} were reduced by 46%, 21%, 57% and 35% compared with that leached from 5 mmol kg^{-1} of EDDS application, respectively. For the treatment of 1 mmol of EDDS, the leached metals decreased to the control levels 14 days after the application of EDDS.
9. The application of biodegradable EDDS in hot solutions to soil provides a good alternative in chelate-enhanced phytoextraction.

Lastly, the major findings on the root exudates induced phytoextracton of

metals are as follows:

1. Mix-culture of pea with corn, barley and wheat enhanced the metal concentrations and total phytoextraction in the shoots of pea. The most enhanced effects were observed in the mix-culture system of barley and pea, where the concentrations of Cu, Pb, Zn, Cd and Fe in the shoots of pea reached 1.5-, 1.8-, 1.4-, 1.4- and 1.3-fold of those grown in sole.
2. Adding root exudates of barley to the pea plants grown in the pots resulted in the significant increase of metal accumulation in the shoots of pea. It was thought that root exudates in the mixed culture system played an important role in the process of solubilizing metals in the soil and facilitating metal uptake by plants.
3. Although the improving effects on metal uptake by plants from the root exudates are less effective than the chelate application, the characteristics of lower cost and more environment friendly to the surrounding media will be more attractive to the researchers in the future.

8.2 General Conclusions

The success of phytoremediation is dependent on large plant biomass yields and high metal concentrations in the shoots. Hyperaccumulator plants possess an ability to take up abnormally high concentrations of heavy metals in their shoots (Chaney et al., 1997; Shen et al., 1997). However, most hyperaccumulator

species are not suitable for phytoremediation application in the field due to their small biomass and slow growth. As an alternative, chemically enhanced phytoremediation by high biomass plant species, such as corn (*Zea mays* L.), mung beans (*Vigna radiat* L.), buckwheat (*Fagopyrum esculentum* L.), sunflower (*Helianthus annuus* L.), cabbage (*Brassica rapa* L.), and Indian mustard (*Brassica juncea* L.), has been suggested as a competitive technology in this field (Huang et al., 1997; Shen et al., 2002; Kos et al., 2003; Liphadzi et al., 2003; Chen et al., 2004b; Lim et al., 2004; Meers et al., 2005).

In the process of chelate-enhanced phytoextraction, the metal uptake efficiency by the plant harvestable parts was the first concern. It was reported that among different plant species, even different cultivars within a same species, there were great differences in the metal uptake ability and tolerance (Marschner, 1995). It has been suggested that the growth of and metal accumulation by different plants also showed different sensitivity to the EDTA treatment in soils (Chen et al., 2004a). Screening plants more sensitive to the chelate application is helpful to reduce chelate application dosage and alleviate metal leaching risk to the surrounding environment. Results from the screening experiments in Chapter 3 showed of all the plants tested, garland chrysanthemum showed the greatest growth sensitivity to the application of EDTA and the highest enhancement of the Cu, Pb accumulation in the shoots, which meant this plant may be as a good candidate plant species for metal phytoremediation process in

the field. Further study is needed to clarify the potential mechanisms underlying the high sensitivity to the application of chelate, which will provide useful information on screening more ideal plant species for the purpose of metal phytoremediation in the future.

Besides the metal uptake efficiency by the plant harvestable parts, the potential metal leaching to the surrounding environment with the application of chelates poses a big challenge to the practical application of this technology. Therefore, the possible metal leaching should be minimized to satisfy the environmental safety standard. Although EDTA has been the most widely used chelating agent to facilitate metal uptake by high-biomass plant (Blaylock et al., 1997; Huang et al., 1997; Cooper et al., 1999; Wu et al., 1999; Shen et al., 2002), the toxicity of EDTA and EDTA-heavy metal complexes to plants and soil microorganisms, and the persistence in the environment limited its application in the field (Bucheli-Witschel and Egli, 2001; Nowack, 2002; Grčman et al., 2003). In addition to EDTA, some easily biodegradable chelating agents, such as NTA (nitrilotriacetate) and EDDS (S,S-ethylenediaminedisuccinic acid) have been proposed to enhance the uptake of heavy metals in phytoremediation (Kulli et al., 1999; Kayser et al., 2000; Grčman et al., 2003; Kos and Leštan, 2003 a & b). However, it was concluded that this procedure was far from effective, even at the highest concentrations of heavy metals achieved in the harvestable plant tissues.

The present study showed the application of hot chelate solutions (90°C) was much more efficient than the application of normal chelate solutions (25°C) in improving the uptake of heavy metals by plants. When 1 mmol kg^{-1} of EDDS as a hot solution was applied to soil, the concentrations of Cu, Pb, Zn and Cd and the total phytoextraction by the shoots of the two plant species exceeded or approximated those in the shoots of plants treated with 5 mmol kg^{-1} of normal EDTA solution. However, the leached metal amounts of Cu, Pb, Zn and Cd on the application of EDDS at the rate of 1 mmol kg^{-1} were reduced by 46%, 21%, 57% and 35% compared with that leached from 5 mmol kg^{-1} of EDDS application, respectively. In the treatment of 1 mmol of EDDS, the leached metals decreased to the control levels 14 days after the application of EDDS. In comparison with other proposed strategies combined with the application of chelates to enhance metal phytoextraction efficiency, such as the transplantation (Wu et al, 1999), the addition of electric field around the plants (Lim et al., 2004), and a layer of silicate coated with the solid EDTA (Li et al., 2005), the application of hot EDDS solutions produced significantly higher increased effectiveness in metal removal efficiency. In addition to the higher metal phytoextraction efficiency, applying hot EDDS solution to soils are much easier to manage in practice. Also, the application cost and the risk to the surrounding environment are greatly reduced compared with the normal EDDS application due to the reduced application dosage. Therefore, the application of

biodegradable EDDS in hot solution to soil may be an efficient alternative in chemical-enhanced phytoextraction to increase total metal removal and to reduce possible leaching from soil. Of course, some potential negative effects associated with the hot EDDS application, such as possible deleterious effects on soil microorganisms, need to be critically evaluated before this technology can be applied in the field.

Pot and column studies are useful tools to assess the possibility of this technology under normal environmental conditions in consideration of the potential risk to the surrounding environment associated with the application of chemicals. However, in pot experiments, heavy metal concentrations in plant usually increased more than in field experiments. On average, the field heavy metal removal was only 20% of what was originally expected from the pot results (Kayser et al., 2000). The difference between the initial tests and field experiments should be considered. Therefore, further field tests under real environmental conditions are needed to prove the efficiency of the hot EDDS application on metal phytoremediation.

Besides the application of chelates to desorb metals from soil matrix, under nutrients limiting conditions, graminaceous plants usually increase markedly the release of phytosiderophores (PS) to the rhizosphere, which are capable of chelating not only Fe, but also Cu, Zn and Mn, thus mobilizing these metals

from soils (Treeby et al., 1989). Iron-deficiency is a common problem for calcareous soils because of the extremely low solubility of soil Fe (Mengel, 1994). The present results showed in the calcareous soils, mix-culture of pea with corn, barley and wheat enhanced the metal concentrations and total phytoextraction in the shoots of pea. The most enhanced effects were observed in the mix-culture system of barley and pea, where the concentrations of Cu, Pb, Zn, Cd and Fe in the shoots of pea reached 1.5-, 1.8-, 1.4-, 1.4- and 1.3-fold of those grown sole. Adding root exudates of barley to the pea plants grown in pots resulted in the significant increase of metal accumulation in the shoots of pea. Though the enhancement of metal concentration from the intercropping system was not more than 1.8-fold of the control, the present results pointed out a possible new direction for the naturally enhanced phytoremediation. Also, root exudates are confined to small portion of the soil where roots are located. Therefore, the adverse result of increased leaching of heavy metals by chelate application used in the chemically enhanced phytoremediation can be expected to be negligible in the soil.

The successful transfer of phytoremediation technology from the laboratory study to the field application is a crucial step in the development of phytoremediation technology. The present study mainly focused on the chelate-enhanced phytoextraction at a laboratory scale. As mentioned above, considering the difference between pot experiment and field application, further

field tests are needed to evaluate the practicability of the measures achieved in the present study.

For the chelate-enhanced phytoextraction, studies on screening high-biomass plants sensitive to the application of chelates and exploring new easily degradable and much cheaper chemicals should be continued, which will be very useful to reduce the potential negative effects on the surrounding environments and prompt the extensive operation of this technology in practice. Efficient chelate application methods and water drainage systems should be further tested and adopted in the field operation. More research is required to improve combustion techniques of contaminated biofuels, and the production of marketable products from contaminated biomass for industrial use (mainly oils and fibers) should be evaluated.

Exploring new metal hyperaccumulating plants and metal tolerant plants should be carried out in parallel with the optimizing of chelate-enhanced phytoextraction. The mechanisms responsible for metal hyperaccumulation are worthy further investigation, which will provide vital information to manipulate plants to be more productive and efficient. The results will also provide information for potential gene engineering research to produce a range of crops that can be used for the phytoextraction of several metals in contaminated sites in the future.

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Publications from the Research Project

Luo, C.L., Shen, Z.G., Baker, A.J.M., Li, X.D., 2006. A novel strategy for chemically enhanced phytoremediation of heavy metal-contaminated soils. Plant Soil (*in press*)

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Database of Original Results from All Experiments in the Study

Raw data from this study has been provided in the CD, which includes all the results from every experiment involved in the thesis.