MTT SCIENCE 5

Environmentally benign Fe chelates in plant nutrition

Doctoral Dissertation

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Abstract

The low solubility of iron (Fe) depresses plant growth in calcareous soils. In order to improve Fe availability, calcareous soils are treated with synthetic ligands, such as ethylenediaminetetraacetic acid (EDTA) and ethylenediimonobis(2-hydroxyphenyl)acetic acid (EDDHA). However, high expenses may hinder their use (EDDHA), and the recalcitrance of EDTA against biodegradation may increase the potential of cadmium (Cd) and lead (Pb) leaching. This study evaluated the ability of biodegradable ligands, i.e. different stereoisomers of ethylenediaminedisuccinic acid (EDDS), to provide Fe for lettuce (*Lactuca sativa* L.) and ryegrass (*Lolium perenne* cv. Prego), their effects on uptake of other elements and solubility in soils and their subsequent effects on the activity of oxygen-scavenging enzymes in lettuce. Both EDTA and EDDHA were used as reference ligands.

In unlimed and limed quartz sand both FeEDDS(S,S) and a mixture of stereoisomers of FeEDDS (25% [S,S]-EDDS, 25% [R,R]-EDDS and 50% [S,R]/[R,S]-EDDS), FeEDDS(mix), were as efficient as FeEDTA and FeEDDHA in providing lettuce with Fe. However, in calcareous soils only FeEDDS(mix) was comparable to FeEDDHA when Fe was applied twice a week to mimic drip irrigation. The Fe deficiency increased the manganese (Mn) concentration in lettuce in both acidic and alkaline growth media, whereas Fe chelates depressed it. The same was observed with zinc (Zn) and copper (Cu) in acidic growth media. EDDHA probably affected the hormonal status of lettuce as well and thus depressed the uptake of Zn and Mn even more. The nutrient concentrations of ryegrass were only slightly affected by the Fe availability.

After Fe chelate splitting in calcareous soils, EDDS and EDTA increased the solubility of Zn and Cu most, but only the Zn concentration was increased in lettuce. The availability of Fe increased the activity of oxygen-scavenging enzymes (ascorbate peroxidase, guaiacol peroxidase, catalase). The activity of Cu/ZnSOD (Cu/Zn superoxide dismutase) and MnSOD in lettuce leaves followed the concentrations of Zn and Mn. In acidic quartz sand low availability of Fe increased the cobalt (Co) and nickel (Ni) concentrations in lettuce, but Fe chelates decreased them. EDTA increased the solubility of Cd and Pb in calcareous soils, but not their uptake.

The biodegradation of EDDS was not affected by the complexed element, and [S,S]-EDDS was biodegraded within 28 days in calcareous soils. EDDS(mix) was more recalcitrant, and after 56 days of incubation water-soluble elements (Fe, Mn, Zn, Cu, Co, Ni, Cd and Pb) corresponded to 10% of the added EDDS(mix) concentration.

Key words:
biodegradation, calcareous soil, EDDHA, EDDS, EDTA, Fe deficiency, heavy metals, nutrient uptake, oxygen-scavenging enzymes

Biohajoavat rautakelaatit kasvinravitsemuksessa

Kari Ylivainio

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Tiivistelmä

Kasvit saavat Suomen lievästi happamista peltomaista riittävästi rautaa (Fe) kasvuunsa. Peltoviljelyssä raudan puutosta esiintyy kalkkipitoisissa maissa, koska raudan liukoisuus alenee nopeasti maan pH:n kohotessa. Myös kasvihuoneviljelyssä voi esiintyä raudan puutosta. Raudan liukoisuutta voidaan lisätä käyttämällä synteettisiä kelatointiaineita kuten etyleenidiaminitetraetikkahappoa (EDTA) tai etyleenidiimonobis(2-hydroksyphenyyli)etikkahappoa (EDDHA). Kelatointiaineet ovat kuitenkin kalliita (EDDHA) tai niiden biohajoaminen on hidasta (EDTA). Pysyvyys maaperässä voi lisätä haitallisten raskasmetallien, kuten kadmiumin ja lyijyn, liukoisuutta ja siten niiden huuhtoutumisriskiä. Tässä tutkimuksessa selvitettiin biohajoavien kelatointiaineiden (etyleenidiaminidisukkinaatti, EDDS) muodostamien rautakelaattien soveltuvuutta salaatin ja raiheinän raudan lähteeksi sekä kelaattien vaikutusta muiden alkuaineiden ottoon ja liukoisuuteen maassa. Verranteina käytettiin EDTA:ta ja EDDHA:ta.

Tulokset osoittivat, että biohajoavat kelaatit (Fe-EDDS(S,S) ja stereoisomeerien seos, FeEDDS(mix), jossa 25 % [S,S]-EDDS, 25 % [R,R]-EDDS ja 50 % [S,R]/[R,S]-EDDS) turvasivat salaatin ja raiheinän raudansaannin kvartsihiekassa yhtä hyvin kuin Fe-EDTA ja Fe-EDDHA, riippumatta hiekan pH:sta. Tulosten perusteella biohajoava [S,S]-EDDS voisi olla kasvihuoneviljelyssä potentiaalinen kelatointiaine raudalle, kun kasvualustana on kivivilla. Vaikka [S,S]-EDDS hajoaa maassa nopeasti, voitaisiin kasvihuoneissa yleisesti käytetyn tippukastelun avulla todennäköisesti ylläpitää riittävä Fe-EDDS(S,S):n pitoisuus kasvien raudan saannin turvaamiseksi.

Kalkkipitoisissa maissa Fe-EDDS(S,S):n teho oli heikompi, mutta Fe-EDDS(mix) kasvatti salaatin rautapitoisuuden samalle tasolle kuin Fe-EDDHA, kun lannoite annettiin tippukastelun tapaan kaksi kertaa viikossa. Kalkkipitoisessa maassa [S,S]-EDDS biohajosi 28 vuorokaudessa. EDDS(mix) oli heikommin biohajoava ja 10 % lisätystä määrästä oli jäljellä 56 vuorokauden jälkeeen, kun mittarina käytettiin kelatoituneita raskasmetallipitoisuuksia (Fe, Mn, Zn, Cu, Co, Ni, Cd ja Pb).

Raudan puutos kasvatti salaatin mangaanipitoisuutta kvartsihiekassa ja kalkkipitoisessa maassa, kun taas rautakelaatit pienensivät sitä. Samoin tapahtui sinkin ja kuparin pitoisuuksille happamassa kvartsihiekassa. EDDHA vaikutti todennäköisesti myös salaatin hormonitasapainoon alentaessaan salaatin sinkki- ja mangaani-

pitoisuuksia enemmän kuin muut kelaatit. Raudan saatavuus vaikutti vain vähän raiheinän ravinnepitoisuuksiin.

Salaatilla tutkittiin myös kelatointiaineiden vaikutusta happiradikaaleja vähentävien entsyymien (askorbaattiperoksidaasi, guajakoliperoksidaasi, katalaasi, Cu/Zn-superoksididismutaasi ja Mn-superoksididismutaasi) aktiivisuuksiin. Raudan saatavuus vaikutti salaatin askorbaattiperoksidaasin, guajakoliperoksidaasin ja katalaasin aktiivisuuksiin, ja vaihtelu sinkin, kuparin ja mangaanin pitoisuuksissa puolestaan Cu/Zn- ja Mn-superoksididismutaasin aktiivisuuksiin.

Kalkkipitoisessa maassa EDDS ja EDTA lisäsivät tutkituista raskasmetalleista (Fe, Mn, Zn, Cu, Co, Ni, Cd ja Pb) sinkin ja kuparin liukoisuutta eniten sen jälkeen kun Fe-EDDS ja Fe-EDTA -kompleksit hajosivat, mutta vain sinkkipitoisuus kasvoi salaatissa. EDTA kasvatti kadmiumin ja lyijyn liukoisuutta kalkkimaissa, mutta ei niiden pitoisuutta salaatissa. Happamassa kvartsihiekassa raudan heikko saatavuus lisäsi salaatin koboltti- ja nikkelipitoisuutta, mutta rautakelaatit alensivat pitoisuuksia.

Avainsanat:
antioksidatiiviset entsyymit, biohajoaminen, EDDHA, EDDS, EDTA, kalkkimaa, kasvihuoneviljely, raskasmetallit, raudan puutos, ravinteiden otto

Acknowledgements

I joined the chelating agent project, lead by Docent Reijo Aksela, in February 1998. The project was funded by Kemira Oyj and the National Technology Agency of Finland (TEKES). The main goal for the whole project was to develop new chelating agents to decrease the detrimental effect of free Fe, Mn and Cu on hydrogen peroxide in pulp bleaching. The project had several subprojects, and I was responsible for testing whether these chelating agents could be used as Fe chelates to improve the availability of Fe for plants.

I am grateful to my supervisor, Professor Antti Jaakkola, for his instructions during the project. After Professor Antti Jaakkola retired, Professor Markku Yli-Halla took his place, and I appreciate his work for guiding me through the last steps of this work. I am also indebted to Professor Helinä Hartikainen for her guidance and support during the course of this work.

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Finally, I wish to thank my family for their enduring patience during the work associated with this thesis.

List of original publications

The thesis is a summary and discussion of the following articles, which are referred to by their Roman numerals:

I **Ylivainio, K., Jaakkola, A. & Aksela, R. 2004.** Effects of Fe compounds on nutrient uptake by plants grown in sand media with different pH. Journal of Plant Nutrition and Soil Science 167: 602–608.

II **Ylivainio, K., Jaakkola, A. & Aksela, R. 2006.** Impact of liming on utilization of ^{59}Fe-chelates by lettuce (*Lactuca sativa* L.). Journal of Plant Nutrition and Soil Science 169: 523–528.

III **Ylivainio, K. 2010.** Influence of Iron-Chelates on Trace Element Uptake from Two Calcareous Soils and the Activity of Oxygen-Scavenging Enzymes in Lettuce. Journal of Plant Nutrition. In press.

IV **Ylivainio, K.** Effects of iron(III)-chelates on the solubility of heavy metals and their uptake by lettuce (*Lactuca sativa* L.) in calcareous soils. Environmental Pollution. Submitted.

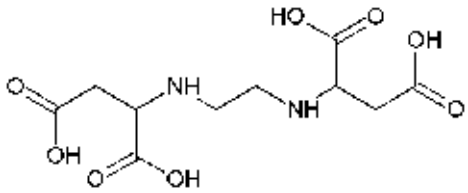
In addition some unpublished data are presented

The author's contribution in joint publications

I, II Kari Ylivainio was responsible for planning and conducting the experiments, and for preparing the manuscripts. Professor Antti Jaakkola took part in planning the experiments and commenting on the manuscripts. Docent Reijo Aksela provided the ligands for the experiments and commented on the manuscripts.

Symbols and abbrevations

Symbols:



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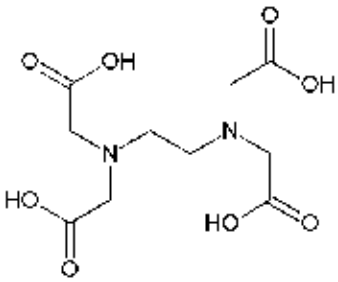

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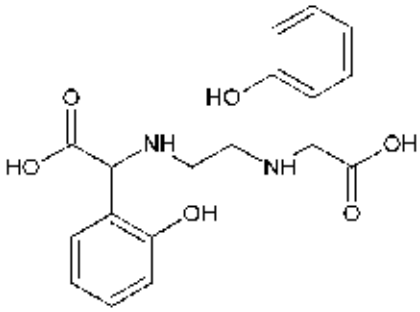

EDDHA

Abbreviations:

EDDS	ethylenediaminedisuccinic acid
EDTA	ethylenediaminetetraacetic acid
EDDHA	ethylenediiminobis(2-hydroxyphenyl)acetic acid
EDDHMA	ethylendiaminedi(2-hydroxy-4-methylphenylacetic) acid
DTPA	diethylenetriaminepentaacetic acid
HEEDTA	hydroxyl-2-ethylenediaminetriacetic acid
IRT1	iron-regulated transporter 1
SOD	superoxide dismutase
GPX	guaiacol peroxidase
APX	ascorbate peroxidase
CAT	catalase

Contents

1 Introduction 11
1.1 Content and solubility of Fe in soils 11
1.2 Iron in plant nutrition 13
1.2.1 Environmental factors affecting Fe uptake 13
1.2.2 Plant strategies for Fe acquisition 14
1.2.3 Functions of Fe in plants 16
1.3 Chelating agents in plant nutrition 17
1.3.1 Utilization of Fe chelates by plants 18
1.3.2 Reactions of chelating agents in soils 18
1.3.3 Effects of chelates on element uptake 19
1.3.4 Biodegradation 20
1.3.5 Prevalence of Fe deficiency in Finland 20
1.4 Objectives of the study 21

2 Materials and methods 22
2.1 Experiments 22
2.1.1 Ligands and preparation of Fe chelates 22
2.1.2 Growth experiments 23
2.1.3 Incubation experiment 25
2.2 Methods of analyses 26
2.3 Quality control 27

3 Results and discussion 28
3.1 Yield responses to Fe chelates 28
3.2 Effects of Fe chelates on element uptake 30
3.2.1 Uptake of Fe, Mn, Zn and Cu by lettuce and ryegrass 30
3.2.2 Uptake of Ni, Co, Cd and Pb by lettuce and of Ni and Co by ryegrass 33
3.3 Activity of oxygen-scavenging enzymes 34
3.4 Solubility of heavy metals in calcareous soils after Fe chelate addition 37
3.4.1 Biodegradation 41

4 Conclusions 43

References 45

Appendices 57

1 Introduction

1.1 Content and solubility of Fe in soils

The average iron (Fe) concentration in the soil surface is 3.5% (see Chesworth 1991). It is the fourth most common element in the earth's crust, after oxygen, silicon and aluminium. In spite of its abundance in soils, the deficiency of Fe restricts plant growth due to its low solubility. The solubility of Fe is strongly dependent on soil pH. The activity of Fe^{3+} decreases 1000–fold for each unit increase in soil pH, while its solubility is lowest in a pH range of 7.4–8.5 (Lindsay 1979). Therefore, the Fe deficiency is greatest in calcareous and alkaline soils. It is estimated that calcareous soils cover about 30% of world's land surface and are therefore susceptible to Fe deficiency (Vose 1982).

In primary minerals, Fe occurs mostly in ferromagnesian minerals. Upon weathering, Fe^{2+} is oxidized to Fe^{3+}, which after release from mineral lattices precipitates and forms ferric oxides and hydroxides, such as ferrihydrite, hematite and goethite (Carlson 1982; Schwertmann 1991). The most stable and prevalent Fe^{3+} oxides are goethite, α-FeOOH, and hematite, α-Fe_2O_3 (Lindsay 1979; Schwertmann 1991). They are also the least soluble, and freshly precipitated Fe^{3+} sustains a soluble Fe^{3+} concentration that is 3630 times higher than that sustained by goethite (Figure 1).

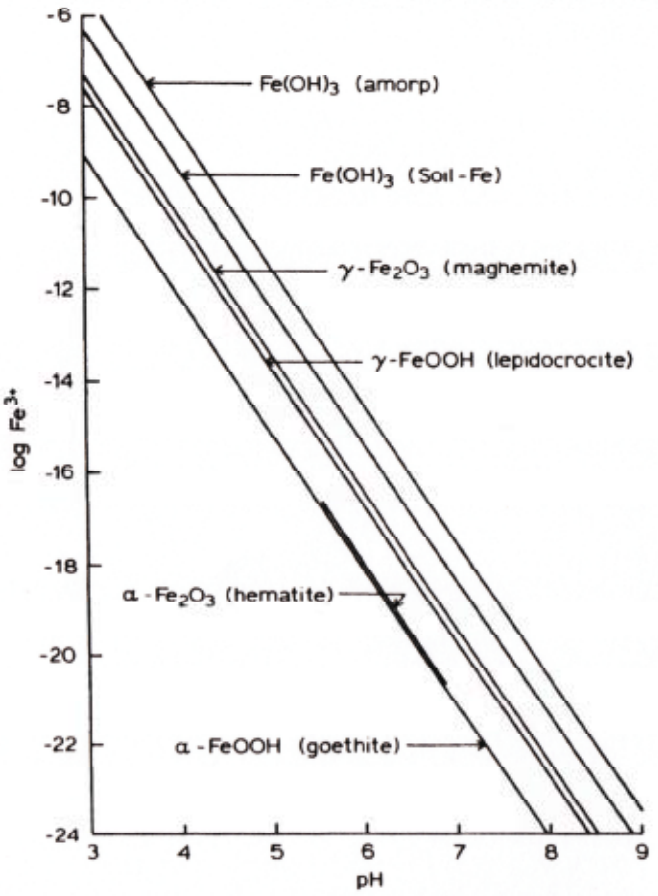

Figure 1. Effect of different Fe(III) oxides on the activity of Fe^{3+} as a function of pH (Lindsay 1979. With the kind permission of The Blackburn Press and John Wiley & Sons, Inc).

In aqueous solutions, Fe^{3+} is complexed by six water molecules, forming a Fe^{3+} aqua complex, $Fe(H_2O)_6^{3+}$. For the sake of simplicity, H_2O molecules are usually omitted from the formula. With increasing pH, H^+ is removed step by step from the coordinated water molecules, producing a series of hydrolysis species, namely $FeOH^{2+}$, $Fe(OH)_2^+$, $Fe(OH)_3$ and $Fe(OH)_4^-$, which form the total concentration of soluble Fe. In soils with a pH between 4.0–7.4, the main hydrolysis species is $Fe(OH)_2^+$, and at the pH range of 7.4–8.5, which is the minimum solubility range of ferric hydroxides, $Fe(OH)_3^0$ is the dominant form. At the pH range of 7.4–8.5, the sum of Fe(III) hydrolysis species is about $10^{-10.4}$ M (Lindsay 1984). Above pH 8.5 the main hydrolysis species is $Fe(OH)_4^-$. The activity of Fe in soil solution is also increased by the complexes and ion pairs formed between anions and Fe (Lindsay 1979). However, the hydrolysis species of Fe dominate the total soluble Fe concentrations, and $FeHPO_4^+$ increases the total Fe in solution only in acid soils (Lindsay 1979).

Ferric oxides provide soluble Fe through protonation, reduction and complexation (Lindsay 1979; Schwertmann 1991). During protonation, protons are adsorbed on Fe-OH groups, leading to the weakening of the Fe-O bond and causing detachment of Fe (Schwertmann 1991). In reductive dissolution of Fe, the mechanism is rather similar, except that the reducing agent provides electrons for Fe reduction, leading to Fe detachment as Fe^{2+} (Schwertmann 1991). There are two different viewpoints on how complexation increases the solubility of Fe. According to Lindsay (1979), ligands complex Fe already present in the soil solution, which causes solid phase to replenish the activity of Fe^{3+} in the soil solution. On the other hand, according to the hypothesis of ligand-promoted disso-

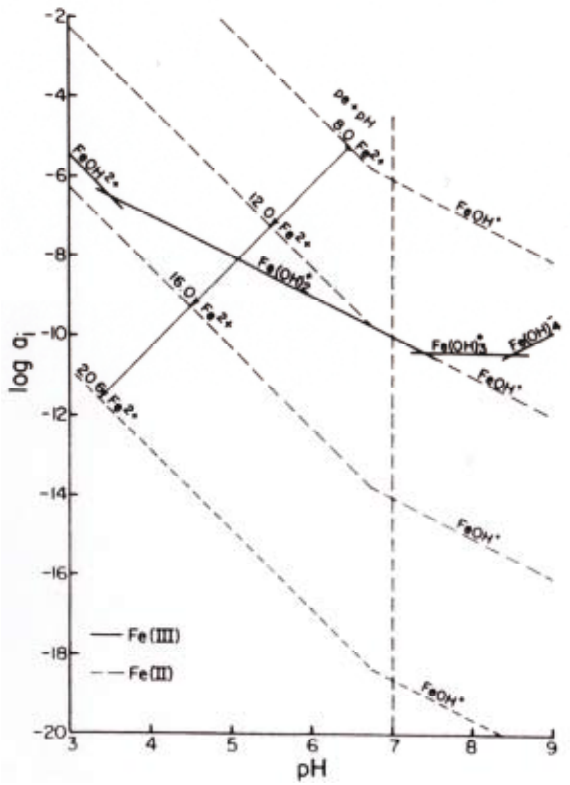

Figure 2. The effect of pe+pH on the activity of Fe^{2+} and $FeOH^+$ in comparison with Fe(III) hydrolysis species in equilibrium with soil-Fe (Lindsay and Schwab 1982, Reproduced with a kind permission of Taylor & Francis Ltd).

lution, organic ligands complex metals that are bonded to the surface of Fe oxides, causing the metal to be detached (Borggaard 1991).

In well oxidized soils with a pe+pH value of 12–16, Fe(III) hydrolysis species are the dominant form of soluble Fe, and the solubility of Fe^{2+} increases significantly after pe+pH drops below 12 (Figure 2). The redox potential may be depressed near the respiring roots or via the metabolism of micro-organisms, the situation being more drastic in submerged soils (Lindsay and Schwab 1982). Organic acids, such as citrate and oxalate, released by roots, can detach Fe from the Fe(III) oxides after complexation. In addition, soil organic matter can increase the solubility of Fe by complexation. Solubility of Fe(III) oxides is also affected by the type of oxide, surface area, crystallinity and crystal chemistry (Schwertmann 1991). Only on very old soils (oxisols) with a stable redox situation, can other minerals besides $Fe(OH)_3$ control the solubility of Fe (Lindsay 1979; Lindsay and Schwab 1982).

Soil organic matter and clay may retain Fe oxides in poorly crystalline form, thus maintaining a higher Fe activity in the soil solution than do old, more crystalline forms (e.g. ferrihydrite vs. goethite; Loeppert and Hallmark 1985).

1.2 Iron in plant nutrition

1.2.1 Environmental factors affecting Fe uptake

High soil pH and high bicarbonate concentration have been reported to be the main causes of Fe deficiency (Boxma 1972; Mengel et al. 1984; Lucena 2000) by restricting Fe uptake and translocation to shoot (Nikolic et al. 2000) or by inactivating Fe within the leaves and hampering Fe translocation from apoplast to symplast (Gruber and Kosegarten 2002). Factors that increase soil bicarbonate content, e.g. high soil moisture and compaction, may increase the incidence of Fe deficiency (Boxma 1972; Mengel et al. 1984; Inskeep and Bloom 1986). However, it has also been suggested that the low solubility of Fe in calcareous soil is not the main cause of Fe deficiency in plants, but rather the Fe movement from root apoplast to symplast (Kosegarten and Koyro 2001). In a recent experiment, bicarbonate blocked the expression of genes responsible for Fe efficiency reactions (Lucena et al. 2007).

The balance of cation-anion uptake has a significant effect on rhizosphere pH. With an excess of anion uptake, net efflux of OH^- occurs, whereas excess cation uptake induces H^+ extrusion uniformly along the roots, thus acidifying the rhizosphere and enhancing Fe solubility (see Haynes 1990). The balance of cation-anion uptake is determined by nitrogen nutrition, because either NH_4^+ or NO_3^- is the predominant cation or anion adsorbed, respectively. If NO_3^- is the sole source of nitrogen, alkalinization of the rhizosphere can cause chlorotic symptoms (Lucena 2000). Nitrate nutrition may also depress Fe utilization within the leaves by increasing the pH in the apoplast (Kosegarten and Englisch 1994; Kosegarten et al. 2001). Increased availability of heavy metals has been shown to increase the likelihood of Fe deficiency (Römheld and Marschner 1986b; Wallace et al. 1992). A difference in phosphorus (P) uptake efficiency also affects Fe utilization efficiency within the plant (Brown et al. 1977).

Although Fe deficiency is most prevalent in calcareous soils, it can also occur in acidic soils (Stewart and Leonard 1952; Heinonen 1961; O'Toole 1966; Van Dijk and Bienfait 1993). A deficiency of boron (B) has been shown to cause Fe deficiency by restricting root growth and thus depressing Fe utilization (Heinonen 1961). Because the mobility of Fe in soils is diffusion-controlled (O'Connor et al. 1971;

Chaney 1984), Fe uptake is dependent on root growth, and unfavorable growing conditions may depress Fe uptake (Römheld and Marschner 1986b).

1.2.2 Plant strategies for Fe acquisition

The minimum required concentration of soluble Fe in soil solution for plants is about 10^{-8} M (Lindsay 1984). Without any modifying factors of the plant roots, a deficiency of Fe can occur even at pH 5 (Lindsay 1995). For this reason, plants have developed mechanisms for increasing Fe availability (Römheld and Marschner 1986b). These mechanisms can be divided to non-specific and specific, with the specific mechanisms being dependent on the Fe nutritional status of the plant (Römheld and Marschner 1986b).

It has been proposed that control of Fe efficiency reactions takes place in the roots (Bienfait et al. 1987; Maas et al. 1988) via signals from shoots to roots, such as phloem Fe concentration (Maas et al. 1988), a shoot-borne signal (Schikora and Schmidt 2001), auxin (Romera and Alcántara 1994), ethylene (Romera et al. 1999) or NO (Murgia et al. 2002).

Non-specific mechanisms

The non-specific mechanisms for increasing Fe availability are "acidic" nutrient uptake (cations > anions) and extrusion of organic acids and other photosynthetically bound carbon from the roots (Römheld and Marschner 1986b). The main nutrients that affect the balance of cation/anion uptake are NH_4^+ and NO_3^- (Riley and Barber 1971; Marschner and Römheld 1983), form of K nutrition (Oertli and Opoku 1974) and the nutritional status of P in the plant (Hedley et al. 1983). Genotypic differences also affect the root-induced changes in the rhizosphere (Römheld and Marschner 1986b). In a recent study, root exudates of Fe deficient red clover selectively influenced the microbial community, which enhanced Fe acquisition by producing siderophores and auxin (Jin et al. 2006). Microbial siderophores may thus have an important role in Fe acquisition by plants (Masalha et al. 2000; Jin et al. 2006). Although utilization of siderophores may be low because of their high stability, they may increase Fe concentration in the rhizosphere for later utilization (Bar-Ness et al. 1992).

Specific mechanisms

Plants have adopted two different strategies for Fe acquisition, i.e. Strategy I and Strategy II. Strategy I employed by dicots and nongraminaceous monocots and Strategy II by graminaceous monocots are both enhanced after Fe deficiency stress (Römheld and Marschner 1986b).

Strategy I plants reduce Fe^{3+} to Fe^{2+} prior to uptake by roots (Chaney et al. 1972; Römheld and Marschner 1983). The roots of Strategy I plants undergo both physiological and morphological changes in order to enhance Fe^{3+} reduction. These Fe efficiency reactions have been shown to take place before the so-called Fe deficiency symptoms become visible (Yi and Guerinot 1996). Reduction of Fe^{3+} is carried out by ferric chelate reductase (FCR), and the activity of FCR is increased during Fe deprivation (Bienfait 1985; Moog and Brüggemann 1994). It has been argued that roots contain two separate reductases, FeCN (standard) and Fe(III)EDTA (Turbo) (Bienfait 1985; Moog and Brüggemann 1994). Standard reductase is suggested to operate in a whole root system, whereas turbo reductase operates only in the epidermal cells of young roots and becomes active during a deficiency of Fe (Bienfait 1985). Recently the genes responsible for encoding FCR were isolated (Robinson et al. 1999; Waters et al. 2002). The optimum pH for FCR is about 5 (Römheld et al. 1982; Römheld and Marschner 1983; Moog and Brüggemann 1994; Kosegarten et al. 2004) and its activity decreases with increasing pH (Susín

et al. 1996; Lucena and Chaney 2007). A deficiency of Cu has been shown to increase the activity of FCR as well as rhizosphere acidification (Welch et al. 1993). FCR also operates in the leaf plasma membrane; reduction of Fe is required prior to Fe uptake, and light increases its activity (Brüggemann et al. 1993).

The increased activity of H^+-ATPase in roots during Fe deprivation (Rabotti and Zocchi 1994) acidifies the rhizosphere by excreting H^+ and may decrease the pH of the rhizosphere by 0.5–1 pH units (Marschner et al. 1989). Acidification of rhizosphere is confined to the apical root zones (Römheld et al. 1984), whereas with an excess cation uptake, acidification is uniformly distributed to the whole root system (Römheld et al. 1984). Localization of acidification may amplify plants' ability to increase Fe solubility (Marschner 1995) and stimulate FCR activity (Bienfait et al. 1983; Marschner et al. 1986).

A deficiency of Fe increases the production and concentration of organic acids in the roots, mostly citrate and malate. This is related to enhanced proton extrusion from apical root zones (Bedri et al. 1960; Landsberg 1981; Abadía et al. 2002) and to the formation of rhizodermal transfer cells (Römheld et al. 1984). Increased citrate concentration in leaves after Fe deficiency has been noted as well (see Abadía et al. 2002).

Organic acids may acidify the rhizosphere or complex Fe and thus increase the availability of Fe as well as of other heavy metals (Marschner 1995). However, the significance of citrate and malate in complexing Fe and dissolving Fe oxides decreases as soil pH increases and these acids are rapidly mineralized by microorganisms (Jones 1998). A deficiency of Fe induces plants to release reducing compounds, mainly phenolics (Römheld and Marschner 1983). Phenols have little influence on the reduction of chelated Fe(III) in comparison to FCR (Römheld and Marschner 1983). The reducing compounds provided at most only 4% of the total reducing capacity of the roots (Grusak and Pezeshgi 1996). However, a recent experiment showed that phenolics may enhance the utilization of apoplastic Fe (Jin et al. 2007).

Morphological changes in roots include formation of lateral roots and rhizodermal transfer cells (Landsberg 1982; Römheld et al. 1984), and increased root hair formation (Landsberg 1982; Schikora and Schmidt 2001). Transfer cells are considered to be responsible for rhizosphere acidification by excreting protons (Römheld and Kramer 1983; Römheld et al. 1984).

An increase in the Fe efficiency reactions in the roots of Strategy I plants increases the solubility of other elements as well. The solubility and uptake of Mn and Cu increased during Fe deficiency (Römheld et al. 1982; Welch et al. 1993; Cohen et al. 1998). Zn (Römheld et al. 1982; Yi and Guerinot 1996; Cohen et al. 1998) and Cd (Cohen et al. 1998) uptake have also been shown to increase during Fe deficiency, whereas heavy metals (Co, Ni, Cu, Cd, Mn, Pb, Zn, Mo) may depress both the induction and functioning of FCR (Alcántara et al. 1994).

Uptake of Fe^{2+} takes place through a specific Fe^{2+} transporter (Zaharieva and Römheld 2000) and is metabolically controlled (Fox et al. 1996). Eide et al. (1996) have shown that the IRT1-gene (iron-regulated transporter) is responsible for coding the Fe(II) transporter in *Arabidopsis thaliana*. However, although IRT1 is inducible by Fe^{2+}, it can transport Mn, Zn, Cd and Co as well (Eide et al. 1996; Korshunova et al. 1999). Translocation of Fe has been suggested to be in the form of Fe citrate (Tiffin 1970).

The **Strategy II** mechanism is considered to be more efficient than the Strategy I mechanism for acquisition of Fe (Römheld

1987; Curie and Briat 2003). The Strategy II Fe uptake mechanism has a lower sensitivity for high soil pH and bicarbonate concentration than the Strategy I Fe uptake mechanism. Plants with Strategy II are therefore better adapted to calcareous environments (Römheld and Marschner 1986a). Strategy II mechanisms include the release of phytosiderophores, which are chelators that have a high affinity for Fe(III) (Takagi et al. 1984; Awad et al. 1994) and effectively solubilize sparingly soluble inorganic Fe(III) compounds, forming Fe(III) phytosiderophores (Römheld and Marschner 1986a). Phytosiderophores are characterized as nonproteinogenic amino acids, such as mugineic and avenic acid (Takagi et al. 1984; Sugiura and Nomoto 1984). Methionine is suggested to be the precursor of the phytosiderophores (Mori and Nishizawa 1987). Uptake of Fe(III) phytosiderophores to the roots occurs through a specific transporter (Curie et al. 2001) that is located in the plasma membrane of the roots (Marschner and Römheld 1994).

The release of phytosiderophores from roots as well as of their transporter in roots, YS1 (yellow stripe 1), increased during Fe shortage (Mori 1999; Curie et al. 2001). The release of phytosiderophores may increase up to 20-fold under Fe deficiency (Marschner et al. 1989), and it has been proposed that the release of phytosiderophores as well as the uptake of Fe(III) phytosiderophore complexes takes place in the apical zone of the roots (Marschner et al. 1987). Susceptibility to Fe deficiency is related to the ability of a plant to excrete phytosiderophores, not to the rate of uptake (Römheld and Marschner 1986a). The roots of Strategy II plants also demonstrate Fe(III) chelate reductase activity, but it cannot be induced by Fe deficiency (Moog and Brüggemann 1994). Unlike plants with Strategy I Fe uptake mechanisms, grasses do not acidify the rhizosphere by increasing proton extrusion under Fe deficiency (Römheld and Marschner 1986b).

Phytosiderophores also complex Zn, Cu and Mn (Treeby et al. 1989). However, only Fe(III) phytosiderophores are preferentially taken up by the roots (Marschner et al. 1989), and Fe(III) phytosiderophores are better source of Fe than synthetic Fe chelates for Strategy II plants (Mino et al. 1983; Marschner et al. 1987). Occurrence of Fe deficiency in calcareous soils with Strategy II uptake mechanisms is not related to bicarbonate content, but to the content of amorphous Fe(III) oxides (Marschner and Römheld 1994).

1.2.3 Functions of Fe in plants

Among the visible symptoms of Fe deficiency are yellowing of the youngest leaves (Terry and Abadía 1986) and depressed leaf growth (Kosegarten et al. 1998). Chlorosis is caused by the depressed biosynthesis of δ-aminolevulinic acid and protochlorophyllide, precursors of chlorophyll molecules (Marschner 1995). Yellowing of leaves in a calcareous environment was first related to Fe by Gries in 1843 (cited by Wallace 1982). Chlorosis occurs in the interveinal area of the leaves, while the veins remain green. This is probably related to the observation that Fe is preferentially located in the midrib and veins in Fe deficient leaves (Jimémez et al. 2009).

The functions of Fe in plants are mainly based on the plant's reduction/oxidation cycle (Marschner 1995). The critical deficiency Fe concentration ranges between 50–150 mg kg^{-1} DW (Marschner 1995), with the average being 70 mg kg^{-1} DW (Smith et al. 1984) and most of the Fe located in the chloroplasts (Terry and Low 1982). The critical deficiency concentration varies depending on the development stage of the leaves. The highest total Fe concentrations have been found in the youngest leaves with the highest growth rate (Häussling et al. 1985). The youngest leaves seem to be less efficient in using Fe (Kosegarten et al. 1998). This may lead to the "chlorosis paradox", a situation where

the Fe concentration is higher in chlorotic than in green leaves (Römheld 2000). A deficiency of Fe has also caused growth retardation without the deficiency being visible (Gruber and Kosegarten 2002). Extraction of fresh leaves with 1 M HCl was used for the first time by Oserkowsky (1933) in determining the concentration of "active Fe". The concentration of active Fe may be lower in chlorotic than in green leaves (Römheld 2000).

The homeostasis of Fe is under strict control (Hall and Williams 2006) due to its ability to form reactive oxygen species (ROS). In the Fe-catalyzed Haber Weiss cycle, Fe^{3+} is reduced by superoxide anion (O_2^-), and Fe^{2+} can further react with hydrogen peroxide (H_2O_2) in a Fenton-like reaction, forming hydroxyl radicals ($OH^- + OH^\cdot$), which are more destructive of cells than the preceding oxygen radicals (see Elstner 1982). Photosynthesis and respiration are the main sources of ROS (Van Breusegem and Dat 2006), and physiological and environmental factors may disrupt the redox state of the plant, causing production of ROS, such as O_2^- (Thompson et al. 1987). Although detrimental for plants at high concentrations, ROS's play an important role in many mechanisms, e.g. as signal molecules (see Schützendübel and Polle 2002).

Plants have evolved a variety of different mechanisms to scavenge ROS. The first barrier against ROS is superoxide dismutase (SOD), a metalloenzyme that catalyzes O_2^- into H_2O_2. SOD contains three different isoenzymes, namely Cu/ZnSOD, MnSOD and FeSOD (see Elstner 1982). Deficiencies of Cu, Zn and Mn have been shown to alter the activity of SOD forms by increasing or decreasing their activity (Yu and Rengel 1999). A deficiency of Fe increased the activity of Cu/ZnSOD (Iturbe-Ormaetxe et al. 1995; Ranieri et al. 1999; Tewari et al. 2005). Catalase and peroxidases, which scavange H_2O_2, contain Fe as a haem group, and deficiency of Fe depresses their activity (Marschner 1995). Catalase operates mainly in peroxisomes/glyoxysomes, whereas ascorbate peroxidase is mainly located in the chloroplasts (Asada 1992). Guaiacol peroxidase is a ubiquitous, non-specific peroxidase that catalyzes a wide range of phenolic substrates (Siegel 1993). The activity of ascorbate peroxidase and catalase has been suggested to be a better indicator of physiologically active Fe than of total Fe (Tewari et al. 2005).

1.3 Chelating agents in plant nutrition

Chelating agents are used in a wide variety of different applications, e.g. in detergents, water treatment, agrochemicals, the pulp and paper industry, photography, electronics, medicine, foods and in the decontamination of nuclear power plants. Chelating agents are normally polydentate organic compounds that form chelate rings with the metal ions. The phrase "chelating agent" is derived from a Greek word "chela", meaning the claw of a crab. The most common chelating agent is EDTA (ethylenediaminetetraacetic acid) and 34,550 metric tons of EDTA were used in Europe in 1999 (see Knepper 2003).

Because of the low solubility of Fe in calcareous environments, synthetic chelating agents are used to sustain Fe in a plant-available form. Before the invention of synthetic chelating agents, organic compounds, such as citrate and tartrate, were used to increase the solubility of Fe in alkaline solutions (Gile and Carrero 1916; Reed and Haas 1924). The use of synthetic chelating agents in plant nutrition dates back to the 1950's, when FeEDTA was used for the first time (Jacobson 1951), and EDDHA (ethylenediiminobis(2-hydroxyphenyl)acetic acid) was synthesized (Kroll et al. 1957). EDTA sustains Fe in a soluble form effectively in moderately acid to near neutral soils, whereas EDDHA sustains Fe in a soluble form in a wider pH range from 4 to 9 (Norvell 1991). Stabili-

ty constants of the chelates (log K) are used to express their effectiveness in sustaining metal in a complexed form:

$$[M] + [L] \leftrightarrow [ML], \qquad \frac{[ML]}{[M]^*[L]}, \text{ where}$$

[M] = metal concentration, [L] = ligand concentration and [ML] = chelate concentration.

Stability constants are based on thermodynamic equilibrium, and the distribution of different metal-ligand complexes is a function of stability constants and the concentrations of the free metals (Nowack 2002). Therefore, a calcareous environment, with a high concentration of Ca^{2+} versus a low concentration of soluble Fe, favors the formation of CaEDTA instead of FeEDTA (Lindsay 1979), although the stability constant of CaEDTA (log K = 11.6) is lower than that of FeEDTA (log K = 26.5, Norvell 1991). In calcareous soils stability constants were not able to predict the concentration of chelated micronutrients in a soil solution (Lucena et al. 1987). In soils with slow element exchange processes, the kinetics of the chelating agents may affect the speciation of metals in soil solution.

1.3.1 Utilization of Fe chelates by plants

Plants with a Strategy I Fe uptake mechanism reduce Fe^{3+} chelate, and Fe^{2+} uptake occurs after chelate splitting (Chaney et al. 1972; Römheld and Marschner 1983), whereas plants with a Strategy II Fe uptake system based on uptake of Fe(III) phytosiderophores are not able to utilize Fe from strong chelates, such as FeEDDHA (Lucena et al. 1988b; Bar-Ness et al. 1992). However, chelates with lower stability may increase apoplastic Fe content after chelate splitting, and phytosiderophores may form Fe(III) phytosiderophore complexes for uptake by roots (Zhang et al. 1991). The reduction rate of Fe from Fe chelates in Strategy I plants as well may be depressed when stability of the Fe chelates increases (Chaney 1989). Leaves are able to reduce foliarly applied Fe chelates as well. The ability of leaves to reduce foliarly applied Fe chelates was increased by light intensity but not by Fe deficiency (Brüggemann et al. 1993; De La Guardia and Alcántara 1996).

After chelate splitting, ligands are free to complex Fe or other elements from the soil (Lindsay 1995; Lucena 2003). This shuttle may provide nutrients for plants until ligand adsorption, leaching or biodegradation. Intact chelates may even be taken up by the plants (Hill-Cottingham and Lloyd-Jones 1965; Jeffreys and Wallace 1968) through a non-selective route, probably through the breakdown of the endodermis after lateral root formation (Marschner et al. 1987). The uptake of metal-EDTA complexes may also be affected by the charge of the molecule (Bell et al. 2003).

The utilization of soil-applied Fe chelates has been reported to be in the range of 0.5–2% (see Chen and Barak 1982). As shown by Lucena and Chaney (2007), only about 1% of the Fe reduced by FCR was taken up and translocated to the shoot. Contrary to soil application, utilization of foliarly applied inorganic Fe is comparable to that of Fe chelates (Brüggemann et al. 1993; Álvarez-Fernández et al. 2004). Spraying the leaves with acidic sprays has cured Fe chlorosis and activated Fe reserves in the leaves (Kosegarten et al. 2001).

1.3.2 Reactions of chelating agents in soils

After Fe chelate splitting via FCR or Fe hydrolysis free ligands can complex Fe again from the soil solution (Lindsay 1979) or they can increase Fe solubility through dissolution or protonation of the solid phase (Schwertmann 1991). Dissolution of Fe(III) oxides is called “ligand-promot-

ed dissolution," and intact EDTA increases Fe solubility more rapidly than if EDTA is complexed with metal ions (Nowack and Sigg 1997). Complexation of metals by free ligands is affected e.g. by the stability of the chelate, soil pH, redox state, organic matter content, exchangeable Ca and adsorption of ligand to soil (Wallace and Lunt 1956; Norvell and Lindsay 1969; Elgala and Maier 1971; Lindsay 1979; Sommers and Lindsay 1979; Cadahía et al. 1988; Lucena et al. 1988a).

The adsorption of ligands onto the solid phase is affected by the type of chelating agent, pH, time, salt concentration and soil texture (Norvell 1991). Of the ligands, EDTA adsorbs on soil more strongly than EDDHA (Wallace et al. 1955). Adsorption of chelates is also affected by the complexed metal as well: FeEDTA is adsorbed more strongly than CaEDTA (Hill-Cottingham and Lloyd-Jones 1957; Norvell and Lindsay 1982) and ZnEDTA least (Wallace and Lunt 1956; Lahav and Hochberg 1975). FeEDDHA is a stable chelate and its adsorption is low (Lahav and Hochberg 1975), especially in calcareous soils (Hernández-Apaolaza and Lucena 2001).

The dissociation constants for the four carboxylic groups of EDTA are: $K_1 = 1.02 * 10^{-2}$, $K_2 = 2.14 * 10^{-3}$, $K_3 = 6.92 * 10^{-7}$ and $K_4 = 5.5 * 10^{-11}$. Therefore, in a pH range of agricultural soils, free EDTA or metal-EDTA species are in an anionic form, and adsorption of EDTA is negatively correlated with soil pH. Adsorption of EDTA on Fe(III) oxides is ligand-like: a binuclear complex is formed at a lower pH value and mononuclear complex at a higher pH value (Nowack and Sigg 1996).

1.3.3 Effects of chelates on element uptake

The agronomic effectiveness of a chelate is defined on the basis of the following four factors (Lucena 2003): 1) Ability to sustain Fe in soluble form in soil and to chelate indigenous Fe for plant uptake, 2) low affinity for other cations, i.e. other cations should not displace Fe from the chelate, 3) the ability of the plant to utilize complexed Fe and 4) low adsorption to soil and resistance against degradation.

After chelate splitting, free ligands are able to complex other elements and act as a shuttle between soil and roots, providing complexed elements for the plant. Although the effects of Fe chelates on Fe uptake have been studied extensively, the effects of ligands on the uptake of other elements have scarcely been explored, especially at levels used to correct nutrient deficiencies. One of the most studied effects of Fe chelate on nutrient uptake is the observed decrease in Mn concentration after FeEDDHA application (Holmes and Brown 1955; Moraghan 1979; Ghasemi-Fasaei et al. 2003). This effect is thought to be the cause of the improved Fe nutrition in plants (Moraghan 1979). FeEDDHA also depressed the concentration of Zn and to lesser extent K, Mg and Ca, but increased that of P in flax (Moraghan 1980).

In phytoremediation studies ligands are used to increase the solubility of heavy metals in soils, most often Pb, Cd, Zn, Cu or Ni, and their subsequent uptake by plants. However, the application levels of the ligands in these studies are higher than those normally used to correct nutrient deficiencies and soils are often contaminated with heavy metals (Huang et al. 1997; Blaylock et al. 1997; Epstein et al. 1999; Lombi et al. 2001). Deficiency of Fe occurs mainly in calcareous and alkaline soils, whereas phytoremediation studies are done mostly in mildly acidic soils, thus restricting the utilization of their results to calcareous environments.

EDTA, a common ligand in a phytoremediation studies, has been found to increase the solubility, uptake and leaching of heavy metals from contaminated soils (Huang et

al. 1997; Kos and Leštan 2003; Grčman et al. 2003; Meers et al. 2005). Although ligands increase the solubility of heavy metals in soils and uptake to roots, their effect on translocation from roots to shoots is less significant (Lombi et al. 2001). At the application level of 2.5 mmol EDTA kg^{-1} soil, Al-, Cd-, Cu-, Co-, Mn-, Ni-, Pb-and ZnEDTA complexes have been found in the xylem of barley (Collins et al. 2001).

1.3.4 Biodegradation

EDTA is rather recalcitrant against biodegradation (Nörtemann 1999; Bucheli-Witschel and Egli 2001), and in rivers EDTA concentrations of 0.01–0.1 µM with a peak concentration of 0.6 µM have been measured (Kari and Giger 1995). The environmental concern with EDTA is related to its ability to increase the solubility of harmful heavy metals, e.g. Cd and Pb, hence, increasing the potential contamination of groundwater with these heavy metals (Lombi et al. 2001; Jiang et al. 2003). Much less is known about EDDHA biodegradation, but it is known to be recalcitrant against photodegradation (Gómez-Gallego et al. 2005), and commercial fertilizers of FeEDDHA have been found to contain impurities due to the manufacturing process (Cremonini et al. 2001). The high price of EDDHA restricts its use only with the most valuable crops (Álvarez-Fernández et al. 2005).

From the environmental point of view, it would be desirable to use biodegradable chelating agents. In phytoremediation studies, a natural complexing agent, S,S-ethylenediaminedisuccinic acid ([S,S]-EDDS), which is produced by microorganisms (Nishikiori et al. 1984; Goodfellow et al. 1997), was used due to its rapid biodegradation. In a sludge-spiked soil the half life of [S,S]-EDDS was 2.5 days, and total biodegradation took place within 28 days (Schowanek et al. 1997). Biodegradation of EDDS has been mainly studied in sludge (Vandevivere et al. 2001) or in soils amended with sludge (Schowanek et al. 1997; Jaworska et al. 1999; Hauser et al. 2005) or contaminated with heavy metals (Meers et al. 2005; Hauser et al. 2005; Tandy et al. 2006). The half life of [S,S]-EDDS was up to 7.5 days (Meers et al. 2005; Tandy et al. 2006), depending on the dosage, whereas in the study of Hauser et al. (2005) biodegradation of [S,S]-EDDS was 18–42% complete within seven weeks. However, to my knowledge no biodegradation studies have been made in calcareous soils with low application rates used to correct nutrient deficiency.

Biodegradation of EDTA has been more thoroughly studied than that of EDDHA or EDDS. A few microorganisms are capable of using EDTA as a sole source of carbon (Thomas et al. 1998; Nörtemann 1999), but biodegradation of EDTA in soils is slow (Tiedje 1975; Means et al. 1980; Bolton et al. 1993). The main route for EDTA degradation is photolysis of FeEDTA complex (Lockhart and Blakeley 1975; Bucheli-Witschel and Egli 2001). [S,S]-EDDS photodegrades at a faster rate than EDTA and it is not species-dependent, i.e. dependent on the metal complexed (Metsärinne et al. 2001).

Ethylenediamine-*N,N´*-disuccinic acid (EDDS) has two asymmetrical carbon atoms, and EDDS has three stereoisomers, i.e. [S,S], [R,R] and ([S,R]/[R,S]). These stereoisomers differ in biodegradability: [S,S] is the most and [R,R] stereoisomer the least biodegradable (Schowanek et al. 1997; Takahashi et al. 1997). Biodegradation of [S,S]-EDDS in activated sludge has shown to be species-dependent (Vandevivere et al. 2001).

1.3.5 Prevalence of Fe deficiency in Finland

Because cultivated soils in Finland are slightly acidic, with an average pH of 6.0, Fe availability is generally not considered a problem in plant nutrition (Sil-

lanpää 1990). On a field scale, chelates are used mainly as foliar fertilizers in order to improve the Mn, Cu, or Zn status of cereals. However, the low availability of other nutrients may depress root development and cause Fe deficiency, as has been shown with boron in recently reclaimed peat soil (Heinonen 1961). The main concern in securing Fe availability for plants is in greenhouse production, where all the plant nutrients are added to a growth medium due to its low nutrient concentration and intensive growth of plants. The main growth media are peat and rockwool.

In Finland the main greenhouse production plants are tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*). In 2007, slightly more than half of the greenhouse acreage was used for vegetable production, the total value of greenhouse production was 225 million € (http://www.kauppapuutarhaliitto.fi). Because all of the above-mentioned plants have a Strategy I Fe uptake mechanism, fluctuations in Fe availability may have effects on the uptake of other elements. Chelating agents may also have an impact on the uptake of essential as well as of harmful heavy metals. In greenhouse production, nutrients are provided mainly in solution, and e.g. EDTA, EDDHA, DTPA, EDDHMA and HEEDTA are used as chelating agents to sustain Fe in a soluble form.

1.4 Objectives of the study

The low availability of Fe in calcareous and alkaline soils restricts plant production worldwide. The availability of Fe for plants can be increased with synthetic chelating agents, e.g. EDTA and EDDHA. However, recalcitrance of EDTA and its ability to increase the solubility of harmful heavy metals in soils have increased the concern for the potential leaching of heavy metals to groundwater, whereas the high price of EDDHA restricts its use.

In phytoremediation studies a biodegradable chelating agent, [S,S]-EDDS, is used to increase the solubility of heavy metals for subsequent plant uptake, but its use for agronomic purposes, such as to correct nutrient deficiencies, has not been tested. In phytoremediation studies the application rates of ligands are considerably higher than those used for correcting nutrient deficiencies. Because the main concern in these studies related to the presence of harmful heavy metals in non-calcareous soils, the results are not applicable to agricultural practices in calcareous environment.

Although [S,S]-EDDS forms weaker complexes with Fe than does EDTA or EDDHA, [S,S]-EDDS has a lower affinity for Ca than does EDTA (Tandy et al. 2004), thus supporting its use as an alternative chelating agent for Fe compared to using EDTA in calcareous environments. After Fe chelate splitting, free ligands are able to complex other elements as well according to their stability constant. However, a deficiency of Fe modifies the pH and redox state in the rhizosphere, thus affecting the solubility of the elements. This may affect the ability of ligands to complex elements as well, and thus also their uptake. However, little is known about the effects of ligands on the uptake of other elements after Fe chelate splitting under Fe deficiency.

The purpose of this study was to determine the ability of biodegradable ligands, when complexed with Fe, to provide Fe and other elements for plants with Strategy I (Lettuce) and Strategy II (Ryegrass) Fe uptake mechanisms in both non-calcareous and calcareous environments. The effects of the Fe chelates on the physiologically active Fe content in lettuce were evaluated by analyzing the activity of ROS scavenging enzymes. The solubility of elements in calcareous soils after the application of Fe chelates at rates used to correct Fe deficiency and subsequent biodegradation of the ligands were determined in an incubation experiment. The evaluat-

ed ligands were [S,S]-EDDS, a mixture of EDDS (25% [S,S], 25% [R,R] and 50% [S,R]/[R,S]), EDTA and EDDHA. In pot experiments the growth media were quartz sand and calcareous soils. To mimic a calcareous environment, limed quartz sand was used, while unlimed quartz sand was used since it resembles rockwool, which is commonly used as a growth medium in greenhouse production.

2 Materials and methods

This study consists of four pot experiments and an incubation experiment. The results of three pot experiments, EXP 1 (Paper I), EXP 2 (Paper II) and EXP 3 (Paper III) have been published, whereas the results of EXP 4 are presented in this thesis for the first time. In addition, some unpublished results for concentrations of Co, Ni, Cd and Pb in lettuce in EXP 1 and EXP 2 and for concentrations of Co and Ni in ryegrass in EXP 1 are also presented. In the following text the pot experiments are referred to as EXP 1, EXP 2, EXP 3 and EXP 4. The incubation experiment, which was undertaken to estimate the biodegradation of ligands, was conducted on the same soil as that used in EXP 3 (Paper IV).

2.1 Experiments

2.1.1 Ligands and preparation of Fe chelates

In the growth and incubation experiments, [S,S]-stereoisomer of ethylenediaminedisuccinic acid, [S,S]-EDDS (except in EXP 1), and a mixture of EDDS isomers, EDDS(mix); 25% [S,S], 25% [R,R] and 50% [S,R]/[R,S] were used. Both [S,S]-EDDS and EDDS(mix) were in alkaline solution (NaOH) in the form of sodium salt with a content of 40.1 and 49.3% on a weight basis, respectively. They were provided by Kemira Oyj. The purity of these ligands was tested by Kemira using ^{13}C-NMR and ^{1}H-NMR techniques. Both H_4EDTA (ethylenediaminetetraacetic acid, purity 99%) and H_4EDDHA (ethylenediiminobis(2-hydroxyphenyl)acetic acid, purity 98%, E4135) were provided by Sigma.

Ethylenediaminedisuccinic acid, a structural isomer of EDTA, has two chiral carbon atoms and three stereoisomeric forms: [S,S], [R,R] and [S,R]/[R,S]. The forms of [S,S] and [R,R] are mirror images, whereas [S,R]/[R,S] form is a meso isomer, i.e. non-optically active. EDDHA is manufactured in the purification of commercial fertilizer and no information is available on its chirality. However, it may be assumed that it contains 50% of both meso isomer and racemic mixture (Cerdán et al. 2006). All of the specific ligands used in the different experiments were of the same origin.

All the Fe chelates were prepared in a metal-to-ligand ratio of 1:1. This ratio has given the best results in growth experiments (Guinn and Joham 1962). The ligand solutions were prepared in deionized water by weighing appropriate amounts of ligands. EDDS(mix) solution was neutralized by adding 1 M HCl. Both H_4EDTA and H_4EDDHA were solubilized in 0.1 M NaOH. Iron solutions were prepared by dissolving either $FeSO_4$*$7H_2O$ (EXP 1),

Table 1. Properties of calcareous soils used in the growth (EXP 3) and incubation experiment (Paper IV).

Properties	Soil 1	Soil 2
Particle size distribution, g kg^{-1}		
Clay	170	250
Silt	320	500
Sand	510	250
pH (water, 1:2.5 v/v)	8.4	7.9
Carbon content, g kg^{-1}		
Total carbon	33	87
Organic carbon	9	16
Carbonate content, g kg^{-1}	204	584
Water holding capacity (pF 2), g kg^{-1}	184	205
Electrical conductivity, dS m^{-1}	1.7	2.2
Sodium adsorption ratio, %	9.1	1.1

$^{59}FeCl_3*6H_2O$ (EXP 2) or $Fe_2(SO_4)_3$ (EXP 3, EXP 4 and incubation experiment) into deionized water, added to a ligand solution, and stored in darkness to prevent photodegradation. The pH of the FeEDDS(S,S), FeEDDS(mix) and FeEDTA solutions was about 3 and that of FeEDDHA about 7. In EXP 2 labelled ^{59}Fe was used in order to evaluate the uptake efficiency of ^{59}Fe from ^{59}Fe chelates. The stability constants of FeEDDS(S,S), FeEDDS(mix), FeEDTA and FeEDDHA are 20.6, 20.1, 26.5 and 35.4, respectively (Norvell 1991; Orama et al. 2002). An inorganic Fe source, $FeSO_4*7H_2O$, was also used in every experiment.

2.1.2 Growth experiments

The experiments were conducted either in quartz sand (EXP 1, 2, 4) or in calcareous soils (EXP 3). Particle size distribution of quartz sand was determined by sieving to the following fractions: < 0.063, 0.063–0.2, 0.2–0.63 and 0.62–2 mm, consisting of 1, 89–93, 6–9 and 0–3% of the material, respectively. The two calcareous soils, Soil 1 and Soil 2, originated from Cyprus and were tentatively classified as Haplic Calcisols (FAO 1998). The properties of the calcareous soils are presented in Table 1.

DTPA extraction showed that the calcareous soils used here had a low plant-available Fe concentration (Table 2). The critical DTPA-extractable Fe concentration for sorghum has been estimated as 2.4–4.5 mg kg^{-1}, causing Fe deficiency without yield reduction (Lindsay and Norvell 1978). The plant-available Mn, Zn and Cu concentrations were above the critical level considered to cause deficiency in corn (Lindsay and Norvell 1978). The total concentrations of Cd, Pb, Co and Zn in calcareous soils met the criteria for good quality for soil according to Dutch legislation (Table 2), whereas the concentration of Ni in both soils (35 mg kg^{-1} DW) and Cu (36 mg kg^{-1}) in Soil 1 were slightly above the limit for good quality (see Adriano 2001).

In the growth experiments with quartz sand the total weight of the growth medium was 10 kg (EXP 1, 2, 4) and with calcareous soils 8 kg (EXP 3). To mimic a calcareous environment, 2–4% $CaCO_3$ was added to the quartz sand. All growth experiments were done in a greenhouse on lettuce (*Lactuca sativa* cv. Australian gelber; EXP 1 and 2. *Lactuca sativa* cv. Waldmann's dark green; EXP 3 and EXP 4) and ryegrass (*Lolium perenne* cv. Prego; EXP 1) grown in Kick-Brauckmann

Table 2. Total and DTPA-extractable metal contents of calcareous soils used in the growth (EXP 3) and incubation experiment (Paper IV).

	Soil 1	Soil 2
Total content, mg kg^{-1}		
Fe	28,000	13,000
Mn	654	1,151
Zn	81.8	49.0
Cu	49.2	33.4
Ni	41.4	36.2
Co	15.9	11.3
Pb	4.8	9.2
Cd	0.14	0.17
DTPA extraction, mg kg^{-1}		
Fe	4.4	2.0
Mn	11.7	4.5
Zn	2.0	1.4
Cu	1.0	2.8
Ni	0.24	0.67
Pb	0.36	0.89
Cd	0.02	0.01

type pots. Prior to seeding, nutrients were mixed into the whole soil volume (Table 3). The sand was watered with 500 ml of deionized water, after which 300 mg ryegrass and about 20 seeds of lettuce were covered with 300 g of sand and moistened with water and covered with plastic to prevent water evaporation until germination. After germination the lettuce was thinned to three (EXP 1) or two (EXP 2–4) plants per pot. Then, 29 and 24 days from seeding in EXP 3 and EXP 4, respectively, an additional 100 mg kg^{-1} dose of N [NH_4NO_3/$Ca(NO_3)_2$] and K (KCl) was applied. In EXP 2 an additional 100 mg kg^{-1} dose of N [$Ca(NO_3)_2$] was applied half-way through the experiment.

In EXP 1, Fe solutions were added into quartz sand at the time of seeding, whereas in the other experiments Fe was added after the plants were thinned to 2 plants per pot. The total amount of applied Fe was 2.8 mg kg^{-1} (0.05 mmol kg^{-1}) in EXP 1, 2 and 4, and 5.6 mg kg^{-1} (0.1 mmol kg^{-1}) in EXP 3. The control treatment did not receive Fe. Foliar application of Fe was done for lettuce and ryegrass grown in limed quartz sand (EXP 1) 7 and 12 days before harvest, respectively. The total amount of applied Fe was 2 mg pot^{-1}. This amount was divided into four 2 ml portions and applied early in the morning and late in the evening on two successive days. In every experiment the treatments were replicated four times.

The plants were watered with deionized water. The quartz sand was watered to water holding capacity, and calcareous soils were watered to field capacity (pF 2) by weighing. In EXP 2, supplemental light was provided with Osram Viatox Nav-T 400 W lamps (Holland) to provide a minimum of 160 µmol m^{-2} s^{-1} quantum flux density at the top of the plants during the 16-h photoperiod, whereas other experiments were started in spring in adequate natural light. For EXP 3 and 4, the day/night temperature was adjusted according to day length and was 16/13°C during germination and 20/15°C thereafter for the rest of the growth period. Temperatures were not adjusted in EXP 1 and EXP 2.

The plants were harvested 2 cm above the soil surface and their fresh weight was recorded. Lettuce was harvested 35, 53, 47 and 44 days after planting in EXP 1, EXP 2, EXP 3 and EXP 4, respectively. Ryegrass was harvested 40 days after planting in EXP 1. The plants were washed briefly with a detergent (0.05% Tween 80) and rinsed three times with deionized water and then blotted dry. Fresh plant samples were taken from the edge of the first full mature leaf (lettuce), excluding the outermost edge and middle rib of the leaves, wrapped in aluminium foil and frozed in liquid nitrogen, and stored at -70°C until analysis. The rest of the plant material was dried at 65°C for 48 hours. Dried plant material was ground in a mill with a titanium rotor.

Table 3. Total amounts of nutrients in mg kg^{-1} mixed to growth media in unlimed (U) and limed (L) quartz sand (EXP 1, 2 and 4) and in calcareous soils (EXP 3) at the beginning of the experiments.

		EXP 1		EXP 2		EXP 3	EXP 4	
Nutrient	Compound	U	L	U	L		U	L
N	NH_4NO_3	100	100				33	
	$Ca(NO_3)_2$			200	200	200	167	200
P	KH_2PO_4	40	40	40	40	50	40	40
K	KH_2PO_4	50	50	50	50	63	50	50
	KCl	50	50	50	50	137	150	150
Mg	$MgSO_4*H_2O$	20	20	20	20	20	20	20
Ca	$CaCO_3$	20	8,000		16,000			16,000
	$Ca(NO_3)_2$			140	140	286	191	286
S	$MgSO_4*7H_2O$, $MnSO_4*H_2O$, $ZnSO_4*7H_2O$, $CuSO_4*5H_2O$	28	28	28	28	30	28	28
Mn	$MnSO_4*H_2O$	1.1	1.1	1.1	1.1	2.2	1.1	1.1
Zn	$ZnSO_4*7H_2O$	1.3	1.3	1.3	1.3	2.6	1.3	1.3
Cu	$CuSO_4*5H_2O$	0.6	0.6	0.6	0.6	1.3	0.6	0.6
Mo	$Na_2MoO_4*2H_2O$	0.5	0.5	0.5	0.5	0.5	0.5	0.5
B	H_3BO_3	1.0	1.0	1.0	1.0	1.0	1.0	1.0

At the end of the growth experiments, the pH of the growth medium was determined from water (EXP 3 and 4) or from 0.01 M $CaCl_2$ suspensions (EXP 2) at a 1:2.5 ratio (v/v). In EXP 1, the pH of percolating water was measured with a Piccolo HI 1280 pH meter 19 and 33 days after planting.

Moist soil samples were taken from the total length of the soil profile from calcareous soils (EXP 3) after the growth experiment and extracted with Milli-Q water (Millipore, Bedford, MA, USA) at a soil-to-water ratio of 1:1 in a horizontal position with a reciprocal shaker for 30 minutes at 500 rpm, filtered through a blue ribbon filter (589^3 Blueband, Schleicher & Schuell, Dassel, Germany). The filtrates were acidified with HNO_3 (J.T. Baker Nitric Acid 69–70%, Baker Instra-Analyzed Reagent) to reach a 2% acid concentration for subsequent analysis of Fe, Mn, Zn, Cu, Ni, Co, Cd and Pb. After EXP 2, quartz sand was extracted with deionized water (1:1) and filtered through a blue ribbon filter to analyze the activity of ^{59}Fe.

2.1.3 Incubation experiment

The calcareous soils were incubated for 1, 3, 5, 7, 28 and 56 days in an experiment to estimate the biodegradation of the ligands (Paper IV). Before the incubation experiment the soils were air-dried and sieved (< 2 mm), 20 g samples of soils were weighed into 100 ml plastic bottles, and a total of 100 µmol Fe kg^{-1} was added (1 ml) as $FeSO_4$, FeEDDS(S,S), FeEDDS(mix), FeEDTA or FeEDDHA. The control did not receive any Fe. All the treatments received Mn (40 µmol kg^{-1} as $MnSO_4*H_2O$), Zn (40 µmol kg^{-1} as $ZnSO_4*7H_2O$) and

Cu (20 µmol kg^{-1} as $CuSO_4*5H_2O$) in one solution (1 ml). Finally, the soils were watered to reach field capacity (pF 2) and the bottles were closed with caps, but loosely to ensure air circulation. The bottles were stored in cardboard boxes at a controlled temperature of 24.5 ± 0.2°C in darkness. Air temperature was recorded with a data logger at 2–3 hour intervals, and readings were verified with a precision thermometer (Amarell Certificate #2623). Soil moisture was checked by weighing and evaporated water was replenished with Milli-Q water once a week. After the incubation periods, moist soils were extracted as in EXP 3 after the growth experiment.

Biodegradation of ligands in the calcareous soils were estimated indirectly by calculating the decrease of water-soluble Fe, Mn, Zn, Cu, Co, Ni, Cd and Pb concentrations during the incubation experiment. As shown by Tandy et al. (2006), the decrease of soluble Fe, Zn, Cu, Ni, Cd and Pb concentrations over time was coupled with the biodegradation of [S,S]-EDDS.

2.2 Methods of analyses

The methods of plant and soil analyses are presented in Table 4. The plant samples (1 g) were ashed in a muffle furnace in porcelain crucibles at 500°C for two hours, after which about 10 drops of Milli-Q water was added to retain the ash. Then 3 ml of 6 M HNO_3 (J.T. Baker Nitric Acid 69–70%, Baker Instra-Analyzed Reagent) was added and evaporated in a sand bath. Thereafter, the samples were heated to 500°C for one hour, and ash residues were dissolved in 10 ml of 6 M HNO_3 and decanted to 50 ml glass bottles. The samples were stored in plastic bottles.

For the elemental analyses of the plant samples and water extracts of the soil samples, standard solutions of 2, 10, 50, 200 and 500 µg l^{-1} of Fe, Mn, Zn, Cu, Ni, Co, Pb and Cd were prepared from MERCK IV stock solution (1000 mg l^{-1}). For Fe, Mn and Zn analyses 10, 50, 200 and 500, for Cu analyses 2, 10 and 50 and for Co, Ni, Cd and Pb analyses 2 and 10 µg l^{-1} standard solutions were used.

The elemental composition of the plant samples and the water-soluble elements of the soil samples (Fe, Mn, Zn, Cu, Co, Ni, Cd, Pb) were analyzed with an inductively coupled mass spectrometer (ICP-MS, Perkin Elmer Sciex Elan 6000). Analysis of the elements with ICP-MS is based on measurements of invidual isotopes according to the ratio of atomic mass and charge (m/z) by a mass spectrometer. Atoms are ionized by plasma that is provided by argon (Ar) at a temperature of 6,000–10,000°C. When the elements are ionized they form ion pairs, which may interfere measurement, and ^{40}Ar can form ion pairs with e.g. nitrogen (^{14}N) and oxygen (^{16}O), producing $^{40}Ar^{14}N^+$ and $^{40}Ar^{16}O^+$, interfering the measurements of ^{54}Fe and ^{56}Fe, respectively. Due to this isobaric interference, ^{54}Fe isotope was analyzed, although it is less abundant than ^{56}Fe (5.8 vs. 91.8%). However, background interference was at a lower level when analyzing ^{54}Fe. For the analyses, the samples were diluted to obtain an intensity of the elements below 2 million counts per second, because at that range measurement is based on the observed counts. The following isotopes were measured: ^{54}Fe, ^{55}Mn, ^{59}Co, ^{62}Ni, ^{63}Cu, ^{64}Zn, ^{114}Cd and ^{208}Pb.

The assay mixtures contained 2% HNO_3 and 10 µg l^{-1} of rhodium (^{103}Rh) as an internal standard to correct temporal variations in signal intensity. The intensity of ^{103}Rh varied between 200,000–480,000 counts per second. Blank samples were included in the series of analyses and subtracted from the sample concentrations. The activity of ^{59}Fe in lettuce and water extracts (Paper II) was analyzed with a gamma counter (Ultro gamma 1280, 3" x 3"

Table 4. Methods used in the plant and soil analyses.

Property	Method	Paper
Plant		
Dry weight	Drying at 65ºC for 48 h	I–III
Fe, Mn, Zn, Cu, Co, Ni, Cd, Pb	Dry combustion at 500ºC, dissolving residues in 6 M HNO_3	I–III
Chlorophyll concentration	Extraction with 80% acetone (Arnon 1949)	I–III
Ascorbate peroxidase (EC 1.11.1.11)	Phosphate buffer extraction, H_2O_2, 290 nm (Klapheck et al. 1990; Asada 1984)	III
Guaiacol peroxidase (EC 1.11.1.7)	TRIS-maleate extraction, guaiacol, H_2O_2, 470 nm (Burel et al. 1994)	III
Catalase (EC 1.11.1.6)	Phosphate buffer extraction, H_2O_2, 240 nm (Klapheck et al. 1990)	III
Superoxide dismutase (EC 1.1.15.1)	Photochemical assay (Giannopolitis and Ries 1977)	III
Soil		
pH	Extraction with water or 0.01 M $CaCl_2$.	I–IV
Soil texture	Pipette method (Elonen 1971).	III
Fe, Mn, Zn, Cu	DTPA extraction (Lindsay and Norvell 1978), ICP-MS determination.	III
Fe, Mn, Zn, Cu, Co, Ni, Cd, Pb	Total analysis (EPA 3051), ICP-MS determination.	III
Fe, Mn, Zn, Cu, Co, Ni, Cd, Pb	Water extraction, ICP-MS determination.	III, IV
Electrical conductivity	Saturation paste (Zhang et al. 2005).	III
Sodium adsorption ratio	Saturation paste (Zhang et al. 2005), ICP-AES determination.	III
Carbonate content	Acetic acid extraction, pH change (Loeppert et al. 1984).	III
Total carbon	LECO	III

NaI(Tl), Wallac Oy, Turku, Finland). The counting efficiency of the gamma counter was 10.4%.

2.3 Quality control

All the laboratory hardware that came into contact with the samples was soaked in 2% acid (65% HNO_3 diluted with Milli-Q water) in at least over night, rinsed thoroughly with Milli-Q water, dried in a drying chamber and sealed in a plastic bag until further use. For ICP-MS analyses HNO_3 (J.T. Baker Nitric Acid 69–70%, Baker Instra-Analyzed Reagent) was used. All dilutions were done with Milli-Q water and the quality of the Milli-Q water was checked by measuring its electrical conductivity.

In order to control the quality of the plant analyses, standard reference material was included in every sample batch (Dried tomato leaves, NIST, SRM 1573a). Certified and analyzed values are presented in Table 5. During the analysis with ICP-MS, every 10^{th} sample was a reference sample, and if the Fe concentration differed by more than 5% from the certified value, the samples after the previous reference sample were reanalyzed. Re-calibration was done at least every two hours.

Table 5. Certified values of a reference sample (Dried tomato leaves, NIST, SRM 1573a) and analyzed concentrations of the elements ± SD with ICP-MS, mg kg^{-1} DW.

Element	Certified values	Analyzed values
Fe	368 ± 7	366 ± 11
Mn	246 ± 8	228 ± 10
Zn	30.9 ± 0.7	29.5 ± 1.1
Cu	4.70 ± 0.14	6.78 ± 1.59
Co	0.57 ± 0.02	0.57 ± 0.02
Ni	1.59 ± 0.07	2.50 ± 0.24
Cd	1.52 ± 0.04	1.34 ± 0.05
Pb	-	0.86 ± 0.09

Table 6. Average element concentration in the blank samples (n = 50) and measured concentrations in a 100 µg l^{-1} standard solution ± SD measured as a sample, µg l^{-1}.

Element	Blank sample	100 µg l^{-1} standard
Fe	68.3 ± 28.9	102.1 ± 6.2
Mn	0.1 ± 0.1	103.3 ± 4.5
Zn	2.6 ± 1.7	98.4 ± 4.7
Cu	0.6 ± 0.4	98.0 ± 5.7
Co	0.02 ± 0.03	105.4 ± 6.8
Ni	0.08 ± 0.07	99.6 ± 2.2
Cd	0.02 ± 0.04	99.6 ± 1.5
Pb	0.2 ± 0.2	115.7 ± 6.2

Elemental concentrations of the blank samples in the incubation experiment were used to calculate the limit of quantitatum (LOQ). LOQ was determined to be ten times the standard deviation in 50 blank samples measured during the courses of analyses. LOQ values for Fe, Mn, Zn, Cu, Co, Ni, Cd and Pb were 357.1, 1.2, 19.2, 4.2, 0.3, 0.8, 0.4 and 2.4 µg l^{-1}, respectively. In the series of analysis about every 10^{th} sample was a standard solution and the concentrations of the elements were 100 µg l^{-1}. The measured values are shown in Table 6.

3 Results and discussion

3.1 Yield responses to Fe chelates

The growth of lettuce in limed quartz sand was depressed by the low availability of Fe (Table 7). In EXP 4, the pH of limed sand was 8.1–8.3. The lower chlorophyll concentration in lettuce indicated Fe deficiency (Table 7). Unlike with lettuce, ryegrass growth was not depressed (Table 7), supporting the view that the Strategy II Fe uptake mechanism is better adapted to calcareous environments than the Strategy I Fe uptake mechanism (Römheld 1987).

The growth of lettuce in quartz sand was increased by FeEDDS(S,S) and FeEDDS(mix) as efficiently as by FeEDTA and FeEDDHA (Table 7) despite their lower stabilities compared to those of FeEDTA and FeEDDHA (Norvell 1991; Orama et al. 2002). Prior to Fe uptake from Fe chelate, Strategy I plants weaken the Fe(III) chelate bond by reducing Fe^{3+} to Fe^{2+} by FCR, causing Fe(II) chelate to split with a subsequent uptake of Fe^{2+} (Chaney et al. 1972). However, the reduction rate by FCR is lower with Fe chelates with higher stabilities, hence the less stable Fe chelates may be better Fe sources

(Lucena and Chaney 2006; Lucena and Chaney 2007). Inorganic Fe was a poorer Fe source in limed quartz sand than Fe chelates (Table 7). This is due to the precipitation of Fe as insoluble Fe(III) oxides (Lindsay 1979).

Foliar application of Fe had no effects on either lettuce or ryegrass yields (Table 7). This was probably due by the fact that foliar application was done only 7 and 12 days prior to lettuce or ryegrass harvest, respectively. Application of FeEDTA even decreased lettuce yield due to foliar necrosis, whereas FeEDDS(mix) or FeEDDHA caused no visible damage to lettuce. This outcome is in line with the observation made in a phytoremediation study with a application rate of 10 mmol kg^{-1} EDDS or EDTA (Grčman et al. 2003), where EDDS was less phytotoxic than EDTA and no yield reductions were observed. EDDS was less phytotoxic also to soil microorganisms.

The lower lettuce yields in EXP 1 compared to EXP 2 and EXP 4 in unlimed sand (Table 7) may be partly related to the growth inhibition caused by a low pH in quartz sand. In EXP 1 the pH of the percolating water decreased as the experiment progressed, reaching values of 4.1–4.6 (Paper I), whereas in EXP 2 the sand pH at the end of the growth experiments was 7.4–7.6 (0.01 M $CaCl_2$ suspension). In EXP 4 the pH of sand in $FeSO_4$ and control treatments after the growth experiment was 5.6 and 6.1, respectively, and in all Fe chelate treatments 6.2 (Water suspension, 1:2.5 v/v). The differences in EXP 4 were not statistically significant. The variation in pH is probably related to different ratios of supplemented NH_4-N/NO_3-N, because excess uptake of cations acidifies and excess uptake of anions alkalinizes the growth medium (see Van Breemen et al. 1983).

Nitrate nutrition in EXP 2 did not depress lettuce yields significantly in unlimed quartz sand (Table 7) although the pH of

Table 7. Lettuce (EXP 1, 2, 3 and 4) and ryegrass (EXP 1) yields, g, and chlorophyll concentrations, mg g^{-1} FW, in unlimed (U) and limed (L) quartz sand and in calcareous soils (EXP 3).

	EXP 1						EXP 2		EXP 3		EXP 4	
	Soil application				Foliar application		Soil application					
	Lettuce		Ryegrass		Lettuce	Ryegrass	Lettuce					
Yields	U	L	U	L	L	L	U	L	Soil 1	Soil 2	U	L
Control	4.4^{ab}	3.4^{a}	16.4^{a}	18.2^{a}	3.4^{ab}	18.2^{a}	11.7^{a}	3.7^{ab}	16.8^{a}	23.5^{a}	13.2^{a}	7.1^{a}
$FeSO_4$	3.8^{a}	4.6^{ab}	15.6^{a}	18.6^{a}	3.7^{ab}	17.7^{a}	12.1^{a}	2.5^{a}	16.8^{a}	23.7^{a}	10.6^{a}	8.9^{ab}
FeEDDS(S,S)							15.7^{a}	10.3^{c}	16.9^{a}	23.2^{a}	14.2^{a}	12.0^{b}
FeEDDS(mix)	6.2^{b}	7.8^{c}	16.5^{a}	19.4^{a}	4.2^{b}	17.6^{a}	14.2^{a}	5.0^{abc}	17.1^{a}	25.0^{a}	12.9^{a}	10.6^{b}
FeEDTA	4.9^{ab}	7.0^{bc}	16.8^{a}	18.6^{a}	2.8^{a}	17.3^{a}	12.7^{a}	6.5^{abc}	17.2^{a}	23.9^{a}	13.2^{a}	11.6^{b}
FeEDDHA	4.8^{ab}	6.1^{abc}	16.5^{a}	18.4^{a}	4.3^{b}	17.4^{a}	13.8^{a}	9.7^{bc}	17.7^{a}	26.8^{a}	13.8^{a}	10.5^{b}
Clorophyll												
Control	0.6^{a}	0.5^{a}	1.4^{a}	1.4^{a}	0.5^{a}	1.4^{a}	0.7^{ab}	0.5^{a}	1.2^{a}	1.2^{a}	1.5^{ab}	1.2^{a}
$FeSO_4$	0.6^{a}	0.6^{a}	1.5^{a}	1.3^{a}	0.5^{a}	1.5^{a}	0.6^{a}	0.5^{a}	1.2^{a}	1.2^{ab}	1.6^{b}	1.2^{a}
FeEDDS(S,S)							0.9^{c}	0.7^{b}	1.3^{a}	1.3^{abc}	1.6^{b}	1.3^{b}
FeEDDS(mix)	0.7^{a}	0.6^{a}	1.4^{a}	1.4^{a}	0.6^{a}	1.4^{a}	0.8^{bc}	0.7^{ab}	1.4^{a}	1.5^{a}	1.4^{a}	1.4^{b}
FeEDTA	0.7^{a}	0.6^{a}	1.5^{a}	1.5^{a}	0.5^{a}	1.5^{a}	0.9^{c}	0.7^{b}	1.3^{a}	1.4^{cd}	1.4^{a}	1.4^{b}
FeEDDHA	0.7^{a}	0.6^{a}	1.6^{a}	1.3^{a}	0.6^{a}	1.5^{a}	0.8^{abc}	0.8^{b}	1.3^{a}	1.4^{bcd}	1.7^{b}	1.3^{b}

Means within a column followed by a common letter do not differ significantly ($p < 0.05$, Tukey HSD test). Unlimed and limed quartz sand and Soil 1 and 2 were tested separately.

the quartz sand at the end of the experiment was at the level of lowest Fe solubility (Lindsay 1979). Nitrate nutrition has been suggested to cause Fe deficiency by depressing Fe uptake due to the increased pH at the root surface (Nikolic and Römheld 2003) and together with bicarbonate inhibits Fe uptake most (Kosegarten et al. 2004). This phenomenon was observed in EXP 2.

The ability of lettuce to cope with a low Fe availability (Welkie and Miller 1992) may be the reason that yield reductions were not observed in calcareous soils, although plant-available Fe concentrations were low as shown by DTPA extraction (Table 2). However, the lower chlorophyll concentration in the control treatment indicates that the lettuce was probably suffering from latent Fe deficiency (Table 7).

3.2 Effects of Fe chelates on element uptake

3.2.1 Uptake of Fe, Mn, Zn and Cu by lettuce and ryegrass

Soil application of FeEDDS(mix) increased the Fe concentration in lettuce at least equally as efficiently as did FeEDTA and FeEDDHA in quartz sand and in calcareous soils (Table 8). Foliarly applied FeEDDS(mix) was even more efficient (Table 8). In quartz sand FeEDDS(S,S) increased the Fe concentration in lettuce, whereas the same phenomenon was not observed in calcareous soils (Table 8). This may be due to the lower microbial activity in quartz sand compared to calcareous soils, thus slowing down the biodegradation of [S,S]-EDDS, or FeEDDS(S,S) was split faster in calcareous soils. Indeed, the concentration of water-soluble Fe after FeEDDS(S,S) application dropped rapidly in calcareous soils (Paper IV). According to analysis of variance with a factorial treatment structure (SAS 9.1), soil type had a statistically significant interaction on lettuce Fe concentration in FeEDDS(S,S) and $FeSO_4$ treatments ($p < 0.01$). These Fe sources probably retained Fe in a plant available form better in Soil 1 than in Soil 2. This is evident with the higher water-soluble Fe concentration in Soil 1 than in Soil 2 after FeEDDS(S,S) addition in an incubation experiment (Paper IV, see section 3.4).

Liming of quartz sand depressed the concentration of Fe in ryegrass slightly but it had no significant effect between treatments (Paper I; 84-97 µg Fe g^{-1} DW). In acidic soils EDTA has been shown to increase the Fe uptake of ryegrass when EDTA was supplied with irrigation water (Hartikainen 1981), but the application dosages (5.4–11.3 mmol kg^{-1}) were far greater than in the current study (0.05 mmol kg^{-1}).

EDDHA was notably the most efficient ligand in making ^{59}Fe available for uptake by lettuce (Paper II). However, other chelates increased the Fe concentration in lettuce equally to ^{59}FeEDDHA (EXP 2, Table 8). This indicates that after ^{59}Fe chelate splitting, free ligands were able to increase the uptake of indigenous Fe. Intact chelates have also been detected in plants (Hill-Cottingham and Lloyd-Jones 1965; Blaylock et al. 1997) and they may thus enhance Fe translocation from roots to shoots. The uptake of intact chelates or ligands may occur along an apoplastic pathway after lateral root penetration through the endodermis (Marschner et al. 1987). When applied foliarly, FeEDDS(mix) increased Fe concentration in lettuce (Table 8) and in ryegrass most (Paper I), suggesting that it penetrated into the leaf cells as such and facilitated Fe translocation (Ferrandon and Chamel 1989). Inorganic Fe was a poor Fe source for lettuce when applied through soil, but when foliarly applied it was comparable to FeEDTA and FeEDDHA (Table 8). For ryegrass Fe chelates with a lower stability are a better source of Fe, because stable chelates retain Fe in an unavailable form that cannot be complexed by phytosi-

Table 8. Concentrations of Fe, Mn, Zn and Cu in lettuce grown in unlimed (U) or limed (L) quartz sand (EXP 1, 2 and 4) and in calcareous soils (EXP 3), μg g^{-1} DW. Foliar application was done in EXP 1 in a limed quartz sand (L*).

		EXP 1			EXP 2		EXP 3		EXP 4	
		U	L	L*	U	L	Soil 1	Soil 2	U	L
Fe	Control	112^{a}	58^{a}	58^{a}	79^{a}	79^{a}	70^{a}	65^{a}	103^{a}	63^{a}
	$FeSO_4$	104^{a}	78^{b}	134^{b}	81^{a}	79^{a}	72^{ab}	61^{a}	118^{ab}	67^{ab}
	FeEDDS(S,S)				104^{b}	116^{b}	73^{ab}	65^{a}	127^{b}	81^{ab}
	FeEDDS(mix)	160^{a}	102^{c}	190^{c}	109^{b}	116^{b}	78^{ab}	76^{b}	127^{b}	86^{b}
	FeEDTA	128^{a}	103^{c}	121^{b}	105^{b}	106^{b}	71^{a}	74^{b}	129^{b}	80^{ab}
	FeEDDHA	130^{a}	105^{c}	127^{b}	108^{b}	119^{b}	80b	78^{b}	112^{ab}	83^{ab}
Mn	Control	266^{b}	240^{c}	240^{c}	282^{b}	380^{c}	48^{bc}	78^{c}	184^{b}	130^{c}
	$FeSO_4$	280^{b}	186^{b}	202^{bc}	266^{b}	402^{c}	47^{bc}	74^{c}	183^{b}	118^{c}
	FeEDDS(S,S)				138^{a}	129^{ab}	49^{c}	62^{b}	135^{a}	50^{a}
	FeEDDS(mix)	185^{a}	62^{a}	162^{b}	140^{a}	157^{ab}	52^{c}	58^{b}	134^{a}	51^{a}
	FeEDTA	191^{a}	78^{a}	177^{b}	150^{a}	210^{b}	44^{b}	62^{b}	138^{a}	73^{b}
	FeEDDHA	181^{a}	61^{a}	99^{a}	118^{a}	127^{a}	37^{a}	29^{a}	137^{a}	54^{ab}
Zn	Control	233^{b}	64^{bc}	64^{b}	106^{c}	122^{abc}	17^{a}	24^{a}	148^{b}	60^{b}
	$FeSO_4$	264^{b}	58^{b}	58^{ab}	103^{c}	114^{ab}	17^{a}	25^{a}	144^{b}	58^{b}
	FeEDDS(S,S)				82^{b}	156^{bcd}	20^{a}	29^{b}	94^{a}	98^{c}
	FeEDDS(mix)	151^{a}	65^{bc}	62^{b}	82^{b}	188^{cd}	33^{b}	32^{c}	93^{a}	90^{c}
	FeEDTA	154^{a}	81^{c}	63^{b}	74^{ab}	228^{d}	31^{b}	42^{d}	88^{a}	93^{c}
	FeEDDHA	153^{a}	34^{a}	48^{a}	57^{a}	82^{a}	18^{a}	25^{a}	99^{a}	40^{a}
Cu	Control	22.6^{c}	8.8^{a}	8.8^{a}	13.8^{a}	13.3^{a}	9.7^{a}	10.2^{a}	17.4^{de}	9.2^{a}
	$FeSO_4$	21.5^{bc}	9.5^{a}	8.6^{a}	12.8^{a}	16.3^{ab}	10.3^{a}	9.3^{a}	17.6^{e}	11.3^{ab}
	FeEDDS(S,S)				9.9^{a}	12.9^{a}	9.9^{a}	8.9^{a}	9.3^{ab}	7.8^{a}
	FeEDDS(mix)	10.8^{a}	9.3^{a}	10.4^{a}	10.4^{a}	16.2^{ab}	10.1^{a}	11.2^{a}	8.4^{a}	15.9^{bc}
	FeEDTA	16.1^{ab}	10.0^{a}	14.5^{b}	14.0^{b}	18.0^{ab}	9.8^{a}	10.7^{a}	12.5^{bc}	9.3^{a}
	FeEDDHA	16.1^{ab}	14.3^{b}	10.5^{ab}	27.1	25.5^{b}	11.8^{a}	10.3^{a}	13.8^{cd}	20.8^{c}

Means within a column followed by a common letter do not differ significantly ($p < 0.05$, Tukey HSD test). Columns were tested separately.

derophores (Lucena et al. 1988b). In limed quartz sand the Fe concentration of ryegrass was increased most by FeEDDS(mix) and least by FeEDDHA, but the difference was not statistically significant (Paper I).

The deficiency of Fe increased the Mn concentration of lettuce more when grown in quartz sand than in calcareous soils (Table 8). The solubility of Mn is controlled by soil pH and redox state and therefore increases in the Fe efficiency reactions in roots; rhizosphere acidification and the release of reducing compounds increase Mn solubility and enhance its uptake, whereas the activity of FCR is decreased when the Fe status of the plant improves (Römheld and Marschner 1986b; Lucena and Chaney 2006). Unlike the solubility of Mn, the solubility of Zn and Cu is controlled mainly by soil pH (Lindsay 1979), and therefore the Fe deficiency increased the Zn and Cu concentrations of lettuce only in unlimed quartz sand (Table 8). Although the dry matter yields of lettuce were not depressed significantly in the control treatment in unlimed quartz sand (Table 7), the increased uptake of Mn, Zn and Cu indi-

cates a latent Fe deficiency. This is probably due to the enhanced Fe efficiency reactions before a Fe deficiency affected yield (Yi and Guerinot 1996). The uptake of Mn and Zn may have taken place through an Fe^{2+} transporter encoded by the IRT1 gene (Eide et al. 1996), which is also able to transport Mn and Zn (Korshunova et al. 1999).

In quartz sand all the Fe chelates decreased the Mn concentration of lettuce (Table 8) probably due to depressed Fe efficiency reactions in the roots. Among the chelates, FeEDTA depressed the Mn concentration of lettuce least and FeEDDHA most (Table 8). The increased Mn concentration in lettuce after FeEDTA application compared to other Fe chelate treatments was probably due to increased Mn complexation by EDTA after FeEDTA splitting (Paper III, IV). Although the DTPA-extractable Mn concentration was at a higher level in Soil 1 than in Soil 2 (Table 2), the Mn concentration in lettuce was increased to a higher level when grown in Soil 2 in all treatments except the FeEDDHA treatment (Table 8). This is probably related to the fact that among chelates FeEDDHA sustained Fe in the water-soluble form most efficiently (Paper III, IV) and thus probably depressed Fe efficiency reactions most. The more rapid decrease of water-soluble Fe concentration in Soil 2 compared to Soil 1 after addition of FeEDDS(S,S) and FeEDDS(mix) (Paper IV) may also increase the strength of the Fe efficiency reactions.

The concentrations of Fe and chlorophyll in lettuce were at the same level in FeEDDHA and FeEDDS(mix) treatments, indicating equal amounts of physiologically active Fe concentration within the leaves. However, in most cases FeEDDHA depressed the concentration of Mn and Zn in lettuce when grown in quartz sand and the Mn concentration when grown in calcareous soils to a lower level than FeEDDS(mix) (Table 8). These results are in line with the earlier observations that EDDHA depresses Zn uptake (Essington et al. 1962; Moraghan 1980). The depressed Zn uptake after EDDHA addition was partly related to low mobility of Zn due to the Mn toxicity caused by an Fe deficiency (Moraghan 1980). Fixaton of ZnEDDHA (Wallace and Lunt 1956) is unlikely to have caused the decreased Zn uptake, because EDDHA depressed Zn uptake in quartz sand as well.

The above observations indicate that other mechanisms in addition to Fe concentration were controlling the Fe efficiency reactions in the roots of lettuce. The controlling mechanisms may also be based on hormone activity. During Fe deficiency auxin concentration is known to increase in the roots (Landsberg 1981; Römheld and Marschner 1986b). Auxin is also involved in lateral root formation (Reed et al. 1998). Therefore, the increased activity of IAA oxidase or an effect on auxin biosynthesis by phenols (Kefeli and Kutacek 1977), such as EDDHA, may depress Fe efficiency reactions in lettuce roots. The fact that foliarly applied FeEDDHA also depressed Mn and Zn concentrations in lettuce (Table 8), suggests that EDDHA penetrated into the leaf cells and affected the hormone regulation of lettuce.

In reducing conditions, EDDHA is an efficient chelator of Cu (Norvell 1991). Enhanced Fe efficiency reactions in the lettuce roots may have thus facilitated the formation of CuEDDHA in the rhizosphere, especially in limed quartz sand, whereas this was not observed in calcareous soils (Table 8). This may be due to Cu complexation by organic matter, an unavailable form for complexation by EDDHA. In quartz sand Cu occurred probably mostly in an inorganic form and was thus available for complexation.

Concentrations of Mn, Zn and Cu in ryegrass were affected less by Fe chelates compared to those in lettuce (Paper I). This may be due to the specificity of Fe(III)

phytosiderophore uptake by Strategy II plants (Marschner et al. 1989). Although phytosiderophores also mobilize Mn, Zn and Cu (Treeby et al. 1989), their uptake is less specific (Römheld 1991). In corn, a Strategy II plant, the uptake of Zn was controlled by the free Zn^{2+} concentration, and corn was not able to utilize ZnEDTA (Halvorson and Lindsay 1977). The high stability of FeEDDHA in a calcareous environment depressed Fe availability and caused increased uptake of Mn and Zn by ryegrass (Lucena et al. 1988b). An indication of the same phenomenon was observed in limed quartz sand when comparing Mn and Zn concentrations of ryegrass in FeEDDHA and other Fe chelate treatments (Paper I). Foliar application of Fe chelates did not depress Mn or Zn concentration in ryegrass (Paper I).

3.2.2 Uptake of Ni, Co, Cd and Pb by lettuce and of Ni and Co by ryegrass

Liming decreased the Ni and Co concentrations of lettuce, while Fe chelates decreased their concentrations both in unlimed and limed quartz sand (Table 9). The solubility of Ni and Co in soil is pH dependent, i.e. solubility decreases as pH

Table 9. Concentrations of Ni, Co, Cd and Pb in lettuce grown in unlimed (U) or limed (L) quartz sand (EXP 1, 2 and 4) and in calcareous soils (EXP 3), μg g^{-1} DW. Foliar application was done in EXP 1 in a limed quartz sand (L*).

		EXP 1			EXP 2		EXP 3		EXP 4	
		U	L	L*	U	L	Soil 1	Soil 2	U	L
Ni	Control	4.0^{bc}	0.9^{a}	0.9^{a}	1.3^{b}	1.4^{b}	0.6^{a}	0.7^{a}	1.9^{c}	0.9^{b}
	$FeSO_4$	4.5^{c}	0.8^{a}	0.8^{ab}	1.4^{b}	1.4^{b}	0.7^{a}	1.1^{a}	1.8^{c}	1.0^{b}
	FeEDDS(S,S)				0.4^{a}	0.4^{a}	0.8^{ab}	1.0^{a}	0.9^{a}	0.3^{a}
	FeEDDS(mix)	1.8^{a}	0.7^{a}	0.7^{ab}	0.5^{a}	0.4^{a}	1.2^{b}	0.9^{a}	0.7^{a}	0.4^{a}
	FeEDTA	1.3^{a}	0.7^{a}	1.3^{b}	0.5^{a}	0.7^{ab}	0.6^{a}	0.7^{a}	1.0^{a}	0.6^{a}
	FeEDDHA	2.7^{ab}	0.7^{a}	0.7^{a}	0.6^{a}	0.6^{a}	0.5^{a}	0.5^{a}	1.4^{b}	0.6^{a}
Co	Control	0.92^{c}	0.14^{c}	0.14^{a}	0.16^{bc}	0.09^{ab}	0.11^{ab}	0.08^{ab}	0.37^{c}	0.13^{c}
	$FeSO_4$	1.19^{d}	0.12^{bc}	0.12^{a}	0.18^{c}	0.08^{ab}	0.10^{ab}	0.08^{ab}	0.45^{c}	0.12^{c}
	FeEDDS(S,S)				0.05^{a}	0.04^{a}	0.10^{ab}	0.08^{b}	0.10^{a}	0.04^{a}
	FeEDDS(mix)	0.19^{a}	0.04^{a}	0.18^{a}	0.06^{a}	0.06^{a}	0.11^{b}	0.08^{ab}	0.09^{a}	0.04^{a}
	FeEDTA	0.46^{b}	0.06^{a}	0.14^{a}	0.10^{ab}	0.09^{ab}	0.09^{a}	0.09^{b}	0.20^{ab}	0.06^{ab}
	FeEDDHA	0.55^{b}	0.10^{b}	0.17^{a}	0.08^{a}	0.12^{b}	0.10^{ab}	0.07^{a}	0.22^{b}	0.07^{b}
Cd	Control	0.18^{a}	0.11^{c}	0.11^{a}	0.15^{b}	0.05^{a}	0.15^{a}	0.15^{b}	0.14^{a}	0.02^{a}
	$FeSO_4$	0.29^{b}	0.07^{b}	0.09^{a}	0.15^{b}	0.05^{a}	0.20^{a}	0.16^{b}	0.16^{a}	0.02^{a}
	FeEDDS(S,S)				0.15^{b}	0.04^{a}	0.15^{a}	0.17^{b}	0.12^{a}	0.02^{a}
	FeEDDS(mix)	0.17^{a}	0.05^{ab}	0.10^{a}	0.18^{b}	0.04^{a}	0.19^{a}	0.17^{b}	0.13^{a}	0.01^{a}
	FeEDTA	0.27^{b}	0.03^{a}	0.11^{a}	0.07^{a}	0.05^{a}	0.16^{a}	0.14^{b}	0.12^{a}	0.02^{a}
	FeEDDHA	0.26^{b}	0.03^{a}	0.09^{a}	0.12^{ab}	0.03^{a}	0.14^{a}	0.09^{a}	0.14^{a}	0.01^{a}
Pb	Control	0.20^{a}	0.18^{a}	0.18^{ab}	0.27^{a}	0.44^{a}	0.25^{a}	0.23^{ab}	0.33^{a}	0.17^{a}
	$FeSO_4$	0.23^{a}	0.19^{a}	0.11^{a}	0.25^{a}	0.56^{a}	0.29^{a}	0.18^{ab}	0.26^{a}	0.10^{a}
	FeEDDS(S,S)				0.32^{a}	0.34^{a}	0.17^{a}	0.23^{ab}	0.22^{a}	0.15^{a}
	FeEDDS(mix)	0.32^{a}	0.24^{a}	0.16^{ab}	0.51^{a}	0.36^{a}	0.19^{a}	0.24^{b}	0.18^{a}	0.13^{a}
	FeEDTA	0.39^{a}	0.18^{a}	0.29^{b}	0.34^{a}	0.40^{a}	0.29^{a}	0.20^{ab}	0.16^{a}	0.17^{a}
	FeEDDHA	0.31^{a}	0.19^{a}	0.14^{a}	0.64^{a}	0.29^{a}	0.16^{a}	0.11^{a}	0.22^{a}	0.19^{a}

Means within a column followed by a common letter do not differ significantly ($p < 0.05$, Tukey HSD test). Columns tested separately.

increases. The increased Fe efficiency reactions of the roots may therefore have increased the solubility of Ni and Co in the rhizosphere and subsequent uptake by the Fe^{2+} transporter, which takes up other heavy metals as well (Eide et al. 1996). For plants, Ni (Brown 2007) and Co (Talukder and Sharma 2007) are essential and beneficial elements, respectively, In this study the concentration of Ni in plants was within an adequate range in all treatments (Brown 2007). The uptake of Ni and Co by ryegrass was depressed most efficiently by chelates with lower stability, i.e. by FeEDDS(mix) and FeEDTA (Table 10). In calcareous soils, [S,S]-EDDS, EDDS(mix) and EDTA increased complexed Ni and Co concentrations most (Paper IV). The same may have taken place in quartz sand, thus decreasing free Co^{2+} and Ni^{2+} concentrations in soil solution for ryegrass uptake. In calcareous soils contaminated with Ni and Cd, phytosiderophores increased the solubility and uptake of Ni and Cd by wheat and sorghum (Römheld and Awad 2000).

At the end of EXP 3, EDDS(mix) had increased the water-soluble Ni and Co to the highest level in calcareous soils (Paper IV). EDTA had a similar effect on Cd and Pb concentrations under similar conditions, but only EDDS(mix) had increased the Ni concentration in lettuce significantly (Table 9). Of the three pot experiments done in quartz sand with soil application of Fe chelates, the concentration of Cd in lettuce was increased by FeEDTA and FeEDDHA only in EXP 1. None of the ligands increased concentrations of Ni, Co and Pb statistically significantly in those experiments (Table 9). The higher Cd concentration in lettuce in acidic sand after FeEDTA and FeEDDHA treatments (EXP 1, Table 9) may be partly due to the damage to the roots caused by the chelates at a low soil pH. This damage may have enhanced the uptake of Cd. In the study of Lombi et al. (2001), EDTA (2.7 mmol kg^{-1}) increased the solubility of Ni, Cd and Pb in a soil contaminated with these heavy metals and enhanced their accumulation in roots, but translocation to maize shoot was poor. However, with a comparable application level of EDTA as above, the uptake of heavy metals has been found to increase (Huang et al. 1997; Blaylock et al. 1997). In this study chelates were applied below the level required to enhance translocation of Cd and Pb to shoots. Among the foliarly applied chelates, FeEDTA injured lettuce leaves most and increased Pb uptake compared to $FeSO_4$ and FeEDDHA treatments (Table 9). This may be due to the enhanced translocation of Pb from roots to shoots by EDTA.

3.3 Activity of oxygen-scavenging enzymes

The activity of SOD in lettuce grown in quartz sand was greater than in lettuce

Table 10. Concentrations of Ni and Co in ryegrass (EXP 1) grown on an unlimed (U) or limed (L) quartz sand, µg g^{-1} DW. Foliar application was done in limed quartz sand (L*).

	Ni			Co		
	U	L	L*	U	L	L*
Control	5.0^{ab}	2.5^{a}	2.5^{a}	0.42^{c}	0.23^{c}	0.23^{a}
$FeSO_4$	5.9^{b}	2.7^{a}	3.4^{a}	0.42^{c}	0.22^{c}	0.25^{a}
FeEDDS(mix)	2.6^{a}	1.8^{a}	2.4^{a}	0.08^{a}	0.04^{a}	0.24^{a}
FeEDTA	4.9^{ab}	1.7^{a}	2.5^{a}	0.27^{b}	0.07^{ab}	0.28^{a}
FeEDDHA	5.8^{b}	2.0^{a}	2.7^{a}	0.27^{b}	0.11^{b}	0.29^{a}

Means within a column followed by a common letter do not differ significantly ($p < 0.05$, Tukey HSD test). Columns were tested separately.

grown in calcareous soils (Table 11). This indicates either a greater need for removing ROS from lettuce grown in quartz sand or that higher concentrations of Mn, Zn and Cu are utilized in forming isozymes of SOD. The need for greater SOD activity may be related to the presence of higher concentrations of Fe and Cu in lettuce grown in quartz sand (Table 8). These elements are phytotoxic when in excess and may cause formation of ROS's (Elstner 1982). However, the activity of Cu/Zn-SOD and MnSOD may be related also to the biologically active Mn, Zn and Cu concentrations within the plant (del Río et al. 1978; García et al. 1981).

Most of the total activity of SOD (tot-SOD, about 70–80%) originated from the activity of Cu/ZnSOD (Table 11). This is in line with the share of the activity of Cu/ZnSOD found earlier (del Río et al. 1978; Cakmak and Marschner 1993). Most of this activity is found in the chloroplasts (del Río et al. 1992). In calcareous soils the activity of SOD was not affected by the Fe status of the lettuce, and the activity was at the lowest level in those treatments with the lowest and highest Fe concentration (Table 11), whereas in quartz sand, a deficiency of Fe led to increased activity of SOD. This is in line with the results of studies done in a solution culture, where an Fe deficiency increased the activity of SOD, mostly Cu/Zn-SOD (García et al. 1981; Iturbe-Ormaetxe et al. 1995; Ranieri et al. 1999). This was suggested to be related to the decreased electron transport activity in thylakoids of chloroplasts, thus forming O_2^- (Ranieri et al. 1999), while the concentration of Zn was not taken into account when evaluating the reasons for the increased activity of Cu/ZnSOD (Iturbe-Ormaetxe et al. 1995; Ranieri et al. 1999).

Table 11. Activities of SOD (tot-SOD, Cu/ZnSOD and MnSOD) of lettuce grown in calcareous soils (EXP3) or in unlimed (U) and limed (L) quartz sand (EXP 4), U g^{-1} FW.

		EXP 3		EXP 4	
		Soil 1	Soil 2	U	L
tot-SOD	Control	506^{ab}	617^{a}	1249^{c}	964^{a}
	$FeSO_4$	663^{c}	757^{bc}	916^{a}	905^{a}
	FeEDDS(S,S)	583^{abc}	707^{ab}	989^{ab}	882^{a}
	FeEDDS(mix)	670^{c}	641^{a}	996^{ab}	825^{a}
	FeEDTA	641^{bc}	847^{c}	1135^{abc}	884^{a}
	FeEDDHA	470^{a}	607^{a}	1158^{bc}	903^{a}
Cu/ZnSOD	Control	390^{ab}	492^{a}	841^{b}	650^{ab}
	$FeSO_4$	509^{ab}	617^{c}	686^{a}	573^{a}
	FeEDDS(S,S)	503^{ab}	598^{bc}	685^{a}	658^{ab}
	FeEDDS(mix)	541^{b}	506^{ab}	736^{ab}	559^{a}
	FeEDTA	492^{ab}	720^{d}	824^{b}	610^{ab}
	FeEDDHA	362^{a}	497^{a}	846^{b}	758^{b}
MnSOD	Control	116^{ab}	125^{a}	412^{b}	337^{cd}
	$FeSO_4$	154^{b}	141^{a}	236^{a}	359^{d}
	FeEDDS(S,S)	79^{a}	128^{a}	294^{ab}	202^{ab}
	FeEDDS(mix)	129^{ab}	135^{a}	245^{a}	273^{bc}
	FeEDTA	148^{b}	110^{a}	287^{ab}	298^{cd}
	FeEDDHA	108^{ab}	110^{a}	309^{ab}	171^{a}

Means within a column followed by a common letter do not differ significantly ($p < 0.05$, Tukey HSD test). Columns were tested separately.

The concentrations of Zn and Cu in lettuce were at a higher level when lettuce was grown in quartz sand than when it was grown in calcareous soils (Table 8). In addition, concentrations of Zn and Cu were at a higher level in unlimed than in limed quartz sand, as was also the activity of Cu/ZnSOD (Table 11). The concentration of Mn in lettuce was increased more in quartz sand than in calcareous soils after Fe deficiency (Table 8) as was also the activity of MnSOD in lettuce grown in quartz sand (Table 11). Thus, the activity of MnSOD seemed to follow the concentration of Mn in lettuce. In calcareous soils the activity of Cu/ZnSOD was affected by the concentration of Zn in lettuce (Paper III). This is in line with the results of a study done López-Millán et al. (2005) where an increase in the availability of Zn increased the activity of Cu/ZnSOD. The activity of Cu/ZnSOD was affected more by the availability of Zn than that of Cu (Vaughan et al. 1982; Yu et al. 1998).

These results indicate that the activity of SOD was an expression of bioavailability of the elements included in the isozymes of SOD, as was suggested by del Río et al. (1978), although it has been shown that tissue Mn concentration correlates weakly with MnSOD (López-Millán et al. 2005). In this study I was not able to detect FeSOD activity, and if it was present it was included in the activity of MnSOD (Paper III).

The activity of peroxidases (APX and GPX) and catalase (CAT) was depressed by the low availability of Fe in both quartz sand and in calcareous soils (Table 12). The activity of these enzymes was at its highest level in unlimed quartz sand, but liming of quartz sand depressed the activities of GPX and CAT to a level comparable to that in lettuce grown in calcareous soils. The decrease in the activity of peroxidases and catalase after Fe deprivation is related to the fact that Fe is a cofactor of heme in these enzymes. The reduction of the activities in these enzymes has been found by other researchers after Fe deficiency as well (DeKock et al. 1960; Agarwala et al. 1965; Del Rio et al. 1978; Iturbe-Ormaetxe et al. 1995; Ranieri et al. 1999). The activity of APX, CAT and GPX was depressed by the low availability of Fe in unlimed quartz sand although no significant decrease in yield was observed (Table 7). These results affirm the view that lettuce was experiencing a latent Fe deficiency, and the activity of CAT and APX is a good indicator of the Fe nutritional status of plants (Del Rio et al. 1978; Iturbe-Ormaetxe et al. 1995).

In calcareous environments only FeEDDHA increased the activity of APX significantly (Table 12). The activity of CAT was increased most by FeEDDHA as well, but not significantly (Table 12). These results are probably due to the steady supply of Fe by FeEDDHA, whereas the other Fe chelates were not able to retain Fe in a soluble form throughout the experiments. In limed quartz sand, FeEDDS(S,S) increased the activity of APX equal to other Fe chelates, whereas in calcareous environments the activity was at the level of the control, probably due to more rapid biodegradation or chelate splitting in calcareous soil than in inert quartz sand.

In a calcareous environment, the activity of APX was depressed more by Fe deficiency than the activity of CAT (Table 12). This is probably related to the different location of APX and CAT within the cell: APX is located in cytosol and chloroplasts, while CAT is located in peroxisomes (Asada 1992). During Fe deficiency, the concentration of Fe is decreased most in chloroplasts (Terry and Abadía 1986), probably therefore decreasing APX most (Paper III). In addition APX requires a non-haem Fe in addition to a haem Fe (Kubo et al. 1992). In unlimed quartz sand (EXP 4) the lowest yields were obtained with an $FeSO_4$ treatment (Table 7), and concentrations of Mn, Zn and Cu were at the same level as in the control treatment, indicating enhanced Fe

Table 12. Activity of ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPX) in lettuce grown in calcareous soils (EXP 3) or in unlimed (U) and limed (L) quartz sand (EXP 4), μmol H_2O_2 min^{-1} g^{-1} FW.

		EXP 3		EXP 4	
		Soil 1	Soil 2	U	L
APX	Control	0.082[a]	0.079[a]	0.329[a]	0.125[a]
	$FeSO_4$	0.114[ab]	0.080[a]	0.415[a]	0.146[ab]
	FeEDDS(S,S)	0.089[a]	0.128[ab]	0.371[a]	0.228[ab]
	FeEDDS(mix)	0.124[ab]	0.117[ab]	0.508[a]	0.206[ab]
	FeEDTA	0.118[ab]	0.117[ab]	0.445[a]	0.196[ab]
	FeEDDHA	0.158[b]	0.156[b]	0.482[a]	0.244[b]
CAT	Control	104[a]	119[a]	207[a]	98[a]
	$FeSO_4$	109[a]	105[a]	409[c]	99[a]
	FeEDDS(S,S)	120[a]	105[a]	263[ab]	111[a]
	FeEDDS(mix)	123[a]	129[a]	367[bc]	106[a]
	FeEDTA	116[a]	125[a]	237[a]	146[a]
	FeEDDHA	143[a]	145[a]	287[abc]	170[a]
GPX	Control	2.3[a]	2.7[a]	4.1[a]	2.9[a]
	$FeSO_4$	3.1[ab]	3.0[a]	6.6[c]	3.0[a]
	FeEDDS(S,S)	2.9[ab]	2.8[a]	4.7[ab]	3.5[a]
	FeEDDS(mix)	3.2[b]	3.6[a]	4.4[a]	3.7[a]
	FeEDTA	3.1[ab]	3.0[a]	6.4[bc]	3.2[a]
	FeEDDHA	4.4[c]	3.8[a]	4.3[a]	3.2[a]

Means within a column followed by a common letter do not differ significantly ($p < 0.05$, Tukey HSD test). Columns were tested separately.

efficiency reactions in the roots and thus Fe deficiency. Nevertheless, the activities of CAT and GPX were increased most (Table 12). However, Del Rio et al. (1978) found that an Fe deficiency increased the activity of peroxidase in pea leaves. They suggested that this may be a result of hormonal control after depressed growth. This may be an explanation for the observed increase in the activity of GPX and for CAT in this study.

3.4 Solubility of heavy metals in calcareous soils after Fe chelate addition

The concentrations of water-soluble FeEDDS(S,S) and FeEDDS(mix) in calcareous soils were less than 10% of the added Fe chelate concentration after one day of incubation (Figure 3). [S,S]-EDDS sustained the solubility of Fe at a higher level than EDDS(mix) after one day, following the stability of the respective Fe chelates (Orama et al. 2002). The concentration of water-soluble FeEDTA was at most about 30% of the added concentration after one day of incubation (Paper IV). However, the solubility of FeEDTA decreased rapidly, resembling that found by Garcia-Mina et al. (2003). Only FeEDDHA sustained the concentration of water-soluble Fe above the limit of quantitatum for the whole incubation period of 56 days (Paper IV), which is in line with its high stability (Norvell 1991). About 70% of the added FeEDDHA was in a water-soluble form after one day, which is about the same level as that reported by Hernández-Apaolaza et al. (2007). The solubility of FeEDDHA decreased steadily with time, and after 8 weeks of incubation complexed heavy met-

als, mainly Fe, corresponded to 22 and 14% of the added FeEDDHA content in Soil 1 and Soil 2, respectively. This is in line with the results of previous research (Elgala and Maier 1971; Schenkeveld et al. 2008). Although complexed Fe concentration was taken as a difference between Fe chelate and $FeSO_4$ treatment, Garcia-Mina et al. (2003) showed that water-extractable Fe and chelated (FeEDTA and FeEDDHA) Fe concentrations were almost the same in an incubation study done in calcareous soil.

In a calcareous environment FeEDTA is the main adsorbed EDTA species (Nowack et al. 1996), and Ca displaces Fe efficiently from FeEDTA complex (Aboulroos 1981; Tandy et al. 2004), whereas [S,S]-EDDS formed weak complexes with Ca in soils with less than 2% $CaCO_3$ (Tandy et al. 2006). However, higher amounts of $CaCO_3$ may have depressed Fe solubility more rapidly in Soil 2 than in Soil 1 (Figure 3). The weaker Ca-binding capacity of [S,S]-EDDS compared to EDTA may have caused [S,S]-EDDS to extract indigenous Fe as efficiently as did EDTA (Paper IV). Regardless, only EDDHA was able to extract indigenous Fe in Soil 2 (Paper IV).

After Fe chelates were added to calcareous soils, the main metals complexed by [S,S]-EDDS and EDDS(mix) were Cu and Zn (Figure 3), corresponding to about 80% of the complexed heavy metals. Among the water-soluble heavy metals, the differences of Zn concentrations between FeEDDS(S,S) and FeEDDS(mix) additions to soils were greatest (Figure 3): when $ZnSO_4$ was added, the concentration of water-soluble ZnEDDS(S,S) was at most 19.7 and of water-soluble ZnEDDS(mix) 11.6 µmol kg^{-1} in Soil 1 (Figure 3). However, the difference was smaller when ligands extracted indigenous Zn, namely water-soluble ZnEDDS(S,S) and ZnEDDS(mix), being 11.3 and 10.2 µmol kg^{-1}, respectively (Paper IV). After FeEDTA splitting, ZnEDTA was the main complex, constituting about 65% of the water-soluble metal-EDTA complexes. The solubility of Mn was only slightly affected by the Fe chelates, most by FeEDTA (Paper IV). In calcareous soils EDTA did not increase the Mn concentration of lettuce more than other ligands, but in quartz sand it did (Table 8). When only ligands were added to calcareous soils, EDDHA increased the solubility of Mn significantly (Paper IV). This was probably related to the oxidation of Mn^{2+} and the formation of Mn^{3+}EDDHA species (Chaney 1988). The same phenomenon probably increased the solubility of Co as well (Chaney 1988).

After splitting of FeEDDS(S,S) and FeEDDS(mix), the solubilities of Co and Ni were increased far more than after splitting of FeEDTA or FeEDDHA during the first week after chelate application (Paper IV). Complexed CoEDDS(S,S) and NiEDDS(S,S) concentrations reached at most 3.0 and 8.3 µmol kg^{-1} in Soil 1 and Soil 2, respectively (Paper IV). The trend in the solubility of Co and Ni after FeEDDS(S,S) and FeEDDS(mix) additions was similar up to [S,S]-EDDS biodegradation, after which recalcitrant forms of EDDS, namely [S,R/R,S] and [R,R] (Schowanek et al. 1997), were able to sustain the solubility of Co and Ni. However, biodegradation of CoEDDS(mix) and NiEDDS(mix) complexes depressed their concentration after 28 days of incubation. Of the measured metal-EDDS complexes, NiEDDS seemed to be most recalcitrant, which is in line with the findings of Vandevivere et al. (2001).

The solubility of Ni increased steadily after FeEDTA application to soils; after 8 weeks, NiEDTA and ZnEDTA were the main complexes and NiEDTA made up about 45% of the analyzed water-soluble metal-EDTA complexes (Paper IV), accounting for 7.6% of the added EDTA concentration. Although [S,S]-EDDS complexed Ni at a higher level during the first week of incubation than the level reached by EDTA

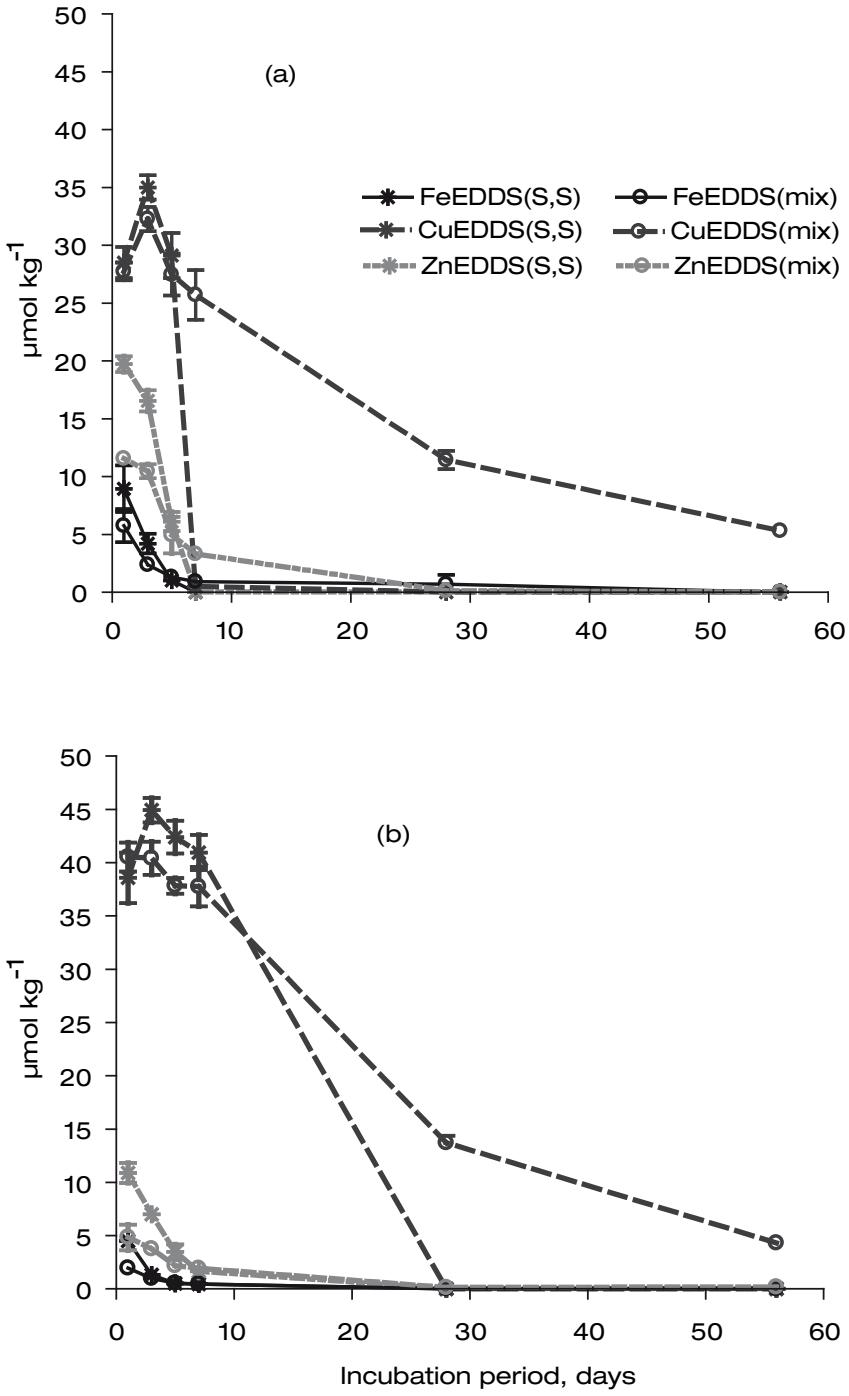

Figure 3. Water-soluble concentrations of Fe, Cu and Zn in Soil 1 (a) and Soil 2 (b) after addition of FeEDDS(S,S) and FeEDDS(mix) over an incubation period of 56 days. Error bars ±SD, and if not shown, error bars are within the symbol.

after 56 days of incubation, the solubility of NiEDDS(S,S) was at the level of the control treatment after 28 days. Less biodegradable forms of EDDS sustained the solubility of Ni throughout the 56-day period, but the concentration started to decrease after 28 days of incubation. The slow increase of NiEDTA concentration is related to the slow kinetics of Ni desorption by EDTA (Bryce et al. 1994), and Ni desorption may occur preferentially with free EDTA, thus causing the metal-EDTA complex to split prior to Ni desorption (Bryce and Clark 1996). My results show that even a low ligand application level increases the solubility and thus the potential for heavy metals to leach (Means et al. 1980). Because biodegradation of EDTA is slow (Nörtemann 1999; Bucheli-Witschel and Egli 2001), it sustains Ni in a complexed form for a longer period of time than EDDS.

EDTA was the only ligand that increased the solubility of Cd and Pb in calcareous soils (Figure 4). Of the total Pb concentration in soils, EDTA solubilized at most 0.7%, but of total Cd up to 8.4% was complexed by EDTA. The stabilities of both PbEDTA (log K = 19.0, Norvell 1991) and CdEDTA (log K = 17.4, Norvell 1991) are rather similar, but the more efficient extraction of Cd by EDTA is probably due to its weaker adsorption to soil (Harrison et al. 1981; Labanowski et al. 2008). However, in soils contaminated with Pb, up to 80% of the Pb was extracted with EDTA when using an equal ratio of EDTA:Pb (Kim et al. 2003). In agricultural soils with a low Pb concentration the high concentrations of competing metals probably depressed the formation of PbEDTA. In soils contaminated with heavy metals, also [S,S]-EDDS has increased the solubility of Pb and Cd (Kos and Leštan 2003; Tandy et al.

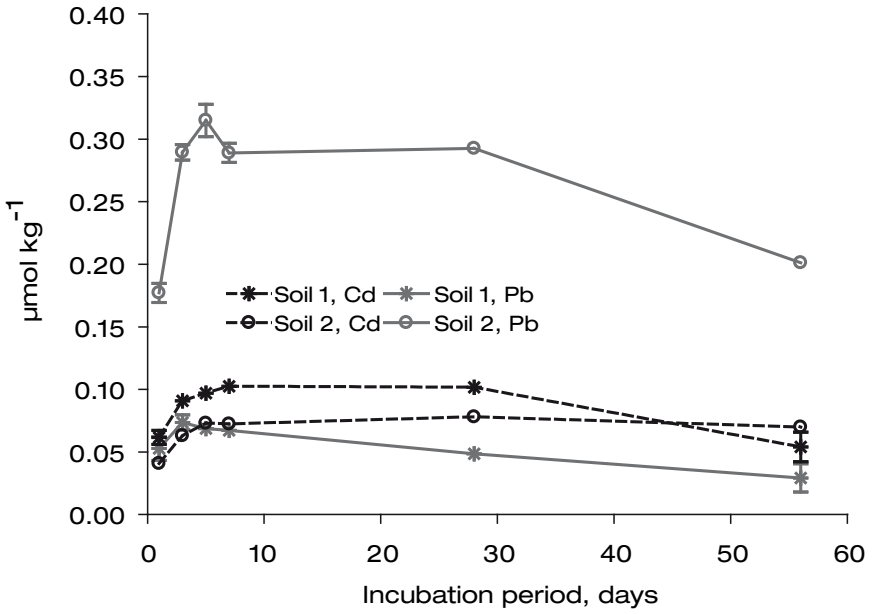

Figure 4. Water-soluble concentrations of Cd and Pb in calcareous soils after addition of FeEDTA over an incubation period of 56 days. Error bars ±SD, if not shown, error bars are within the symbol.

2004; Duquène et al. 2009), but the application rates of [S,S]-EDDS were at a higher level than those used in this study.

3.4.1 Biodegradation

The biodegradation of ligands in calcareous soils was estimated by calculating the decrease of water-soluble heavy metals (Figure 5). The same method was used by Meers et al. (2005) when calculating the biodegradation of [S,S]-EDDS and EDTA in dredged sediment by taking samples from soil solution during a period of 40 days. In that study the lowest application rate of the ligands was 1.8 mmol kg^{-1}. Tandy et al. (2006) criticized this method, arguing that the high application rate of ligands may cause some ligands to be uncomplexed and thus not to be taken into account. In this study the application rates of Fe chelates were 0.1 mmol kg^{-1}, reducing the possibility that ligands would be in an uncomplexed form.

If they are not biodegraded, ligands and metal complexes may be adsorbed onto soil particles. Adsorption of both free ligands and metal-EDTA complexes is similar, being inversely related to pH (Bowers and Huang 1986; Nowack et al. 1996) and at a pH above 7, adsorption of FeEDTA (Bryce et al. 1994), CdEDTA, PbEDTA (Kedziorek et al. 1998), CoEDTA (Brooks et al. 1996), Zn-, Cu-, and MnEDTA (Aboulroos 1981) is low. To my knowledge there are no published results on the adsorption of metal-EDDS to soil, but because EDDS is a structural isomer of EDTA, its adsorption to soil may be sim-

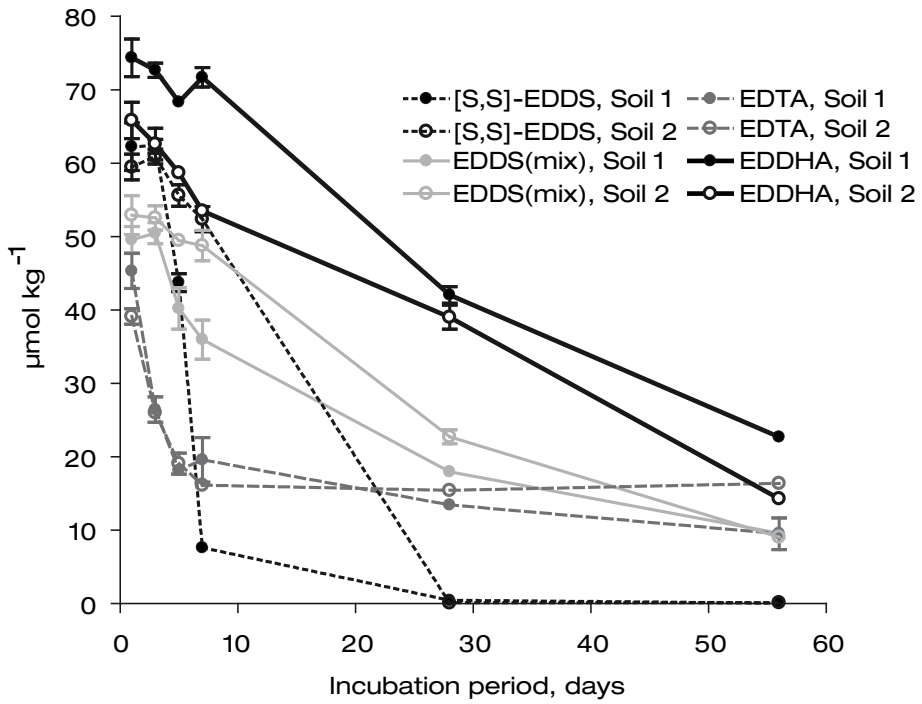

Figure 5. Sum of the water-soluble complexed heavy metals (Fe, Mn, Zn, Cu, Co, Ni, Cd and Pb) after addition of Fe chelates on calcareous soils over an incubation period of 56 days. Error bars ±SD, if not shown, error bars are within the symbol.

ilar. Adsorption of FeEDDHA onto clay particles has not been observed (Wallace and Lunt 1956).

After Fe chelate application to calcareous soils, the sum of the complexed heavy metals (Fe, Mn, Zn, Cu, Co, Ni, Cd and Pb) was increased more by application of FeEDDS(S,S) than of FeEDDS(mix), but more rapid biodegradation of [S,S]-EDDS reversed the situation later on (Figure 5). After one day the sum of water-soluble heavy metals decreased in the following order: FeEDDHA > FeEDDS(S,S) > FeEDDS(mix) > FeEDTA (Figure 5).

The solubility of heavy metals after addition of FeEDDS(mix) and FeEDDS(S,S) to calcareous soils started to decline after a lag phase of three days (Figure 5), indicating ligand biodegradation (Meers et al. 2005; Tandy et al. 2006). A lag phase of up to 11 days for [S,S]-EDDS has been observed in soil contaminated with sewage sludge (Tandy et al. 2006) and 5–10 days in pure sludge (Schowanek et al. 1997; Takahashi et al. 1997). In all these studies the application rates of [S,S]-EDDS were far greater than those used in this study. A decrease in the solubility of heavy metals indicates that [S,S]-EDDS was totally degraded within 28 days in calcareous soils, whereas other enantiomers of EDDS were less biodegradable. This result is in accordance with findings reported in earlier studies (Schowanek et al. 1997; Witschel and Egli 1997).

In a biodegradation study done with activated sludge by Vandevivere et al. (2001), Fe-, Cd-, PbEDDS(S,S) complexes were easily biodegradable, whereas Zn-, Cu-, Ni- and CoEDDS(S,S) complexes were not biodegraded within the incubation period of 15 days. The authors suggested that only intact [S,S]-EDDS was biodegraded, requiring metal-EDDS(S,S) complex splitting prior to biodegradation. In my study, the solubility of Zn- and CuEDDS(S,S) started to decrease before that of Ni- and CoEDDS(S,S), suggesting that Ni- and CoEDDS(S,S) complexes were more recalcitrant against biodegradation. The differences in the biodegradability of metal-EDDS complexes may be affected by their stability: only chelates with lower stabilities are taken up by microbes, and chelates with higher stabilities need to be split prior to biodegradation (Witschel and Egli 1997). However, in this study water-soluble complexed heavy metal concentrations decreased independent of speciation, indicating that all the metal-EDDS(mix) complexes were degraded, regardless of the composition of enantiomers (Paper IV). For EDTA it has been shown that the biodegradation of metal-EDTA complexes is related to the corresponding stability of the chelate (Klüner et al. 1998; Satroutdinov et al. 2000).

Among complexing agents, the solubilities of heavy metals were at the lowest level after addition of FeEDTA or EDTA after one day of incubation (Figure 5, Paper IV). However, this is probably related to the formation of CaEDTA complexes, because EDTA is rather recalcitrant against biodegradation (Nörtemann 1999; Bucheli-Witschel and Egli 2001). Indeed, the sum of analyzed heavy metals was unchanged between incubation days 7 and 56 (Figure 5). Therefore, the results for EDTA biodegradation may be less accurate than the results with other ligands if biodegradation is evaluated without taking into account the concentration of CaEDTA (Paper IV). Calcium complexation after the addition of other Fe chelates may also explain the result that the total sum of complexed heavy metals was below the level of 100 µmol kg^{-1} after one day of incubation. However, from the environmental point of view, increased water-soluble Cu, Zn, Co, Ni, Cd and Pb concentrations are more relevant than an increase in the solubility of Ca.

The results of this study indicate that although EDDS(mix) was less biodegrad-

able than [S,S]-EDDS, solubility of NiEDDS(mix) and CoEDDS(mix) were decreased, whereas EDTA retained the solubility of Co or increased the solubility of Ni as incubation proceeded (Paper IV). In addition, EDTA was the only ligand to increase the solubility of Cd and Pb. Therefore, from the environmental point of view, EDDS(mix) is a suitable alternative ligand for EDTA when a small amount of complexing agents is used to remedy nutrient deficiency in plants grown in calcareous soils.

4 Conclusions

In calcareous soils FeEDDS(S,S) and FeEDDS(mix) were less effective in sustaining a water-soluble Fe concentration compared to FeEDTA and to the most efficient Fe chelate, FeEDDHA. In spite of this, FeEDDS(mix) increased the Fe concentration and the physiologically active Fe pool as efficiently as did FeEDDHA and FeEDTA, respectively, when Fe chelates were applied twice a week to mimic drip irrigation. In unlimed and limed quartz sand, FeEDDS(S,S) increased the concentration of Fe and physiologically active Fe pool in lettuce equally to other Fe chelates. This may be related to the slower chelate splitting or biodegradation of [S,S]-EDDS in inert quartz sand. Ryegrass growth was not depressed by Fe deficiency in limed quartz sand, probably due to its more efficient Fe uptake system (Strategy II) than that of lettuce (Strategy I).

In quartz sand all Fe chelates increased Fe concentration in lettuce equally when applied via soil, whereas FeEDDS(mix) increased Fe concentration more than did FeEDTA or FeEDDHA when applied foliarly. The concentrations of Mn and Zn in lettuce grown in quartz sand and the concentration of Mn when grown in calcareous soils were depressed by Fe chelates, probably due to the depressed Fe efficiency reactions in the roots. In quartz sand EDDHA increased the concentration of Cu in lettuce most among ligands, probably due to formation of CuEDDHA complex in reduced conditions in the rhizosphere after FeEDDHA splitting. The concentration of nutrients in ryegrass was only slightly affected by the availability of Fe.

A deficiency of Fe led to an increase in Co and Ni concentrations in lettuce when grown in quartz sand independent of growth rate. This was also probably related to the increased Fe efficiency reactions of the roots and the resulting increased uptake of these heavy metals. Concentrations of Co and Ni in lettuce were depressed most by FeEDDS(S,S) and FeEDDS(mix). In calcareous soils EDDS increased the solubility of Co and Ni most, but it had only a small effect on the uptake of these elements. Water-soluble concentrations of Cd and Pb in calcareous soils were increased only by EDTA. Nevertheless, EDTA did not increase the concentration of Cd and Pb in lettuce. Therefore, when Fe chelates are applied at low rates in order to correct nutrient deficiencies, concentrations of Co, Ni, Cd or Pb are not increased in spite of the increased solubility of these elements in the soil. The concentrations of Ni and Co in ryegrass were depressed most by EDDS(mix). This may be related to the complexation of these elements, making them unavailable for the uptake.

After FeEDDS splitting in calcareous soil, the main complexed metals were Cu and Zn but their concentration decreased rapidly, whereas Co- and NiEDDS were more recalcitrant chelates. However, the biodegradation of [S,S]-EDDS was not affected by the complexed heavy metal and was biodegraded within 28 days, whereas more recalcitrant stereoisomers of EDDS ([S,R]/[R,S] and [R,R]), retained Cu and Ni concentrations elevated throughout the incubation period of 56 days. Unlike EDDS, EDTA increased the solubility of Ni steadily throughout the incubation period and NiEDTA and ZnEDTA were the major EDTA species after 56 days of incubation.

According to this study, EDDS(mix) is an appropriate alternative chelating agent to EDTA in providing Fe for plants despite the fact that FeEDDS(mix) is less stable than FeEDTA. From environmental point of view EDDS did not increase the solubility of Pb or Cd at low application levels, and Co- and NiEDDS(mix) were biodegradable. The most biodegradable stereoisomer of EDDS, namely [S,S]-EDDS, may be an appropriate alternative nutrient carrier in greenhouse production where the growth medium is mainly rockwool, resembling quartz sand, as both are inert substances and require a continuous flow of nutrients.

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