

Influence of chelating ligands on bioavailability and mobility of iron in plant growth media and their effect on radish growth

著者	Hasegawa Hiroshi, Rahman M.A., Saitou K., Kobayashi M., Okumura C.
journal or publication title	Environmental and Experimental Botany
volume	71
number	3
page range	345-351
year	2011-07-01
URL	http://hdl.handle.net/2297/27093

doi: 10.1016/j.envexpbot.2011.01.004

1 **Influence of Chelating Ligands on Bioavailability and Mobility of**
2 **Iron in Plant Growth Media and Their Effect on Radish Growth**

3
4
5
6
7
8 H. Hasegawa*; M. Azizur Rahman; K. Saitou; M. Kobayashi; C. Okumura

9
10
11
12
13
14
15 Graduate School of Natural Science and Technology, Kanazawa University,
16 Kakuma, Kanazawa 920-1192, Japan

17
18
19
20
21
22
23
24 *Corresponding author

25 E-mail: hhiroshi@t.kanazawa-u.ac.jp

26 Tel/Fax: 81-76-234-4792

27
28
29
30
31
32

33 Abstract:

34 In this study, the effects of chelating ligands on iron movement in growth Medium,
35 iron bioavailability, and growth of radish sprouts (*Raphanus sativus*) were investigated. Iron is
36 an important nutrient for plant growth, yet the insoluble state of iron hydroxides in alkaline
37 conditions decreases its bioavailability. Iron chelates increase iron uptake and have been used
38 in agriculture to correct iron chlorosis. While previous studies have reported the effects of
39 chelating ligands on iron solubility and bioavailability, the present study elucidates the pattern
40 of iron movement by chelating ligands in plant growth Medium. The apparent mobility of iron
41 in growth Medium was calculated using a '4-box' model. Ethylenediaminedisuccinic acid
42 (EDDS) and hydroxy-iminodisuccinic acid (HIDS) produced the highest apparent mobility of
43 iron from the bottom layer of the medium (initially 10^{-4} M Fe(III)) to the upper layer (no iron),
44 followed by glutamicdiacetic acid (GLDA), ethylenediaminetetraacetic acid (EDTA),
45 methylglycinediacetic acid (MGDA), and iminodisuccinic acid (IDS). Iron movement in the
46 growth Medium was influenced by the chelating ligand species, pH, and ligand exposure time.
47 The iron uptake and growth of radish sprouts were related to the iron mobility produced by the
48 chelating ligands. These results suggest that, in alkaline media, chelating ligands dissolve the
49 hardly soluble iron hydroxide species, thus increasing iron mobility, iron uptake, and plant
50 growth. HIDS, which is biodegradable, was one of the most effective ligands studied; therefore,
51 this compound would be a good alternative to other environmentally persistent chelating
52 ligands.

53

54

55

56 **Keywords:** Chelating ligands, HIDS, Iron, Radish sprouts (*Raphanus sativus*), Bioavailability.

57

58

59

60 Introduction

61 Iron is an essential micronutrient for plants (Boyer et al., 1988; Zancan et al., 2008) and
62 plays an important role in respiration, photosynthesis, DNA synthesis, nitrogen fixation,
63 hormone production, and many other cellular functions (Vert et al., 2002). Although abundant
64 in nature, Fe exists in alkaline soil as hardly soluble hydrated oxide states, including
65 $(\text{Fe}_2\text{O}_3 \cdot n\text{H}_2\text{O})$, Fe^{3+} , $\text{Fe}(\text{OH})_3$, and $\text{Fe}(\text{OH})^{2+}$ (Aston and Chester, 1973; Barry et al., 1994).
66 These Fe species are poorly absorbed by plant roots (Cohen et al., 1998; Guerinot and Yi,
67 1994) and cause defective growth of the plant (Robin et al., 2008; Yousfi et al., 2007).
68 Insoluble ferric hydroxide complexes are also known as Fe plaques. Formation of Fe plaques
69 in the rhizosphere results in a deficiency of Fe and other nutrients (including P, Cu, Mn, Zn, Pb,
70 and Cd) in the plants (Batty et al., 2000; Christensen and Sand-Jensen, 1998; Otte et al., 1989;
71 Ye et al., 1998; Ye et al., 2001; Zhang et al., 1998). Under such conditions, plants have two
72 distinct natural strategies to assimilate Fe from the environment. Grasses release
73 phytosiderophores, which are low-molecular-weight, high-affinity Fe(III)-chelate compounds
74 that solubilize ferric Fe in the rhizosphere and are recognized by specific membrane
75 transporters (Bienfait, 1988; Chaney, 1987; Romheld, 1987; Romheld and Marschner, 1986a,
76 b). Fe uptake in dicots and non-grass monocots is mediated by a plasma-membrane-bound
77 ferric reductase that transfers electrons from intracellular NADH (Buckhout et al., 1989) to
78 Fe(III)-chelates in the rhizosphere (Chaney et al., 1972). The ferrous ions released from the
79 chelates by this process are subsequently transported into the cytoplasm via a separate
80 transport protein (Fox et al., 1996; Kochian, 1991). In addition, some rhizospheric microbes
81 exude siderophores at the root-plaque interface. These siderophores solubilize ferric iron in the
82 rhizosphere and are recognized for uptake by specific membrane receptors, thus rendering the
83 iron bioavailable (Bienfait, 1988; Chaney, 1987; Romheld and Marschner, 1986a).

84 Research on the interaction between plants and chelating ligands started in the 1950s
85 with the goal of reducing deficiencies of the essential nutrients Fe, Mn, Cu, and Zn (Wenger et

86 al., 2005). Chelators increase the mobility of iron in alkaline media by dissolving the hardly
87 soluble iron hydroxide species (Lucena, 2006; Lucena, 2003; Lucena et al., 1996; Lucena and
88 Chaney, 2006; Tagliavini and Rombolà, 2001; Villen et al., 2007; Yona et al., 1982). Among
89 all soil-applied Fe fertilizers, synthetic Fe(III) chelates are the most effective and commonly
90 used. These compounds originate mainly from polyaminocarboxylic acids with phenolic
91 groups such as ethylenediamine di(*o*-hydroxyphenylacetic) acid (EDDHA) and ethylenediamine
92 di(2-hydroxy-4-methylphenylacetic) acid (EDDHMA) (Alvarez-Fernandez et al., 2005).
93 Ethylenediaminetetraacetic acid (EDTA) has been a popular choice to achieve this purpose
94 (Claudia and Rodríguez, 2003; Nowack and Sigg, 1997; Urrestarazu et al., 2008), but it does
95 not dissolve easily in water or soil, it persists in the environment (Bucheli-Witschel and
96 Thomas Egli, 2001; Nortemann, 1999; Villen et al., 2007), and it affects the material cycle of
97 various elements. This, in combination with its high affinity for heavy metal complexation,
98 results in an increased risk of leaching. EDTA also severely impairs plant growth, even at very
99 low concentrations (Bucheli-Witschel and Thomas Egli, 2001). Therefore, EDTA use is
100 prohibited in some European countries.

101 Biodegradable chelating ligands, such as ethylenediaminedisuccinic acid (EDDS) and
102 hydroxyl-iminodisuccinic acid (HIDS), would be good alternatives to EDTA. In this study, we
103 investigated the biodegradable chelating ligand hydroxyl-iminodisuccinate (HIDS). The
104 physicochemical properties of EDDS, EDTA, and IDS have already been established by a
105 number of researchers (Evangelou et al., 2007; Helena et al., 2003; Jaworska et al., 1999).
106 However, HIDS is a new chelating ligand introduced by Nippon Shokubai Co. Ltd. It is
107 classified as one of the safest and most biodegradable chelating ligands, with a biodegradation
108 rate of about 22.4% within 48 h. HIDS traps and inactivates various metal ions, particularly
109 Fe³⁺ and Cu²⁺ as well as Ca²⁺ and Mg²⁺, over a wide range of pH values. In addition, HIDS is
110 highly stable in harsh conditions and high temperatures (80°C) and highly soluble in aqueous
111 alkaline solutions (Sokubai, 2009). HIDS forms water-soluble complexes with various metal

112 ions over a wide pH range. In particular, it shows superior performance in chelating Fe^{3+} ions
113 in alkaline solutions (Sokubai, 2009). Because of its high degradation rate and high stability
114 constant with Fe^{3+} , we investigated the effectiveness of HIDS on Fe bioavailability and
115 mobility patterns in growth Medium. EDTA, EDDS, and IDS were also studied for comparison.
116 The effects of both biodegradable and non-biodegradable chelating ligands on the mobility and
117 bioavailability of iron in plant growth medium are discussed using a '4-box' model. This is the
118 first report on Fe mobility due to chelating ligands in plant growth Medium.

119

120 **Materials and Methods**

121 **Culture of radish sprouts**

122 Murashige and Skoog (MS) culture medium (Murashige and Skoog, 1962) was used for
123 radish sprout growth. The concentration of chelating ligands in the medium was 10^{-3} M. After
124 adjusting to pH 10 using 0.1 M NaOH, the medium was sterilized by high-pressure
125 sterilization in an autoclave (120°C, 30 min) and UV irradiation. Before the agar hardened, 4
126 mL of the medium (25 mm depth) was dispensed into a 14-mL sterilized polystyrene tube.

127 Radish seeds were collected from a local market and stored at 4°C until use in the
128 experiment. The seeds were sterilized in a solution of 0.25% NaClO and 25 μM Tween20 for 2
129 minutes, and then rinsed 5 times with 5 mL of deionized water (EPW) using an E-pure system
130 (Barnstead). Germinating seeds were planted in the agar medium and cultured for a week in a
131 20°C growth chamber with 180 μM photon $\text{m}^{-2} \text{s}^{-1}$ light intensity from cool white fluorescent
132 lights on a 14:10 h light/dark schedule.

133

134 **Extraction of extracellular iron fractions and chemical analysis**

135 Intra- and extracellular iron fractions in the radish sprouts were determined by
136 radiochemical measurements of ^{55}Fe . To determine intracellular iron concentrations, samples
137 were successively rinsed with 5 mL of EPW, 5 mL of 0.047 M Ti(III)-citrate-EDTA solution,

138 and again with 5 mL of EPW. Samples used to determine total iron (corresponding to intra- and
139 extracellular iron) were rinsed with 5 mL of EPW. Both types of samples, in which $^{55}\text{Fe(III)}$
140 was retained as a tracer, were directly added to 5 mL of liquid scintillation solution (3.0 g of 2-
141 (4-tert-butylphenyl)-5-(4-biphenyl)-1,3,4-oxadiazole per 500 mL toluene) in 20 mL vials.
142 The radiochemical activity of $^{55}\text{Fe(III)}$ was measured using a liquid scintillation counter (LSC-
143 6101, Aloka, Japan) in tritium mode. The concentration of Fe(III) was calculated from the
144 Fe(III)/ $^{55}\text{Fe(III)}$ ratio in solutions.

145

146 **Determination of Fe mobility**

147 A 2-layered modified MS medium was used to measure Fe mobility. The bottom layer
148 contained 10^{-4} M FeCl_3 with 370 MBq/l of ^{55}Fe , and the upper layer contained no FeCl_3 (Fig.
149 1). The MS agar medium was collected after 48, 96, and 144 h during the experiment to
150 measure iron concentrations. The tubes were divided into 5 mm sections, and the agar was
151 removed from each section and dried for 24 h in an electric oven. The iron content was
152 measured by a 370 MBq/l radioactive tracer ^{55}Fe using a liquid scintillation counter.

153 Fe mobility in the nutrient medium was calculated from the transfer coefficient of iron
154 movement using a 4-box model. The details of the model are described in the Results and
155 Discussion.

156

157 **Chemicals**

158 A stock solution of Fe(III) was prepared by dissolving $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Nacalai Tesque,
159 Kyoto) in 1 M HCl (TAMAPURE-AA-100, Tama Chemicals, Tokyo) and standardized using
160 inductively coupled plasma atomic emission spectrometry (Optima 3300XL, Perkin-Elmer,
161 USA). A stock solution of $^{55}\text{Fe(III)}$ was prepared by dissolving $^{55}\text{FeCl}_3$ (PerkinElmer Life &
162 Analytical Sciences, specific activity; 370 MBq/l) in 1 M HCl (TAMAPURE-AA-100). The
163 solutions were diluted to the desired concentration ratios of Fe(III)/ $^{55}\text{Fe(III)}$. Stock solutions of

164 EDTA, HIDS, IDS, MGDA, GLDA and EDDS were prepared by dissolving ethylenediamine-
165 N,N,N',N'-tetraacetic acid (Dojindo Molecular Technologies, Japan), tetrasodium 3-hydroxy-
166 2,2'-iminodisuccinate (Nippon Syokubai), tetrasodium iminodisuccinate (Bayer),
167 methylglycine-N,N-diacetic acid (BASF), L-glutamate-N,N-diacetic acid, and
168 ethylenediamine-N,N'-disuccinic acid (Chelest), respectively, in 0.1 M sodium hydroxide. The
169 reagents were of analytical grade and used without further purification. All solutions were
170 prepared with purified water (EPW) using an E-pure system (Barnstead).

171

172 **Results**

173 **Iron movement in the growth medium**

174 Radish sprouts were grown in 2-layered culture medium to investigate the effect of
175 chelating ligands on Fe movement in the medium. The layers of the growth medium were
176 distinguished by the initial concentration of Fe(III), which was 10^{-4} M in the bottom layer
177 while the upper layer initially contained no Fe(III) (Fig. 1). A solution of 0.1 mM chelating
178 ligand was added to the bottom layer of semisolid MS-agar culture medium. The medium in
179 the test tubes was divided into 5 mm sections, and samples from each section were collected
180 and analyzed for Fe after 48, 96, and 144 h. The presence of chelating ligands increased Fe
181 movement from the Fe-rich bottom layer to the Fe-free upper layer of the Medium (Fig. 3).

182 To investigate the pattern of Fe movement, a Fe gradient was created across two layers
183 of semisolid MS-agar growth medium in the presence of chelating ligand. Each of the two
184 layers was further divided into two layers, and a '4-box' model was established (Fig. 1) to
185 estimate the amount and pattern of Fe movement in the medium. The highest concentration of
186 Fe was measured in box 3 (B₃), although the initial concentrations of Fe in B₃ and box 4 (B₄)
187 were the same. The Fe adsorbed on the bottom surface of the test tubes, which was not
188 desorbed by the addition of the chelating ligand, could explain this phenomenon. The Fe
189 concentration in B₃ differed greatly from box 2 (B₂), where the initial Fe concentration was

190 zero.

191

192 **Four-box model for the determination of Fe mobility**

193 Fe mobility was calculated from the transfer coefficient of iron movement using a '4-
 194 box' model of the 2-layered growth medium. The transfer rate of total Fe between layers is
 195 related proportionally to the differences in dissolved Fe and inversely to the volume of growth
 196 medium in the corresponding layer. The '4-box' model is shown in [Figure 1](#). Using this system,
 197 the transfer coefficient of total Fe was calculated from the following equations:

$$Q_{t1} = \frac{1}{C_{d2} - C_{d1}} V_1 \frac{\Delta C_{t1}}{\Delta T_1} \dots\dots\dots(1a)$$

198 $Q_{t2} = \frac{1}{C_{d3} - C_{d2}} V_2 \frac{\Delta C_{t2}}{\Delta T_2} \dots\dots\dots(1b)$

$$Q_{t3} = \frac{1}{C_{d4} - C_{d3}} V_3 \frac{\Delta C_{t3}}{\Delta T_3} \dots\dots\dots(1c)$$

199 Where Q_t is the transfer coefficient of total Fe; C_d and C_t are the concentrations of
 200 dissolved and total Fe, respectively; V is the volume of the medium; and T is transfer time. The
 201 four boxes are defined as B_1 , B_2 , B_3 , and B_4 , and the volumes of medium in each box are
 202 labeled as V_1 , V_2 , V_3 , and V_4 , respectively, where $V_1=V_4 = 1.5 \text{ cm}^3$, and $V_2=V_3 = 1.0 \text{ cm}^3$ ([Fig. 1](#)).

203 Iron in growth media can exist as either dissolved ($[\text{Fe}]_{\text{dis}}$) or undissolved fractions
 204 ($[\text{Fe}]_{\text{undis}}$). Therefore, total iron ($[\text{Fe}]_t$) in the medium can be calculated as:

205 $[\text{Fe}]_t = [\text{Fe}]_{\text{undis}} + [\text{Fe}]_{\text{dis}} \dots\dots\dots(2)$

206 The dissolved and undissolved fractions of iron contain both inorganic iron species
 207 ($[\text{Fe(III)}]$), such as Fe^{3+} , Fe(OH)^{2+} , Fe(OH)_2^+ , and so forth, as well as organic iron, as in the
 208 FeL complex. Since agar was used in the preparation of the growth medium, some fractions of
 209 the iron might have adsorbed onto agar particles and become undissolved.

$$210 \quad [\text{Fe}]_t = \left\{ [\text{Fe(III)}']_{\text{undis}} + [\text{FeL}]_{\text{undis}} \right\} + \left\{ [\text{Fe(III)}']_{\text{dis}} + [\text{FeL}]_{\text{dis}} \right\} \dots \dots \dots (3)$$

211 After the addition of chelating ligands, most of the FeL was expected to be in the
212 dissolved form, and the existence of Fe in the insoluble form ($[\text{FeL}]_{\text{undis}}$) was negligible. Thus,

$$213 \quad [\text{Fe}]_t = [\text{Fe(III)}']_{\text{undis}} + [\text{Fe(III)}']_{\text{dis}} + [\text{FeL}]_{\text{dis}} \dots \dots \dots (4)$$

214 The concentrations of Fe^{3+} and undissolved fractions of $[\text{Fe(III)}']$ in the medium were
215 proportional to the concentration of dissolved fractions:

$$216 \quad [\text{Fe(III)}']_{\text{undis}} = f(\alpha) [\text{Fe(III)}']_{\text{dis}} \dots \dots \dots (5)$$

$$217 \quad [\text{Fe}^{3+}] = f(\beta) [\text{Fe(III)}']_{\text{dis}} \dots \dots \dots (6)$$

218 The dissolution of Fe in the medium depended on the conditional stability constant of
219 the chelating ligands with Fe^{3+} . The stability constant of chelating ligands (K_{FeL}) can be
220 defined as:

$$221 \quad K_{\text{FeL}} = \frac{[\text{FeL}]_{\text{dis}}}{[\text{Fe}^{3+}][\text{L}]} \dots \dots \dots (7)$$

222 Subsequently, the total Fe concentration in the medium can be calculated by the
223 following equation derived from equations (4), (5), (6), and (7):

$$224 \quad [\text{Fe}]_t = \{ f(\alpha) + 1 + f(\beta)[\text{L}]K_{\text{FeL}} \} [\text{Fe(III)}']_{\text{dis}} \dots \dots \dots (8)$$

225 Thus, total Fe concentration in B_1 and B_2 can be calculated as

$$226 \quad [\text{Fe(III)}']_{\text{dis1}} = \frac{[\text{Fe}]_{t1}}{F'} \dots \dots \dots (9a) , \text{ and}$$

$$227 \quad [\text{Fe(III)}']_{\text{dis2}} = \frac{[\text{Fe}]_{t2}}{F'} \dots \dots \dots (9b), \text{ where } F' = f(\alpha) + 1 + f(\beta)[\text{L}]K_{\text{FeL}} .$$

228 Furthermore, the transfer coefficient of dissolved Fe from B_1 to B_2 can be calculated
229 from the following equation derived from equation (1a):

$$\begin{aligned}
 230 \quad Q_{t1} &= \frac{1}{\left\{ \left[\text{Fe(III)}' \right]_{\text{dis}2} + [\text{FeL}]_{\text{dis}2} \right\} - \left\{ \left[\text{Fe(III)}' \right]_{\text{dis}1} + [\text{FeL}]_{\text{dis}1} \right\}} V_1 \frac{\Delta C_{t1}}{\Delta T_1} \\
 231 \quad &= \frac{1}{\left\{ \left\{ 1 + f(\beta)[L]K_{\text{FeL}} \right\} \frac{[\text{Fe}]_{t2}}{F'} \right\} - \left\{ \left\{ 1 + f(\beta)[L]K_{\text{FeL}} \right\} \frac{[\text{Fe}]_{t1}}{F'} \right\}} V_1 \frac{\Delta C_{t1}}{\Delta T_1} \\
 232 \quad &= \frac{1}{\frac{1}{F} \cdot \{ [\text{Fe}]_{t2} - [\text{Fe}]_{t1} \}} V_1 \frac{\Delta C_{t1}}{\Delta T_1} \dots\dots\dots(10)
 \end{aligned}$$

233 Where, $F = \frac{f(\alpha) + 1 + f(\beta)[L]K_{\text{FeL}}}{1 + f(\beta)[L]K_{\text{FeL}}}$

234 In addition, the coefficient (Q/F) of Fe movement from B₁ to B₂ in the medium can be defined

235 as - $\frac{Q_{t1}}{F} = \frac{1}{[\text{Fe}]_{t2} - [\text{Fe}]_{t1}} V_1 \frac{\Delta C_{t1}}{\Delta T_1} \dots\dots\dots(11a)$, and the Q/F from B₂ to B₃ and from B₃

236 to B₄ would be –

$$\frac{Q_{t2}}{F} = \frac{1}{[\text{Fe}]_{t3} - [\text{Fe}]_{t2}} V_2 \frac{\Delta C_{t2}}{\Delta T_2} \dots\dots\dots(11b)$$

237

$$\frac{Q_{t3}}{F} = \frac{1}{[\text{Fe}]_{t4} - [\text{Fe}]_{t3}} V_3 \frac{\Delta C_{t3}}{\Delta T_3} \dots\dots\dots(11c)$$

238

239 **Iron movement coefficient by chelating ligands**

240 A ‘4-box’ model was established to calculate the apparent coefficient of Fe movement
 241 due to chelating ligands in the growth medium. Using this model, the apparent coefficient
 242 (Q/F) of Fe movement in the medium was calculated from equations (11a), (11b), and (11c),
 243 and the results are presented in [Table 1](#) and [Fig. 4](#).

244 Iron concentrations in B₄ for all ligands and the control treatment were lower than those
 245 in B₃ ([Fig. 3](#)). This might be attributable to the adsorption of additional Fe on the bottom wall
 246 of the test tubes. All sections of the test tubes had a common surrounding wall, while B₄ had a

247 bottom wall in addition to the surrounding wall. Therefore, some of the Fe in B₄ could have
248 adsorbed on this additional surface, resulting in the inconsistent apparent movement of Fe from
249 B₄ to B₃ (Q_{t1}/F) compared to the movement from B₃ to B₂ (Q_{t2}/F) and B₂ to B₁ (Q_{t3}/F) (Table
250 1). In contrast, the Q_{t2}/F and Q_{t3}/F showed a unique and consistent pattern. While the Q_{t3}/F was
251 higher than the Q_{t2}/F in growth medium that lacked chelating ligand, this outcome was
252 reversed in the ligand-treated samples (Fig. 4): the Q_{t2}/F was significantly higher than the Q_{t3}/F
253 in samples treated with chelating ligands. These results suggest that the Q/F of Fe is favored by
254 chelating ligands, and the Fe movement is high across concentration gradients in growth media.

255 The highest Q_{t2}/F values, representing apparent movement of Fe from B₃ to B₂, were
256 0.0103±0.0012 and 0.0116±0.0026 in growth Medium treated with HIDS or EDDS,
257 respectively, followed by GLDA, MGDA, EDTA, and IDS. The same pattern of Q_{t3}/F for Fe
258 was observed with few exceptions (Fig. 4). The coefficients of Fe movement by chelating
259 ligands in the growth Medium would relate to the conditional stability constant of each ligand
260 (LogK_{FeL}). Therefore, the conditional stability constant of the chelating ligand could be an
261 important indicator of Fe bioavailability and movement in growth Medium.

262

263 **Fe uptake and radish growth**

264 The growth of radish sprouts was correlated with the Fe concentration in the plant
265 tissues. The heights of the radish sprouts increased with higher tissue Fe concentrations (Fig. 5).
266 The Fe concentration in the tissues of the radish sprouts was dependant on the chelating
267 ligands, since the Fe was not readily bioavailable under experimental conditions (at pH 10)
268 before the addition of ligands. Compared to the control, the Fe concentration in the sprouts
269 increased with the addition of chelating ligands (Fig. 5). The Fe uptake in radish sprouts was
270 increased by 79% with the addition of HIDS to the growth medium. Other chelating ligands
271 also significantly increased Fe uptake, as follows: 0.4% with IDS, 28% with MGDA, 37% with
272 EDTA, 56% with GLDA, and 58% with EDDS. This increase in Fe uptake by the chelating

273 ligands correlated with radish growth. Compared to the control, the height of the radish sprouts
274 was increased by 34%, 30%, 22%, and 19% with the addition of HIDS, GLDA, EDDS, and
275 EDTA, respectively.

276

277 **Discussions:**

278 **Effect of chelating ligands on Fe uptake in and growth of radish**

279 Although abundant in nature, iron is often unavailable to plants, especially at neutral or
280 alkaline pH, because of the formation of insoluble ferric hydroxide under oxic conditions
281 (Guerinot and Yi, 1994; Robinson et al., 2006). Precipitation of Fe in the rhizosphere may
282 result in an Fe deficiency in the plants and reduce growth. Chelating ligands have been used in
283 agriculture as an additive in micronutrient fertilizers in order to increase Fe bioavailability
284 (Alvarez-Fernandez et al., 2005), and the growth of all organisms is dependent on the
285 acquisition of the proper quantities of trace elements. Iron is an important micronutrient for
286 plants and plays vital roles in respiration, photosynthesis, and many other cellular functions
287 including DNA synthesis, nitrogen fixation, and hormone production (Vert et al., 2002). Ferric
288 ions and their complexes have low solubility in aquatic systems, but they are extensively
289 buffered by chelation (Morel and Hering, 1993), which increases their dissolved concentration.
290 The dissolved concentration of Fe determines its rate of uptake by organisms. Anderson and
291 Morel (1982) observed that the Fe uptake rate in laboratory cultures of the marine diatom
292 *Thalassosira weissflogii* was a unique function of the free ferric ion (Fe^{3+}) concentration and
293 the presence of various chelating ligands. Although the influence of EDTA and EDDS on Fe
294 uptake and plant growth is not new, HIDS is a new biodegradable chelating ligand that shows
295 improved performance in Fe acquisition and plant growth. When researchers, industries or
296 users are looking for environmentally safe and biodegradable chelating ligands that perform
297 well, HIDS would be a good alternative to the environmentally persistent and widely used
298 EDTA.

299

300 Influence of chelating ligands Iron movement in the growth medium

301 Chelating ligands form a soluble Fe-ligand complex (FeL) in the rhizosphere and
302 increase Fe bioavailability and uptake in plants. Therefore, chelating ligands such as EDTA and
303 EDDS have been widely used in agriculture, to increase Fe levels in crops ([Alvarez-Fernandez
304 et al., 2005](#); [Gil-Ortiz and Bautista-Carrascosa, 2004](#); [Hernandezapaolaza et al., 1995](#); [Ignatova
305 et al., 2000](#); [Lucena, 2006](#); [Marques et al., 2008](#)); however, the pattern and efficiency of Fe
306 movement by chelating ligands is poorly understood. The present study elucidates the
307 enhancement of Fe mobility and bioavailability in growth Medium due to the presence of
308 chelating ligands. A unique pattern of Fe movement in the growth Medium was observed after
309 the addition of chelating ligands. This movement of Fe increased Fe concentration in the
310 rhizosphere soils and assisted the uptake of Fe in plants.

311 The movement of Fe in the growth medium is was dependent upon the type of
312 chelating ligands as well as the pH of the medium. Fe movement was several times higher at
313 pH 6 than at pH 10 ([Fig. 2](#)). The stability constant of the Fe-complexing chelating ligands was
314 another important factor that affected Fe movement in the growth medium. Chelating ligands
315 produce soluble FeL complexes ([Alvarez-Fernandez et al., 2005](#); [Bell et al., 2005](#)) and
316 consequently increase bioavailability of Fe. This study hypothesizes that the Fe moves from
317 the deeper rhizosphere to the shallow rhizosphere as a result of its increased bioavailability.

318 Results indicate an apparent movement of Fe from B₃ to B₂ due to the addition of
319 chelating ligands. Some of the Fe also moved from B₂ to box 1 (B₁), the topmost layer of the
320 medium, which initially had no Fe. These results demonstrate that the increase in Fe
321 bioavailability and uptake by chelating ligands is useful not only for desorption and/or
322 solubilization of Fe oxides ([Lucena, 2003](#); [Schwertmann, 1991](#)) but also for movement of Fe
323 from a higher concentration area to a lower concentration area within growth Medium.

324 Fe movement in the growth medium was influenced by the chelating ligand species.

325 Compared to the control, the highest amount of total Fe moved from the bottom layers (B₄ and
326 B₃) to the upper layers (B₂ and B₁) was achieved using EDDS and HIDS, followed by GLDA,
327 EDTA, MGDA, and IDS (Fig. 3). Both EDDS and HIDS are more biodegradable than EDTA
328 (Table 1). Specifically, the biodegradation rate of HIDS is about 22.4% within 72 h. Iron
329 movement from the bottom layer to the upper layer also increased with an increase in ligand
330 exposure time.

331

332 **Conclusions**

333 Iron deficiency in plants is a common phenomenon in areas of calcareous and/or
334 alkaline soils and produces chlorotic symptoms. Many physiological and biochemical aspects
335 of this nutritional disorder have been studied in order to resolve this problem. Synthetic
336 Fe(III)-chelates, such as EDTA and EDDS, are the most common and effective ligands used to
337 increase Fe bioavailability. An important concern, however, is that most of the commercially
338 used chelating ligands are poorly biodegradable and therefore rather persistent in the
339 environment. EDTA, for example, occurs at higher concentrations in European surface waters
340 than any other anthropogenic organic compounds identified. As a result, the development of
341 more effective and easily biodegradable chelating ligands is essential.

342 HIDS is a new chelating ligand with high biodegradability and a high stability constant
343 with Fe³⁺. The present study revealed that the performance of HIDS with respect to Fe
344 movement in growth Medium and radish growth is higher than that of other chelating ligands
345 tested. Thus, HIDS would be a good alternative to EDTA and other poorly biodegradable
346 chelating ligands.

347

348 **Acknowledgements**

349 This research was supported partly by Grants-in-Aid for Scientific Research (21651101
350 and 20-08343) from the Japan Society for the Promotion of Science (JSPS).

351

352 **References**

353 Alvarez-Fernandez, A., Garcia-Marco, S., Lucena, J.J., 2005. Evaluation of synthetic iron(III)-
354 chelates (EDDHA/Fe³⁺, EDDHMA/Fe³⁺ and the novel EDDHSA/Fe³⁺) to correct iron
355 chlorosis. Eur. J. Agron. 22, 119-130.

356 Anderson, M.A., Morel, F.M.M., 1982. The influence of aqueous iron chemistry on the uptake
357 of iron by the coastal diatom *Thalassiosira Weiss-ogii*. Limnol. Oceanogr. 27, 789-813.

358 Aston, S.R., Chester, R., 1973. The influence of suspended particles on the precipitation of iron
359 in natural waters. Estuar. Coast. Mar. Sci. 1, 225-231.

360 Barry, R.C., Schnoor, J.L., Sulzberger, B., Sigg, L., Stumm, W., 1994. Iron oxidation kinetics
361 in an acidic alpine lake. Water Res. 28, 323-333.

362 Batty, L.C., Baker, A.J.M., Wheeler, B.D., Curtis, C.D., 2000. The effect of pH and plaque on
363 the uptake of Cu and Mn in *Phragmites australis* (cav.) trin ex. steudel. An. Bot. 86,
364 647-653.

365 Bell, P.F., Edwards, D.G., Asher, C.J., Kerven, G.L., 2005. Effects of iron complexation and
366 indiscriminate uptake on shoot iron of barley. J. Plant Nutr. 28, 963-979.

367 Bienfait, H.F., 1988. Mechanisms in Fe-efficiency reactions of higher plants. J. Plant Nutr. 11,
368 605-629.

369 Boyer, R.F., Clark, H.M., LaRoche, A.P., 1988. Reduction and release of ferritin Iron by plant
370 phenolics. J. Inorg. Biochem. 32, 171-181.

371 Bucheli-Witschel, M., Thomas Egli, 2001. Environmental fate and microbial degradation of
372 aminopolycarboxylic acids. FEMS Microbiol. Rev. 25, 69-106.

373 Buckhout, T.J., Bell, P.F., Luster, D.G., Chaney, R.L., 1989. Iron-stress induced redox activity
374 in tomato (*Lycopersicon esculentum* Mill.) is localized on the plasma membrane. Plant
375 Physiol. 90, 151-156.

376 Chaney, R.L., 1987. Complexity of iron nutrition: lessons for plant-soil interaction research. J.
377 Plant Nutr. 10, 963-994.

378 Chaney, R.L., Brown, J.C., Tiffin, L.O., 1972. Obligatory reduction of ferric chelates in iron
379 uptake by soybeans. Plant Physiol. 50, 208-213.

380 Christensen, K.K., Sand-Jensen, K., 1998. Precipitated iron and manganese plaques restrict
381 root uptake of phosphorus in *Lobelia dortmanna*. Can. J. Bot. 76, 2158-2163.

382 Claudia, O., Rodríguez, J., 2003. EDTA: The chelating agent under environmental scrutiny.
383 Quim. Nova 26, 901-905.

384 Cohen, C.K., Fox, T.C., Garvin, D.F., Kochian, L.V., 1998. The role of iron-deficiency stress

- 385 responses in stimulating heavy-metal transport in plants. *Plant Physiol.* 116, 1063-1072.
- 386 Evangelou, M.W.H., Ebel, M., Schaeffer, A., 2007. Chelate assisted phytoextraction of heavy
387 metals from soil. Effect, mechanism, toxicity, and fate of chelating agents.
388 *Chemosphere* 68, 989-1003.
- 389 Fox, T.C., Shaff, J.E., Grusak, M.A., Norvell, W.A., Chen, Y., Chaney, R.L., Kochian, L.V.,
390 1996. Direct measurement of ^{59}Fe -labeled Fe^{2+} influx in roots of pea using a chelator
391 buffer system to control free Fe^{2+} in solution. *Plant Physiol.* 111, 93-100.
- 392 Gil-Ortiz, R., Bautista-Carrascosa, I., 2004. Effects of Fe-EDDHA chelate application on
393 evolution of soil extractable iron, copper, manganese, and zinc. *Commun. Soil Sci.*
394 *Plant Anal.* 35, 559-570.
- 395 Guerinot, M.L., Yi, Y., 1994. Iron: Nutritious, noxious, and not readily available. *Plant Physiol.*
396 104, 815-820.
- 397 Helena, H., Orama, M., Saarinen, H., Aksela, R., 2003. Studies on biodegradable chelating
398 ligands: complexation of iminodisuccinic acid (ISA) with Cu(II), Zn(II), Mn(II) and
399 Fe(III) ions in aqueous solution. *Green Chem.* 5, 410 - 414.
- 400 Hernandezapaolaza, L., Garate, A., Lucena, J.J., 1995. Efficacy of commercial Fe(III)-EDDHA
401 and Fe(III)-EDDHMA chelates to supply iron to sunflower and corn seedlings. *J. Plant*
402 *Nutr.* 18, 1209-1223.
- 403 Ignatova, M., Manolova, N., Rashkov, I., Vassileva, V., Ignatov, G., 2000. Remediating the iron-
404 deficient maize plants by new synthetic macromolecular chelating agents. *Plant Soil*
405 227, 27-34.
- 406 Jaworska, J.S., Schowanek, D., Feijtel, T.C.J., 1999. Environmental risk assessment for
407 trisodium [S,S]-ethylene diamine disuccinate, a biodegradable chelator used in
408 detergent applications. *Chemosphere* 38, 3597-3625.
- 409 Kochian, L.V., 1991. Mechanisms of micronutrient uptake and translocation in plants. *Soil*
410 *Science Society of America*, Madison, WI.
- 411 Lucena, J., 2006. Synthetic iron chelates to correct iron deficiency in plants, in: Barton, L.L.,
412 Abadía, J. (Eds.), *Iron Nutrition in Plants and Rhizospheric Microorganisms*. Springer,
413 Dordrecht, The Netherlands, pp 103-128.
- 414 Lucena, J.J., 2003. Fe Chelates for remediation of Fe chlorosis in strategy I plants. *J. Plant Nutr.*
415 26, 1969 - 1984.
- 416 Lucena, J.J., Barak, P., Hernández-Apaolaza, L., 1996. Isocratic ion-pair high-performance
417 liquid chromatographic method for the determination of various iron(III) chelates. *J.*
418 *Chromatogr. A* 727, 253-264.
- 419 Lucena, J.J., Chaney, R.L., 2006. Synthetic iron chelates as substrates of root ferric chelate

- 420 reductase in green stressed cucumber plants. J. Plant Nutr. 29, 423-439.
- 421 Marques, A.P.G.C., Oliveira, R.S., Samardjieva, K.A., Pissarra, J., Rangel, A.O.S.S., Castro,
422 P.M.L., 2008. EDDS and EDTA-enhanced zinc accumulation by *Solanum nigrum*
423 inoculated with arbuscular mycorrhizal fungi grown in contaminated soil.
424 Chemosphere 70, 1002-1014.
- 425 Morel, F.M.M., Hering, J.G., 1993. Principles and applications of aquatic chemistry:
426 Complexation. Wiley, New York, USA.
- 427 Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with
428 tobacco tissue cultures. Physiol. Plant 15, 473-497.
- 429 Nortemann, B., 1999. Biodegradation of EDTA. Appl. Microbiol. Biotechnol. 51, 751-759.
- 430 Nowack, B., Sigg, L., 1997. Dissolution of Fe(III) (hydr) oxides by metal-EDTA complexes.
431 Geochim. Cosmochim. Acta 61, 951-963.
- 432 Otte, M.L., Rozema, j., Koster, l., Haarsma, M.S., Broekman, R.A., 1989. Iron plaque on roots
433 of *Aster tripolium* L.: interaction with zinc uptake. New Phytol. 111, 309-317.
- 434 Robin, A., Vansuyt, G., Hinsinger, P., Meyer, J.M., Briat, J.F., Lemanceau, P., Donald, L.S.,
435 2008. Chapter 4 iron dynamics in the rhizosphere: consequences for plant health and
436 nutrition, Adv. Agron. Academic Press, pp 183-225.
- 437 Robinson, B., Kim, N., Marchetti, M., Moni, C., Schroeter, L., van den Dijssel, C., Milne, G.,
438 Clothier, B., 2006. Arsenic hyperaccumulation by aquatic macrophytes in the Taupo
439 Volcanic Zone, New Zealand. Environ. Exp. Bot. 58, 206-215.
- 440 Romheld, V., 1987. Different strategies for iron acquisition in higher plants. Physiol. Plant 70,
441 231-234.
- 442 Romheld, V., Marschner, H., 1986a. Evidence for a specific uptake system for iron
443 phytosiderophores in roots of grasses. Plant Physiol. 80, 175-180.
- 444 Romheld, V., Marschner, H., 1986b. Mobilization of iron in the rhizosphere of different plant
445 species, in: Tinker, B., Läuchli, A. (Eds.), Adv. Plant Nutr. Praeger Press, New York, pp.
446 155-204.
- 447 Schwertmann, U., 1991. Solubility and dissolution of iron oxides. Plant Soil 130, 1-25.
- 448 Sokubai, N., 2009. Biodegradable chelating agent: HIDS.
- 449 Tagliavini, M., Rombolà, A.D., 2001. Iron deficiency and chlorosis in orchard and vineyard
450 ecosystems. Eur. J. Agron. 15, 71-92.
- 451 Urrestarazu, M., Alvaro, J.E., Moreno, S., Carrasco, G., 2008. Remediation of iron chlorosis by
452 the addition of Fe-o,o-EDDHA in the nutrient solution applied to soilless culture.
453 Hortscience 43, 1434-1436.
- 454 Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M.L., Briat, J.-F., Curie, C.,

- 455 2002. IRT1, an arabidopsis transporter essential for iron uptake from the soil and for
456 plant growth. *Plant Cell* 14, 1223-1233.
- 457 Villen, M., Garcia-Arsuaga, A., Lucena, J.J., 2007. Potential use of biodegradable chelate N-
458 (1,2-dicarboxyethyl)-D,L-aspartic acid/Fe³⁺ as an Fe fertilizer. *J. Agric. Food Chem.* 55,
459 402-407.
- 460 Wenger, K., Tandy, S., Nowack, B., 2005. Effects of chelating agents on trace metal speciation
461 and bioavailability, in: Nowack, B., VanBriesen, J.M. (Eds.), *Biogeochemistry of*
462 *chelating agents; ACS symposium series.* American Chemical Society, Washington DC,
463 pp. 204-224.
- 464 Ye, Z., Baker, A.J.M., Wong, M.-H., Willis, A.J., 1998. Zinc, lead and cadmium accumulation
465 and tolerance in *Typha latifolia* as affected by iron plaque on the root surface. *Aquat.*
466 *Bot.* 61, 55-67.
- 467 Ye, Z.H., Cheung, K.C., Wong, M.H., 2001. Copper uptake in *Typha latifolia* as affected by
468 iron and manganese plaque on the root surface. *Can. J. Bot.* 79, 314-320.
- 469 Yona, C., Phillip, B., Brady, N.C., 1982. Iron nutrition of plants in calcareous soils, *Adv. Agron.*
470 Academic Press, pp 217-240.
- 471 Yousfi, S., Wissal, M.s., Mahmoudi, H., Abdelly, C., Gharsalli, M., 2007. Effect of salt on
472 physiological responses of barley to iron deficiency. *Plant Physiol. Biochem.* 45, 309-
473 314.
- 474 Zancan, S., Suglia, I., La Rocca, N., Ghisi, R., 2008. Effects of UV-B radiation on antioxidant
475 parameters of iron-deficient barley plants. *Environ. Exp. Bot.* 63, 71-79.
- 476 Zhang, X., Zhang, F., Mao, D., 1998. Effect of iron plaque outside roots on nutrient uptake by
477 rice (*Oryza sativa* L.). Zinc uptake by Fe-deficient rice. *Plant Soil* 202, 33-39.
- 478
- 479
- 480
- 481
- 482
- 483
- 484
- 485
- 486

487 Table 1: Apparent mobility of iron in the growth medium affected by Fe-complexing chelating
488 ligands

489

Chelating ligands	$\frac{Q_1}{F}$	$\frac{Q_2}{F}$	$\frac{Q_3}{F}$
Control	0.0716±0.0052	0.0015±0.0002	0.0038±0.0009
EDTA	-0.1432±0.0017	0.0066±0.0004	0.0026±0.0019
HIDS	0.0214±0.0089	0.0103±0.0012	0.0057±0.0001
IDS	0.0169±0.0156	0.0075±0.0018	0.0027±0.0005
MGDA	0.0006±0.0007	0.0058±0.0014	0.0032±0.0009
EDDS	0.0105±0.0081	0.0116±0.0026	0.0034±0.0017
GLDA	-0.0373±0.0845	0.0105±0.0006	0.0052±0.0005

490

491

492

493

494

495

496

497

498

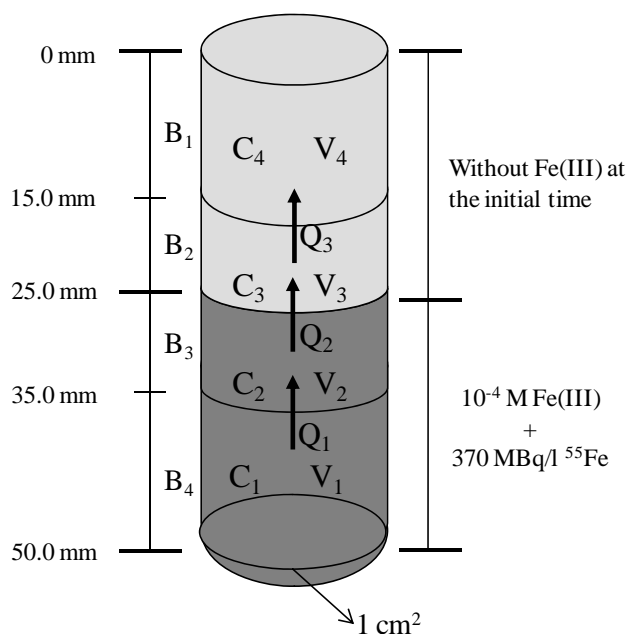
499

500

501

502

503



504

505 Fig. 1: Experimental set up of two-layered culture medium. Initially, the lower layer of the
506 medium contained Fe(III) (10⁻⁴ M) while the upper layer had no Fe. The two-layered
507 medium was divided into four sections and apparent Fe mobility was measured in each
508 section.

509

510

511

512

513

514

515

516

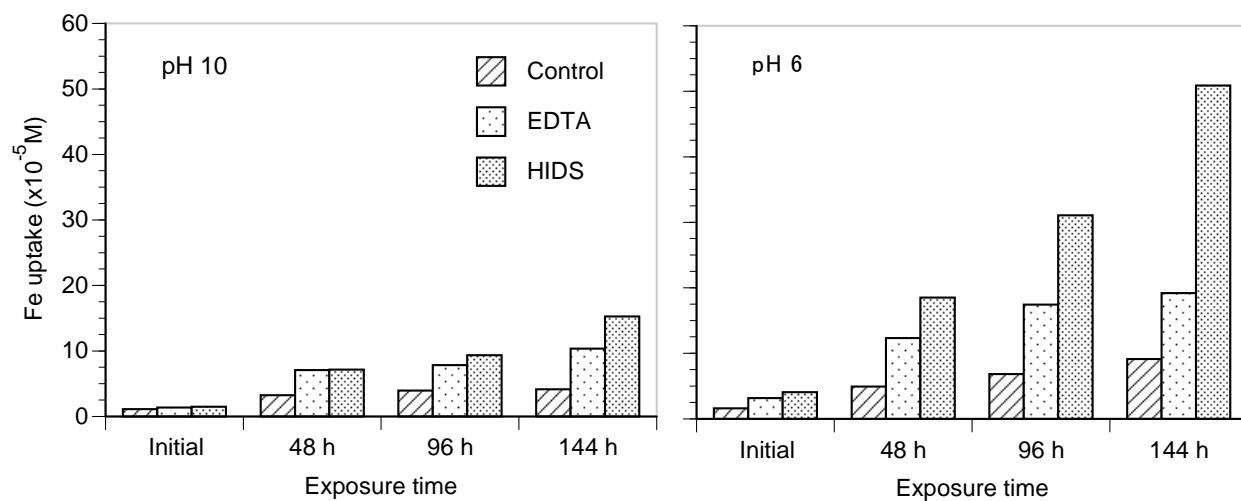
517

518

519

520

521



522

523 Fig. 2: Effect of pH on Fe mobility in the growth Medium.

524

525

526

527

528

529

530

531

532

533

534

535

536

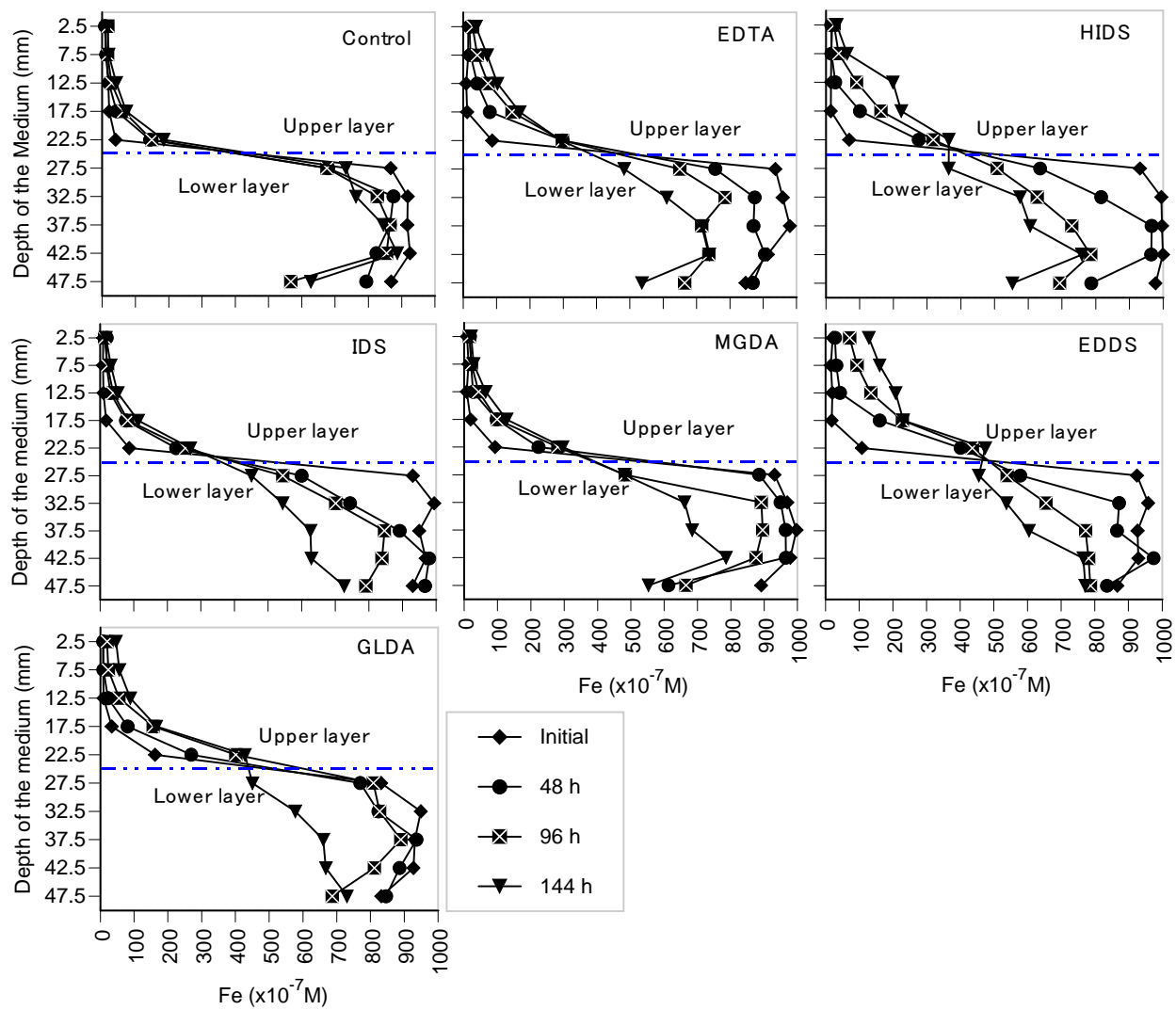
537

538

539

540

541



542

543 Fig. 3: Effect of chelating ligands on Fe movement from lower to upper layers of the culture
 544 medium (pH 10).

545

546

547

548

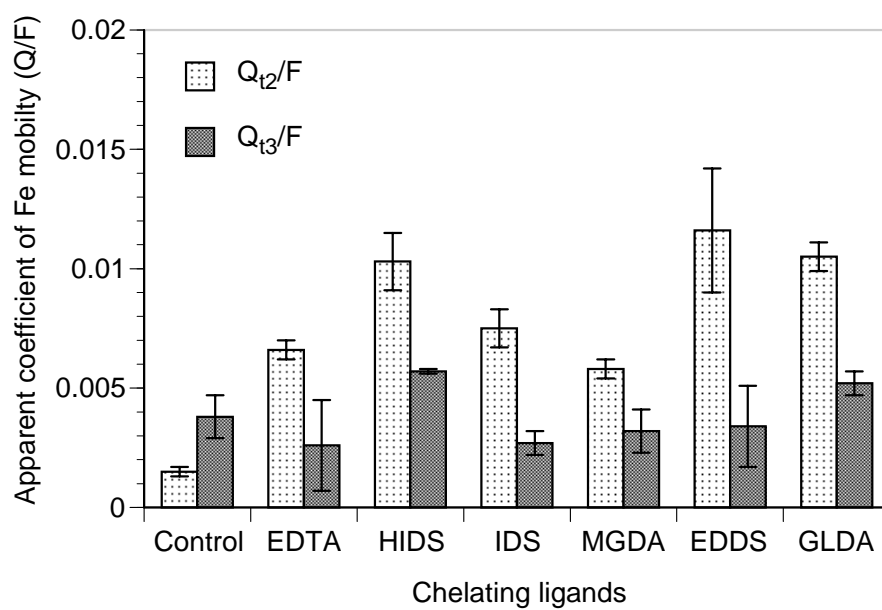
549

550

551

552

553



554

555 Fig. 4: The apparent Fe movement in the growth medium explained by a '4-box' model.

556

557

558

559

560

561

562

563

564

565

566

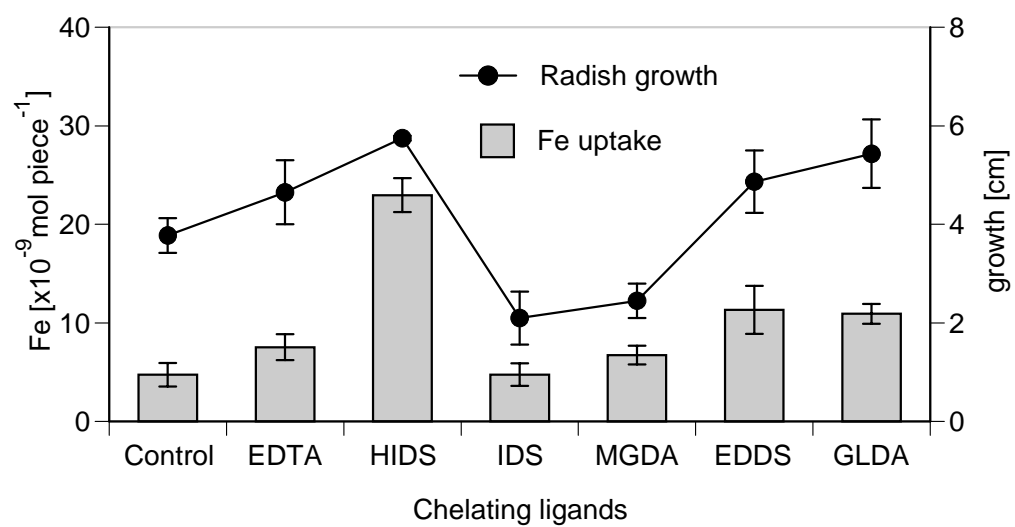
567

568

569

570

571



572

573 Fig. 5: Iron uptake and growth of radish sprouts in Medium with Fe-complexing chelators.

574