Effect of biodegradable chelating ligand on iron bioavailability and radish growth

Effect of Biodegradable Chelating Ligand on Iron Bioavailability and Radish Growth (*Raphanus sativus* L.)

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Abstract:

The effect of chelating ligands on iron uptake and growth of radish (*Raphanus sativus* L.) was investigated. The ethylenediaminetetraacetic acid (EDTA) increased ^{55}Fe uptake in roots of radish though its subsequent translocation from roots to shoots and leaves did not increased. About 70%-80% of the total ^{55}Fe was distributed in the roots while about 5%-15% and 11%-17% were in shoots and leaves, respectively. The EDTA increases iron uptake into the roots of radish, but not in the above ground parts of the plant. The growth of radish (*Raphanus sativus* L.) decreased drastically in alkaline condition (pH > 9), even though the concentration of iron was sufficient in the growth medium. The growth of radish was enhanced successfully by the addition of hydroxyiminodisuccinic acid (HIDS) and EDTA. This might be because HIDS and EDTA solubilize iron from its precipitation with hydroxides at higher pH, and increase iron bioavailability. The influence of EDTA and HIDS on radish growth was comparable. Increase of radish growth by ethylenediaminedisuccinic acid (EDDS) and methylglicinediacetic acid (MGDA) was less than those by EDTA and HIDS. Considering the reproducibility of the radish growth (biomass production) at pH 10, HIDS is supposed to be more effective compared to EDTA.

Keywords: Iron (Fe), Chelating ligands, Radish (*Raphanus sativus* L.), HIDS, Bioavailability.

1. Introduction:

Iron is an essential micronutrient for plants, which plays important roles in respiration, photosynthesis, and many other cellular functions such as DNA synthesis, nitrogen fixation, and hormone production (Vert et al. 2002). Although abundant in nature, iron often is unavailable to plants, especially at neutral or alkaline pH, because it forms insoluble ferric hydroxide complexes in the presence of oxygen (Cohen et al. 1998; Guerinot and Yi 1994). The precipitation of iron hydroxide is also known as iron plaque. Iron plaque formation in the rhizosphere, however, may results iron deficiency to the plants. Plants use two distinct strategies to assimilate iron from the environment. Grasses release low molecular weight and high affinity Fe(III)-chelate compounds called phytosiderophores, which solubilize ferric iron in the rhizosphere and are recognized for uptake by specific membrane transporters (Bienfait 1988; Chaney 1987; Romheld and Marschner 1986). Iron uptake in dicots and nongrass monocots is mediated by a plasma membrane-bound ferric reductase that transfers electrons from intracellular NADH (Buckhout et al. 1989) to Fe(III)-chelates in the rhizosphere (Chaney, Brown, and Tiffin 1972). The ferrous ions (Fe^{2+}) released from the chelates by this process are subsequently transported into the cytoplasm via a separate transport protein (Kochian 1991; Fox et al. 1996).

In iron deficient condition, dicots and nongrass monocots stimulate a number of processes to enhance iron accumulation from the soil. Root-mediated acidification of rhizosphere by iron deficient plants to enhance solubilization of Fe^{3+} from iron hydroxides is an interesting strategy (Chaney, Brown, and Tiffin 1972; Bienfait et al. 1983). In nature, rhizospheric microbes have been reported to exude siderophores to the root-plaque interface. These siderophores solubilize ferric iron in the rhizosphere and are recognized for uptake by specific membrane

receptors and render its phytoavailibility (Bienfait 1988; Chaney 1987; Romheld and Marschner 1986).

Research on the interaction of plants with chelating ligands started in the 1950s with a view to alleviating deficiencies in the essential nutrients Fe, Mn, Cu, and Zn (Wenger, Tandy, and Nowack 2005). EDTA has become very popular to achieve this purpose but has the disadvantage that it is quite persistent in the environment due to its low biodegradability. EDTA also impairs plant growth severely, even at very low concentrations. Therefore, biodegradable chelating ligands could be the best alternatives to EDTA for the increase of iron availability to plants. The biodegradable chelating ligands would solubilize precipitated iron in the rhizosphere without any harmful environmental effects. In this study, we investigated the effects of biodegradable chelating ligands on growth of radish (*Raphanus sativus* L.), and iron uptake by the plant at different pH. Our research approach was to increase iron phytoavailibility using biodegradable chelating ligands, when iron becomes unavailable because of its precipitation with hydroxides.

2. Materials and Methods:

2.1. Experimental setup

Before use in the experiment, seeds of radish were stored in refrigerator at 4˚C. They were sterilized with 0.25% NaClO and 25 µM Tween20 solution for two minutes, and rinsed with 5 ml of deionized water using an E-pure system (Barnstead) for five times. The standard Murashige and Skoog (MS) growth medium was modified using 0 and 10 µM $FeCl_3 \cdot 6H_2O$ for Fe-deficient and -sufficient condition, respectively (Table 1). 10 mM chelating ligand were used in the MS medium before the cultivation of radish. Seeds were grown on 4 ml of modified MS medium in a 14-ml sterilized polystyrene tube. After screening from light for 3 days, the plants

were grown in a growth chamber, where the condition was set as 14:10 h light/dark schedule, 180 μM photon m^{-2} s^{-1} light intensity from cool white fluorescent lights and 20 ºC temperatures. The experiments were run for 2-3 weeks. For the measurement of dry-weight, plant samples were dried at 90 ºC until they reached a constant weight.

2.2. Extraction of extracellular fractions of iron and chemical analysis

Extra and intracellular fractions of iron in radish were determined by radiochemical measurements of ^{55}Fe. For intracellular iron, samples were successively rinsed with 5 ml of deionized water, 5 ml of 0.047 M Ti(III)-citrate-EDTA solution and again with 5 ml of deionized water. For total iron (corresponding to intra and extracellular iron), other samples were rinsed with 5 ml of EPW. Both of the samples, in which ^{55}Fe retained as a tracer, were directly added to 5 ml of liquid scintillation solutions [3.0 g of 2-(4-tert-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole / 500 ml toluene] in 20 ml vials. Radiochemical activity of ^{55}Fe was measured using the tritium mode of the liquid scintillation counter (LSC-6101, Aloka, Japan). The concentration of Fe(III) was calculated from Fe(III)/^{55}Fe(III) ratio in sample solutions.

2.3. Chemicals

Stock solution of Fe(III) was prepared by dissolving $FeCl_3 \cdot 6H_2O$ (Nacalai Tesque, Kyoto) in 1M HCl (TAMAPURE-AA-100, Tama Chemicals, Tokyo) and was standardized by using inductively coupled plasma atomic emission spectrometry (Optima 3300XL, Perkin-Elmer, USA). Stock solution of ^{55}Fe(III) was prepared by dissolving $^{55}FeCl_3$ (PerkinElmer Life & Analytical Sciences, specific activity; >111 GBq/g, 37MBq) in 1M HCl (TAMAPURE-AA-100). They were diluted to the desired concentration ratios of Fe/^{55}Fe. Stock solutions of EDTA, HIDS, IDS, MGDA and EDDS were prepared by dissolving ethylenediamine-N,N,N',N'-tetraacetic acid (Dojindo Molecular Technologies, Japan), tetrasodium 3-hydroxy-2,2’-iminodisuccinate

(Nippon Syokubai, Japan), tetrasodium iminodisuccinate (Bayer), methylglycine-N,N-diacetic acid (BASF) and ethylenediamine-N, N'-disuccinic acid (Chelest) in 0.1 M sodium hydroxide, respectively. Other reagents were of analytical grade or better. All solutions were prepared with deionized water.

3. Results and Discussions

3.1. ^{55}Fe uptake in tissues of radish influenced by EDTA

^{55}Fe uptake in roots of radish seedling was increased by EDTA though its translocation from roots to shoots and leaves was not increased (Fig. 1). About 68%, 15% and 17% of the total iron was accumulated in roots, stems and leaves of radish, respectively, when EDTA was not added to the growth medium. With the addition of EDTA to the growth medium, the amounts changed to 84%, 5% and 11% in roots, stems and leaves, respectively. It is important that iron uptake in roots increased by 22% with the addition of EDTA in the culture medium though iron translocation from roots to stems and leaves decreased by 66% and 33%, respectively after the addition of EDTA to the growth medium. The results elucidate that chelating ligands increase iron uptake into plant's roots, but iron translocation from roots to above ground parts of plants might not be affected by its concentrations in roots. Plants translocate iron from roots to aerial parts according to their needs.

Iron content was higher in leaves of radish compared to that in stems (Fig. 1). It might be due to photosynthesis in leaves of green plants. Green plants translocate most of the iron to the leaves to perform photosynthesis, in which many metabolic processes are activated by iron (Briat et al. 1995; Hendry and Rocklebank 1985; Kampfenkel, Van Montagu, and Inze 1995; Prescott and John 1996; Somers and Shive 1942), and iron itself is a prosthetic group of many enzymes (Janneke and Stéphane 2005; Knaff 2004). Iron is an essential micronutrient for plants, which

plays important roles in respiration, photosynthesis, and many other cellular functions such as DNA synthesis, nitrogen fixation, and hormone production (Vert et al. 2002).

3.2. Radish growth affected by Fe at different pH

Radish (*Raphanus sativus* L.) was grown with and without Fe(III) at different pH ranged between 7 and 10. Biomass productions of radish were almost constant in the pH range of 7-9 in the nutrient medium. However, biomass production of the plant was about 62-73% higher in solutions with 10 µM iron than those without iron (Fig. 2). At pH 10, radish growth decreased drastically. The dry biomass of the plant was 14.04±7.92 mg and 12.52±5.50 mg in nutrient solution with iron and without iron, respectively. The result implies that the influence of iron on plant growth is directly related to the pH of the growth medium.

Iron plays important roles in photosynthesis in plants (Vert et al. 2002). Although iron is sufficient in growth medium, its availability is mostly dependent on pH. In alkaline pH condition iron forms insoluble ferric hydroxide complexes in the presence of oxygen (Cohen et al. 1998). Iron deficiency results chlorosis in green leaves, which retards plant growth, and leads to the reduction of crop yields (Guerinot and Yi 1994). The results of the present study also showed that radish growth decreased drastically at higher pH, which might be the consequence of iron chlorosis.

3.3. Influence of chelating ligands and pH on radish growth

The influence of chelating ligands and pH of the growth medium on biomass production of radish was studied. Results show that biomass production decreased drastically at pH 10, when chelating ligands were not applied (Fig. 3). This was due to the drastic reduction of iron availability to the plant because of insoluble ferric hydroxide formation. The depletion in

biomass production was enhanced up to 29% and 31% by the addition of HIDS and EDTA in the growth medium, respectively. However, EDDS was ineffective in the increase of biomass production of the plant (Fig. 3). The result suggests that the efficiency of HIDS in the increase of iron phytoavailibility at higher pH is comparable with that of EDTA. Thus, HIDS, a biodegradable chelating ligand, would be alternative to environmentally persistent and non-biodegradable EDTA.

Biomass production of radish was about 10-31% worse compared to the control treatments (without chelating ligands) even after the addition of chelating ligands in the growth medium (Fig. 3). The highest depletion (52%) in biomass was observed in EDDS treated radish at pH 9. It can be concluded from the results that chelating ligands are effective in the increase of plant growth at higher pH (>10). The use of chelating ligands at lower pH (<9) would produce negative results, as observed in the present study.

Radish was also grown at pH 10, with and without chelating ligands and iron (Fig. 4). Results show that plant biomass was lowest (13.00±5.50 mg), when both iron and ligand were not applied to the medium. With the addition of 10 µM of Fe(III), biomass of EDDS and MGDA treated radish did not differ from the control treatment (without chelating ligand). The plant biomass production was increased by 17%, 29%, and 31% with the addition of IDS, HIDS, and EDTA, respectively (Fig. 4). Considering the reproducibility with the smaller standard deviation, HIDS appears to be better chelating ligand for the increase of plant growth.

3.4. Effect of chelating ligands on height and fresh biomass production of radish

Radish (*Raphanus sativus* L.) was grown in alkaline growth medium (pH 10) containing 10 mM and 10 µM of chelating ligands and Fe(III), respectively. At this pH, Fe(III) becomes

precipitated, and iron phytoavailibility decreased significantly. In the present study, it was observed that chelating ligands increased iron uptake into radish plant, which results in the increase of the plant growth (biomass production) (Fig. 4). It was also observed that some chelating ligands increased plant height compared to the control one (Fig. 5). It might be due to the increase of iron uptake by chelating ligands.

Plant height is an important parameter of growth. Auxin and gibberellin are mainly responsible for the cell elongation leading to the increase of plant height (Rayle and Cleland 1992; Yang, Davies, and Reid 1996). Although information about the direct role of iron in the plant cell elongation is limited, iron might play important role in plant elongation as it is involved in many metabolic processes of plant (Hendry and Rocklebank 1985; Kampfenkel, Van Montagu, and Inze 1995; Vert et al. 2002), and as it is a prosthetic group of many enzymes (Janneke and Stéphane 2005; Knaff 2004).

5. Conclusion:

The use of chelating ligands, especially EDTA, has been widely applied from long time for the increase of iron phytoavailibility. EDTA used for this purpose for long time. Recently, leaching of metals due to the huge application of EDTA in the crop fields, and the non-biodegradability of EDTA raise the question whether this chelating ligand would be used any more or not. Therefore, ascertain of an effective substitute of EDTA with biodegradable characteristics comes in the focus of scientific community in this field. The present study was initiated keeping this point in mind. This article describes the preliminary findings of the study. From our study, we propose HIDS as the best alternative to EDTA for the increase of iron phytoavailibility at alkaline pH. More investigation is needed to establish this proposal.

6. Acknowledgements:

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296 Table 1: Composition of modified Murashige and Skoog (MS) medium used for radish
297 (*Raphanus sativus* L.) growth.
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Compounds	(mg l^{-1})
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2SO_4	170
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Thiamine hydrochloride	15
Nicotinic acid	25
Pyridoxine hydrochloride	0.25
Sucrose	30000
Agar	18000

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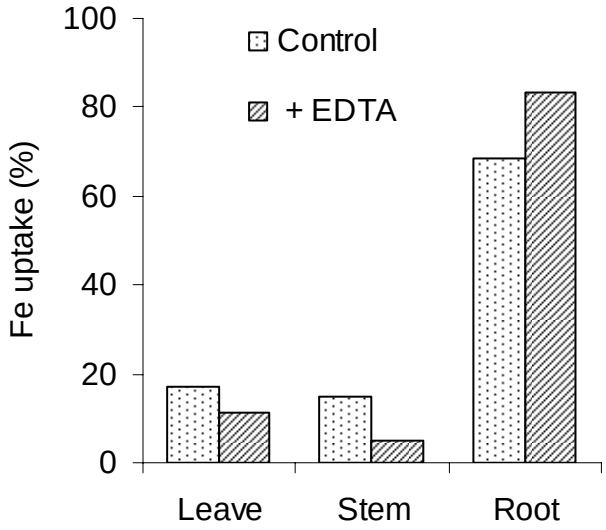


Fig.1: Effect of EDTA on iron (^{55}Fe) uptake in different parts of radish (*Raphanus sativus* L.).

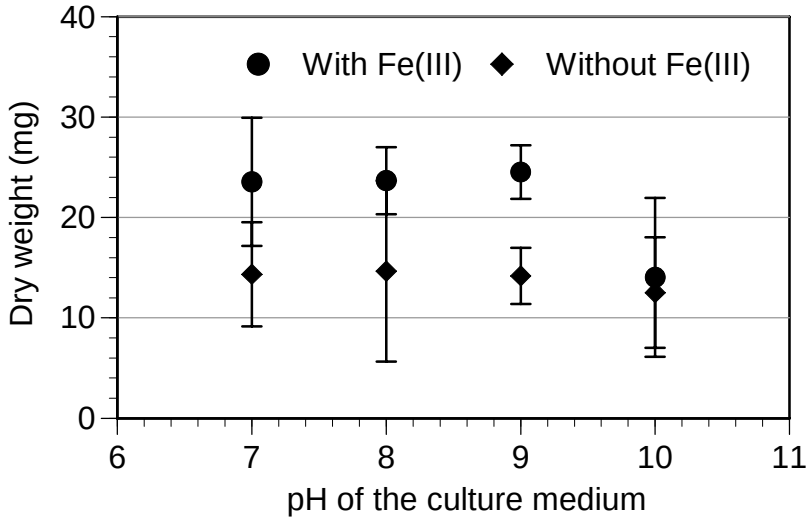


Fig. 2: Growth of radish (*Raphanus sativus* L.) affected by iron at different pH.

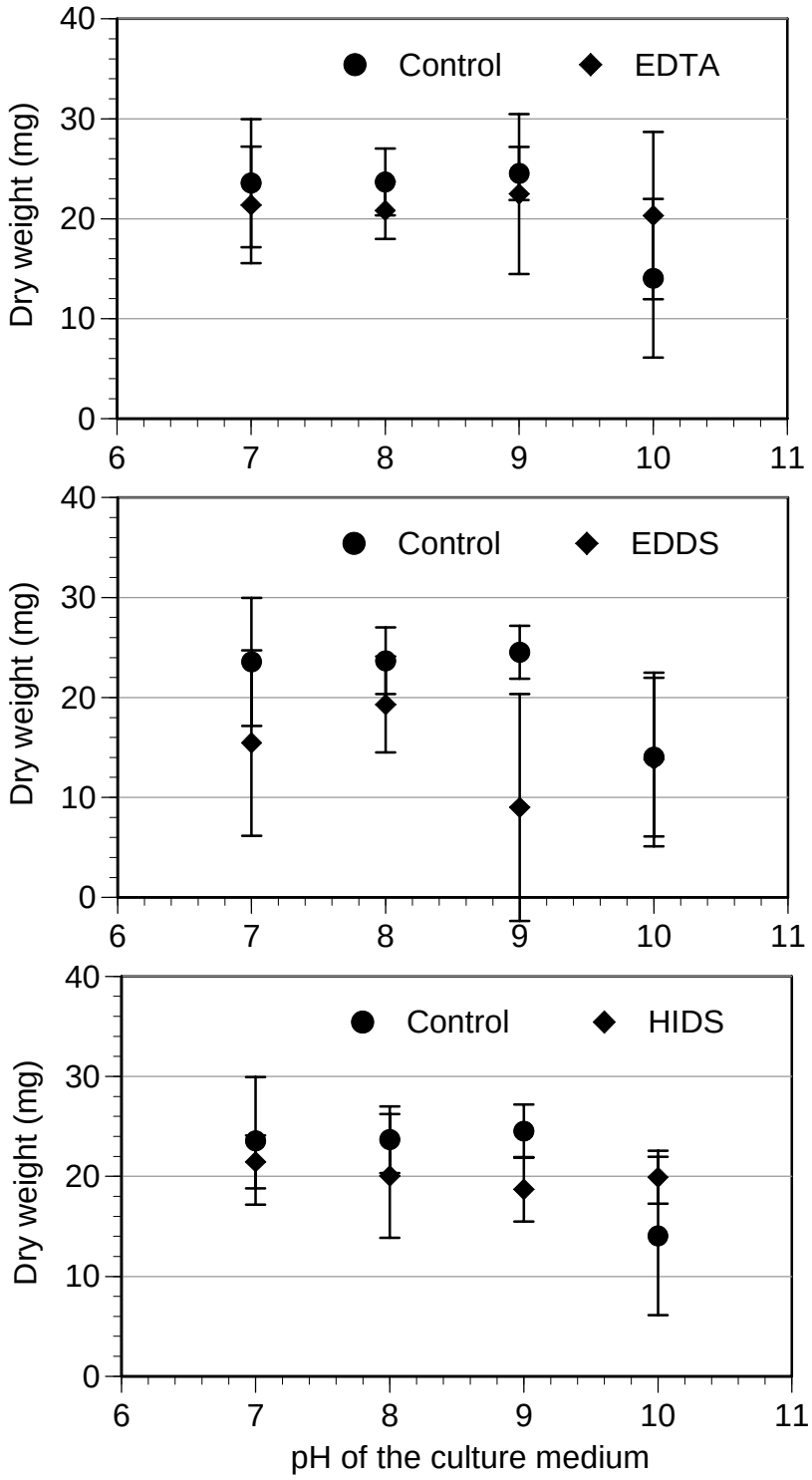
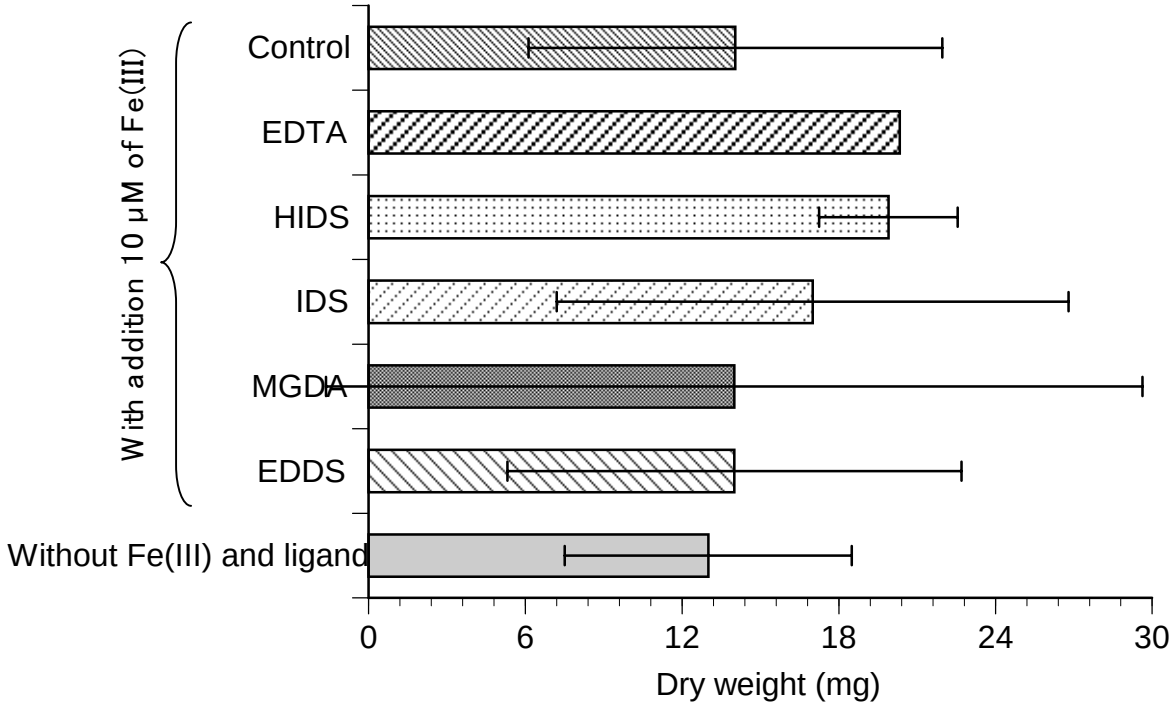


Fig. 3: Effect of chelating ligands on radish (*Raphanus sativus* L.) growth. Concentrations of chelating ligands (EDTA, EDDS, and HIDS) and Fe(III) in growth medium were 10 mM and 10 µM, respectively.



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344 Fig. 4: Effects of chelating ligands and Fe(III) on growth of radish (*Raphanus sativus* L.) in

345 alkaline condition (pH 10).

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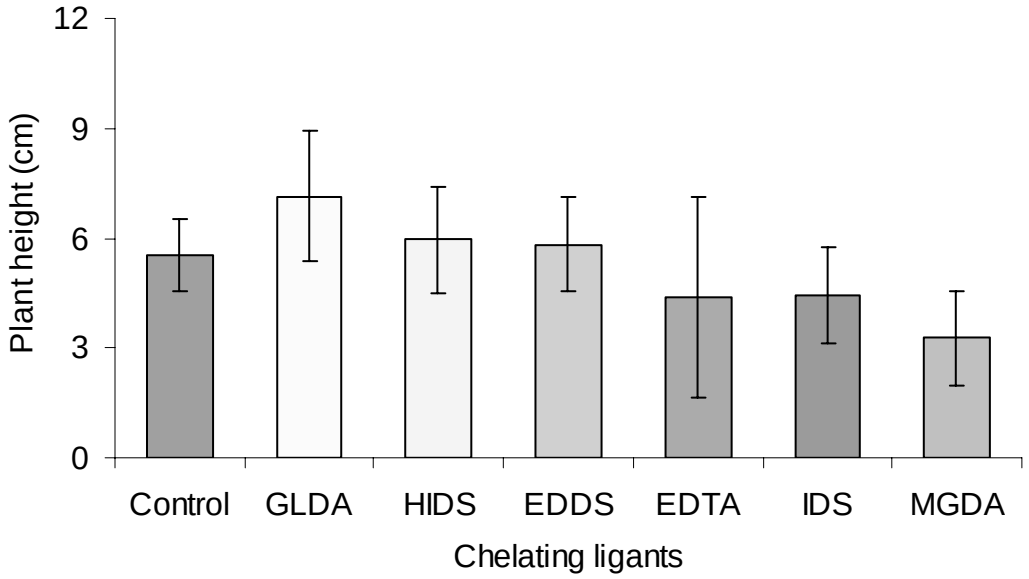

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359 Fig. 5: Effect of chelating ligands on height of radish (*Raphanus sativus* L.) in alkaline condition
360 (pH 10). Concentrations of chelating ligands and Fe(III) in growth medium were 10 mM and 10
361 µM, respectively.

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