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Influence of Chelating Ligands on Arsenic Uptake by Hydroponically Grown Rice Seedling (*Oryza sativa* L.): A Preliminary Study

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Abstract:

Ferric (oxyhydro-)oxides (FeO_x) precipitate in the rhizosphere at neutral or alkaline pH and adsorbed on plant's root surfaces. Consequently, the higher binding affinity of arsenate to FeO_x and low iron phytoavailibility of the precipitated FeO_x makes the phytoremediation of arsenic difficult. In the present study, the influence of chelating ligands on arsenic and iron uptake by hydroponically grown rice seedlings (*Oryza sativa* L.) was investigated. When chelating ligands were not treated to the growth medium, about 63% and 71% of the total arsenic and iron were distributed in root-extract (outer root surfaces) of rice, respectively. On the other hand, Ethylenediaminetetraacetic acid (EDTA), ethylenediaminedisuccinic acid (EDDS) and hydroxyiminodisuccinic acid (HIDS) desorbed a significant amount of arsenic from FeO_x of outer root surfaces. Therefore, the uptake of arsenic and iron into the roots and their subsequent translocation to the shoots of rice seedling increased significantly. The order of increasing arsenic uptake by chelating ligands was; HIDS > EDTA > EDDS. Methylglicinediacetic acid (MGDA) and iminodisuccinic acid (IDS) might not be effective in arsenic solubilization from FeO_x. The results suggest that EDDS and HIDS would be a good and environmentally safe choice to accelerate arsenic phytoavailibility in phytoremediation process because of their biodegradability and would be competent alternative to the widely used non-biodegradable and environmentally persistent EDTA.

1. Introduction:

A large number of sites worldwide are contaminated by arsenic from natural and anthropogenic sources [1, 2]. Elevated levels of arsenic in soil poses a major threat to plant and human health and environment [3-7]. Arsenic enters into the food chains from contaminated agricultural soil and water and affects human health [8-12]. Therefore, remediation of arsenic contaminated soil and water is an important concern. Due to some unavoidable technical and environmental limitations, the traditional remediation technologies lost economic and public acceptance. During the 1980s, the USA government initiated a large scale program for the development of environmental clean-up technologies, which has accelerated the growth of new productive research field. As a result, phytoremediation, a plant based green technology, received huge attention from scientific community for its low cost of implementation and environmental benefits [3, 12-15].

Researchers have come to realize that the development of phytoremediation technologies requires a thorough understanding of the underlying processes at the genetic, molecular, biochemical, physiological and agronomic levels. Some intensive researches have been done on arsenic uptake mechanisms in plants [14, 16-20]. Plants accumulate arsenic primarily into its roots through phosphate uptake pathway i.e., active apoplastic or symplastic mechanisms [18] and subsequently translocated to the above ground parts. The amount of arsenic translocated from roots to shoots indicates the phytoremediation efficiency of the plant. Although few terrestrial plant species such as *Agrostis castellana*; *Agrostis delicatula*, *Bidens cynapiifolia*, and silver fern (*Pityrogramma calomelanos* L.) have been reported to translocate a considerable amount of arsenic from roots to shoots and are regarded as arsenic hyperaccumulators [4, 21], Chinese brake fern (*Pteris vittata* L.) accumulates a formidable amount of arsenic and translocate from root to the shoot [4, 22-24].

The solubility and phytoavailibility of arsenic becomes limited by adsorption to variable charged minerals (Fe and Al) at alkaline pH [25]. An essential requisition for phytoremediation

of contaminated soil is solubilization of the arsenic. Therefore, researchers are concentrating their efforts to increase the solubility and phytoavailibility of arsenic, and its subsequent translocation from roots to shoots. In the past decade, chelant-enhanced phytoremediation has received much attention of scientific community. This technique aims to cleanse arsenic polluted soils by solubilizing arsenic, allowing it to be accumulated in plants that would subsequently remove arsenic from the site. Publications about chelant-enhanced phytoremediation have increased steadily to about 15-20 per year in the last few years, indicating that this is a growing and active research field [26].

Oxygenation of the rhizosphere by wetland plants leads to the precipitation of iron hydroxides in the rhizosphere and on the roots of the plant. The precipitation of iron hydroxide is also known as “iron plaque”. Iron plaque formation in the rhizosphere, however, may results in iron deficiency to plants. It is especially common in soils of neutral or alkaline pH [27]. In nature, plant roots or rhizospheric microbes exude phytosiderophores or siderophores to the root-plaque interface, respectively. These siderophores solubilize ferric iron in the rhizosphere and are recognized for uptake by specific membrane receptors and render its phytoavailibility [28-30]. Research on the interaction of plants with chelating ligands started in the 1950s with a view to reduce the deficiencies of the essential nutrients Fe, Mn, Cu, and Zn [31]. EDTA has been very popular to achieve this purpose, but has the disadvantage that it is quite persistent in the environment because of its low biodegradability. This, in combination with its high affinity for heavy metal complexation, results in an increased risk of leaching. EDTA also impairs plant growth severely, even at very low concentrations [32]. In some cases, the non-biodegradable chelating ligands are toxic to the plants.

Reports on arsenic phytoextraction by biodegradable chelating ligands is limited though a number of investigations have been conducted on chelant-enhanced phytoextraction of Pb, Zn, Hg, Cu and some other heavy metals [33-37]. Biodegradable chelating ligands such as EDDS,

HIDS, MGDA, IDS would be good choice and alternative to EDTA. In the present study, we investigated the synergistic influence of these biodegradable chelating ligands on the increase of iron bioavailability and arsenic phytoextraction. Our research approach is to increase arsenic and iron availability to the plant using biodegradable chelating ligands.

2. Materials and Methods:

2.1. Seed Sterilization

Rice (*Oryza sativa* L.) seeds of BRRI hybrid dhan1 were collected from Bangladesh Rice Research Institute (BRRI). The seeds were surface-sterilized before using them in the experiment. For sterilization, about 100 g seeds were put into 200ml of 1% methyl-1-butylcarbamoyl-2-benzimidazole carbonate for 10 min. After that, the seeds were washed by deionized (DI) water (using an E-pure system (Barnstead)) and put into ID water of 20 ºC for 24 h. The seeds were then transferred into DI water of 45 ºC for 2 min. and of 52 ºC for 10 min.

2.2. Chemicals

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 DI water.

2.3. Nutrient Solution

Sterilized rice seeds were germinated on pre-sterilized bloating paper with standard murashige and skoog (MS) pre-experimental solution (Table 1). Rice seedlings were grown on seed bed (pre-sterilized bloating paper) for two weeks. To prepare the MS solutions, $FeSO_4 \cdot 7H_2O$ was used instead of NaFe(III)-EDTA.

2.4. Experimental Setup:

The plants were transferred into the experimental solution (Table 1) after 3 weeks of growth in pre-experimental solution at pH 6.5. Arsenate ($Na_2HAsO_4 \cdot 7H_2O$) and chelating ligands (EDTA, EDDS, HIDS, MGDS and IDS) concentrations in the experimental solutions were 6 µM and 500 µM, respectively. In experimental solution, iron concentration was increased to 500 µM. The pH of the experimental solution was adjusted to 10 using 0.1 M KOH. About 100 ml solution was taken into 250-ml polystylene bottles and three uniform seedlings were cultivated in each bottle with three replication. The experiment was performed following randomized design (RD). Rice plants were grown in a growth chamber and the conditions in the chamber were set as 14:10 h light/dark schedule, 100-125 µ Em^{-2} s^{-1} light intensity, 22(±2) ºC temperatures. Solutions of each bottle were changed in every 4 days throughout the experiment. Plants were grown in experimental solution for a total of 10 days.

2.5. CBE-Extraction of Fe-plaques

At harvest, the shoots were cut from 1 cm above the roots and separated. Iron plaques from root surfaces were extracted using citrate-bicarbonate-ethylenediaminetetraacetate (CBE)-technique, a modified method of dithionite-citrate-bicarbonate (DCB)-extraction by Taylor and Crowder [38] and Otte et al. [39]. The CBE solution was prepared from 0.03, 0.125 and 0.050 M of sodium citrate, sodium bicarbonate and EDTA, respectively. Roots were treated with 30 ml of

CBE solution for 60 min. at room temperature. The roots were then rinsed with DI water for 3 times, and the rinsed water was added to the CBE-extracts to make a total of 20 ml.

2.6. Sample Preparation

After rinsing with DI water for four times, the root samples were kept on clean absorbent paper to remove the water from the root surfaces. Both root and shoot samples were dried at 65 ºC until they reached a constant weight. Then, 0.10-0.20 g of dried sample was taken into 50-ml polyethylene tubes (*Digi*Tubes, SCP Science, Canada) for digestion. Five ml of 65% HNO_3 were added to the sample and then, left to incubate for 12 hours. The samples were heated on a heating block (*Digi*PREP, SCP Science, Canada) at 95 ºC for 2 hours. After cooling to room temperature, 3 ml of 30% hydrogen peroxide were added and the samples were heated again at 105 ºC for 20 min. Then, the digests were diluted to 10 ml with DI water and taken into 15-ml polyethylene bottles (HDPE, NALGENE®, Nalge Nunc International, Rochester, NY) in readiness for analysis.

2.7. Chemical Analysis

Arsenic and iron were analyzed using graphite-furnace atomic absorption spectrometer (GF-AAS, Z-8100, Hitachi, Japan). For the determination of arsenic, 5 µL of 0.05 M nickel nitrate was added to a 10-µL sample into the cuvette as matrix modifier. Certified standard reference material 1573a (tomato leaf from NIST, USA) was used to check the accuracy of analysis. Arsenic concentration in certified standard reference materials was 0.112±0.004 µg g^{-1} while the measured concentration was 0.111±0.002 µg g^{-1}. The concentrations detected in all samples were above the instrumental limits of detection (≥ 0.01 µM in water sample).

All chemical reagents used in this experiment were of analytical grade. Glassware and dishes were washed with detergent solution and rinsed with DI water for eight times before use. In each analytical batch, at least two reagent blanks and three replicate samples were included.

2.8. Data Analysis

Elemental concentrations in CBE-extracts and plant tissues (roots and shoots) were calculated on dry weight basis. Arsenic and iron concentrations in CEB-extract, root and shoot were calculated as follows:

$$\boldsymbol{T}_{\mathrm{As}} = \boldsymbol{T}_{\text{CBE-extract-As}} + \boldsymbol{T}_{\text{Root-As}} + \boldsymbol{T}_{\text{Shoot-As}}$$

$$\%\ \text{CBE-As} = (\boldsymbol{T}_{\text{CBE-extract-As}} / \boldsymbol{T}_{\mathrm{As}}) \times 100$$

$$\%\ \text{Root-As} = (\boldsymbol{T}_{\text{Root-As}} / \boldsymbol{T}_{\mathrm{As}}) \times 100$$

$$\%\ \text{Shoot-As} = (\boldsymbol{T}_{\text{Shoot-As}} / \boldsymbol{T}_{\mathrm{As}}) \times 100$$

, where $\boldsymbol{T}_{\mathrm{As}}$ means the total arsenic uptake in rice. The $\boldsymbol{T}_{\text{CBE-extract-As}}$, $\boldsymbol{T}_{\text{Root-As}}$ and $\boldsymbol{T}_{\text{Shoot-As}}$ are the total arsenic content in CBE-extract, roots and shoots, respectively.

2.9. Statistical Analysis

The data were subjected to analysis of variance (ANOVA) according to the Duncan Multiple Range Test (DMRT) using SPSS statistical package.

3. Results and Discussions

3.1. As adsorption on ferric (oxyhydro-)oxides of rice roots

Ferric (oxyhydro-)oxides precipitated on root surface of hydroponically grown rice seedlings at higher concentration of iron (500 μM) in the culture solution. A brownish coating was appeared clearly around the root surfaces. High concentration of iron (44.40±2.33 μM g^{-1} dry weight) in the CBE-extract of roots reveals the formation of iron plaque on the root surfaces of rice seedlings (Fig. 1). About 71% of the total iron in rice seedling was distributed to the CBE-extract of roots, whereas 26% and 3% of the total iron were in rice roots and shoots,

respectively (Fig. 2). Formation of iron hydroxides on the roots of wetland plants [38, 40] and hydroponically grown rice seedling [41, 42] have also been reported in literatures. Both in natural conditions and laboratory cultures, the precipitation of ferric (oxyhydro-)oxides (FeO_x) and its association with phytoplankton surfaces has been reported [43]. Robinson et al. [20] found the existence of iron plaque on aquatic macrophytes collected from the Taupo Volcanic zone, New Zealand.

The precipitated FeO_x on plant's root surfaces adsorbs trace elements. Zhang et al. [44, 45] reported that the iron plaque on rice roots could accumulate Zn and P from the growth medium. Ye et al. [46, 47] also observed significantly higher concentrations of Cu on the root surface of *Typha latifilia* grown with iron plaque compared to those without iron plaque. Liu et al. [42] found significant correlation between arsenic concentrations on the root surface of hydroponically grown rice seedlings and the amount of iron plaque on their roots. Field investigation showed that arsenic concentration in DCB-extracts of *Aster tripolium* in flooded treatment was about 40-times higher than that in the aerated treatment [48]. The influence of iron plaque formation around the plant's root surfaces on arsenic uptake is important because of stronger adsorptive affinity of arsenic for iron hydroxides. When rice seedlings were grown in solution without chelating ligands, arsenic in CBE-extracts, which was assumed to be adsorbed on iron plaque, accounted up to 62% of the total arsenic in rice seedlings (Fig. 3). Liu et al. [42] reported that about 75-89% of the total arsenic was adsorbed on iron plaque of rice seedlings. However, reports show that arsenic concentrations on iron plaque of rice root surfaces are much higher than those reported for Cu and Ni in *Typha latifilia* [49]. This might be because the sequestration capacity of iron plaque differed between cations and anions [42].

3.2. Desorption of As from ferric (oxyhydro-)oxides by chelating ligants

Chelating ligands solubilize arsenic from iron plaque of rice root surfaces and increase its uptake in plant tissue. Arsenic concentration in CBE-extract of rice roots without chelating ligands was 0.24±0.05 μmol g^{-1} dry weight, which was decreased by 30% to 50% with the addition of chelating ligands in the culture solutions (Fig. 3). Most of the arsenic (about 62%) was distributed to the iron plaque of rice root surfaces when rice seedlings were grown in solutions without chelating ligands. However, the increase of arsenic concentrations into the roots of rice seedlings were accounted to 51%, 49% and 43% by the addition of EDTA, HIDS and EDDS to the culture solutions, respectively. On the other hand, percent distribution of arsenic in roots, shoots and root extracts (CBE-extracts) of rice seedling show that the solubilizing ability of arsenic by MGDA and IDS was negligible (Fig. 4). Even though MGDA and IDS were applied to the culture solutions, arsenic concentrations in roots and CBE-extracts of roots were about 23% and 65%; 30% and 55%, respectively. The results indicate that HIDS and EDDS could be good alternatives to non-biodegradable chelating ligand (EDTA) in solubilizing arsenic from iron plaque of plant's root surfaces.

3.3. Influence of chelating ligands on As uptake in roots and its translocation to shoots

Despite the fact that the highest arsenic accumulation in rice seedling was 0.38±0.08 μM g^{-1} dry weight without chelating ligands, about 62% was distributed on the root surface (CBE-extract), which is supposed to be associated with FeO_x (Fig. 4). In the present study, it was observed that EDTA, HIDS and EDDS increased the uptake of arsenic into the roots and shoots of rice seedlings. On the other hand, arsenic uptake was not increased by MGDA and IDS (Fig. 3). Results also demonstrate that arsenic concentrations in CBE-extracts of rice roots decreased with the increase of its concentrations in roots and shoots. The increased amount of arsenic in roots and shoots is supposed to be accumulated from iron plaque, which was solubilized by

EDTA, HIDS and EDDS. Therefore, rice seedlings uptake more arsenic into their roots and translocated it to the shoots.

Although iron plaque inhibits the uptake of toxic metals in plants [42, 44, 49, 50], it has also been reported as a pool that increases the uptake of toxic and nutrient elements [45, 46]. The effects of iron plaque on the uptake of nutrient and/or toxic elements depend on the amount of iron plaque on root surfaces [39, 44, 45]. Otte et al. [39] observed that Zn concentrations in roots of *Aster tripolium* L. were significantly higher in roots having 500-2000 nmol Fe cm^{-2} on the root surface compared to those having less than 500 or more than 2000 nmol Fe cm^{-2}. However, Liu et al. [42] demonstrated that even though the increasing amount of iron plaque increased arsenic accumulation on the root surface, they did not affect its uptake in rice shoots. Liu et al. [42] also suggested that iron plaque might act as a "buffer" to prevent the translocation of arsenic from roots to shoots.

Chelating ligands have been used successfully in chemically induced phytoremediation technology to increase the uptake of toxic elements to the above ground parts of plants. The result of the present study demonstrates that EDTA, HIDS and EDDS increase arsenic uptake into the roots of rice and its translocation from roots to shoots. Presently, the lower biodegradability and persistency of EDTA in the environment prevent its use and acceptability worldwide [51]. The results of the present study show that the efficiency of HIDS and EDDS in the increase of arsenic uptake by into rice roots and its translocation to the shoots is comparable to that of EDTA. Therefore, HIDS and EDDS could be an alternative to EDTA as HIDS and EDDS are biodegradable and non-persistent to the environment.

3.4. Influence of chelating ligands on iron uptake in roots and its translocation to shoots

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 [52]. 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 [28, 53]. Iron plaque formation in the rhizosphere, however, may results iron deficiency to the plants.

Chelating ligands, especially EDTA, have been widely used in agriculture as an additive in micronutrient fertilizers worldwide [55]. Biodegradable chelating ligands have been proposed as the alternative to EDTA because of its low biodegradability and risk of leaching. In the present study, about 71% iron was observed to be distributed in CBE-extract of rice roots in treatments without chelating ligands. After the application of EDTA, HIDS and EDDS to the culture solution, iron concentrations in CBE-extract of rice roots decreased by 38%, 36% and 27%, respectively (Fig. 2). The result suggests that the effectiveness of HIDS and EDDS in increasing iron uptake into the rice roots is comparable with that of EDTA. Although EDTA, HIDS and EDDS increased iron uptake into rice roots, the chelating ligands did not increase its translocation from roots to shoots (Fig. 2).

4. Concluding Remarks:

The use of chelating ligands in the phytoextraction of toxic metals and in the increase of essential nutrient elements is not new at all. Especially, the EDTA has been widely used in agriculture for the above purposes. The use of EDTA, however, has the disadvantage that it is quite persistent in the environment due to its low biodegradability. Therefore, biodegradable chelating ligands have been proposed as alternatives to EDTA and other non-biodegradable chelating ligands. But, do the biodegradable chelating ligands are efficient to achieve those purposes? In this preliminary study, HIDS and EDDS are supposed to be effective alternative to EDTA because the uptake efficiency of arsenic and iron in rice seedlings by these two

biodegradable chelating ligands are comparable with that of EDTA. More intensive investigations are needed to confirm the efficacy of HIDS and EDDS.

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Table 1: Composition of Murashige and Skoog (MS) pre-experimental and experimental solutions used for the hydroponic culture of rice seedling (*Oryza sativa* L.)*

Nutrients	Pre-experimental solution (mg l^{-1})	Experimental solution (mg l^{-1})
KNO_3	1900	1900

NH_4NO_3	1650	1650
$CaCl_2 \cdot 2H_2O$	440	440
$MgSO_4 \cdot 7H_2O$	370	370
K_2HPO_4	170	170
$FeSO_4 \cdot 7H_2O$	27.8	500**
$MnSO_4 \cdot 5H_2O$	22.3	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025
$Na_2HAsO_4 \cdot 7H_2O$	-	6.0 (μM)
Chelating ligands	-	500 (μM)
pH	6.50	10.0

* The pre-experimental solution was used to grow the rice seedlings prior to the uptake experiment, and the experimental solution was used for the uptake experiment. Arsenic and chelating ligands were added only to the experimental solution.

** Iron concentration in the experimental solution was modified to 500 μM.

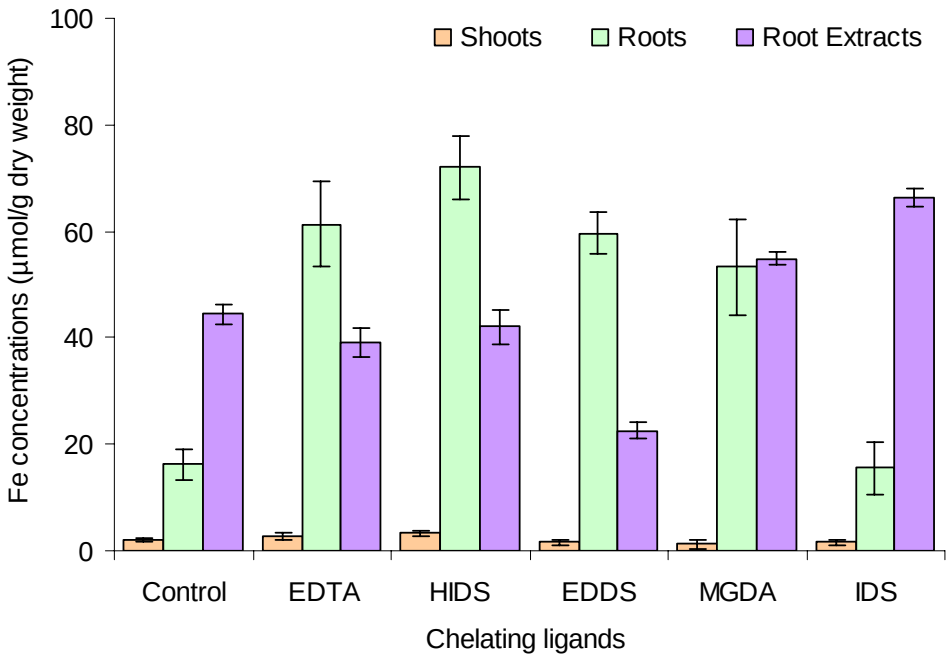

Fig. 1: Influence of chelating ligands on iron uptake by rice seedling (*Oryza sativa* L.).

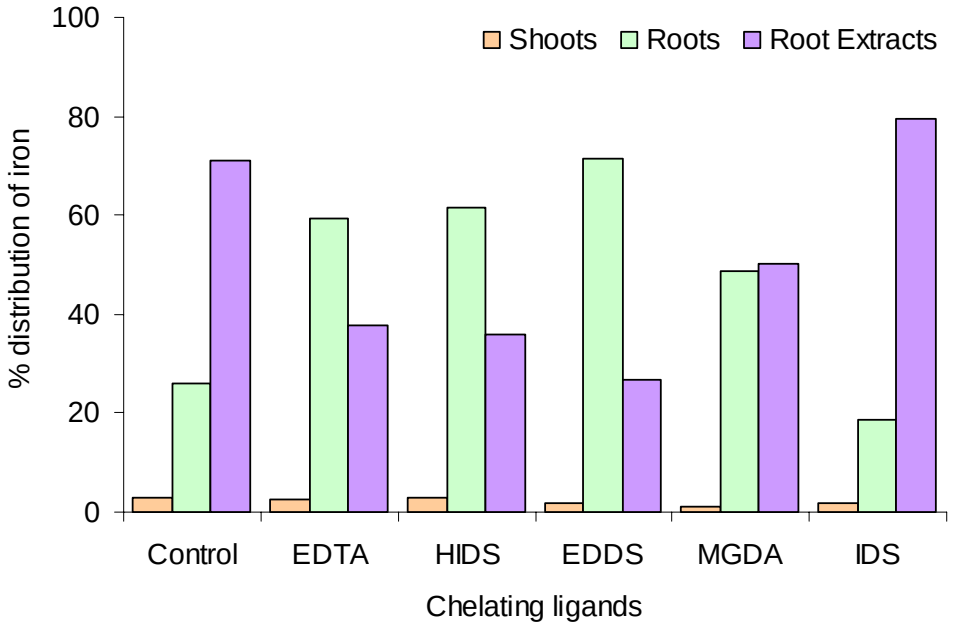

Fig. 2: Percentage uptake of iron in deferent parts of rice seedlings (*Oryza sativa* L.) influenced by chelating ligands.

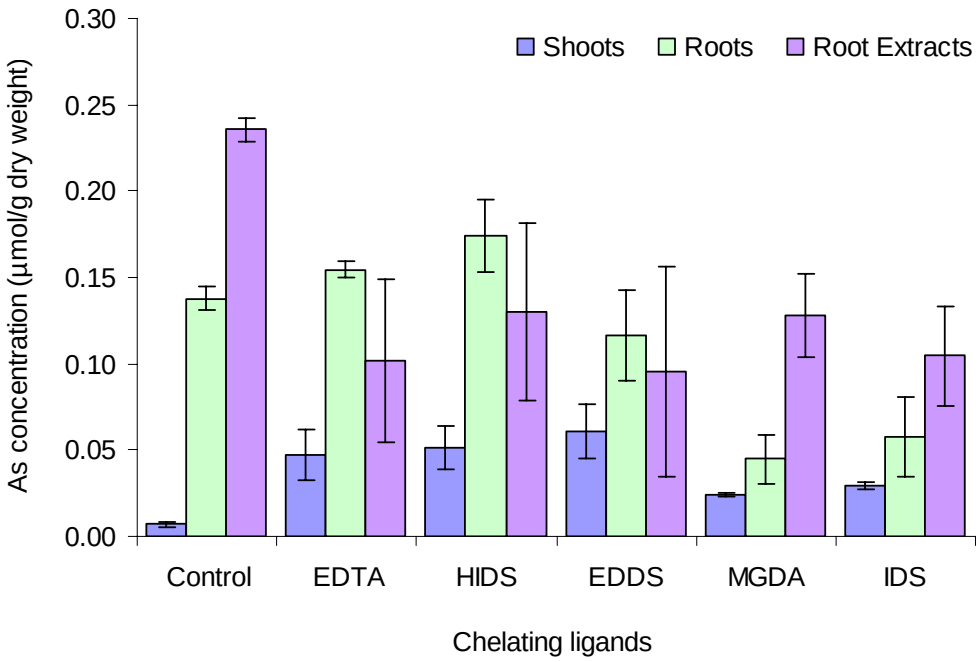

Fig. 3: Influence of chelating ligands on arsenic uptake in different parts of rice seedlings (*Oryza sativa* L.).

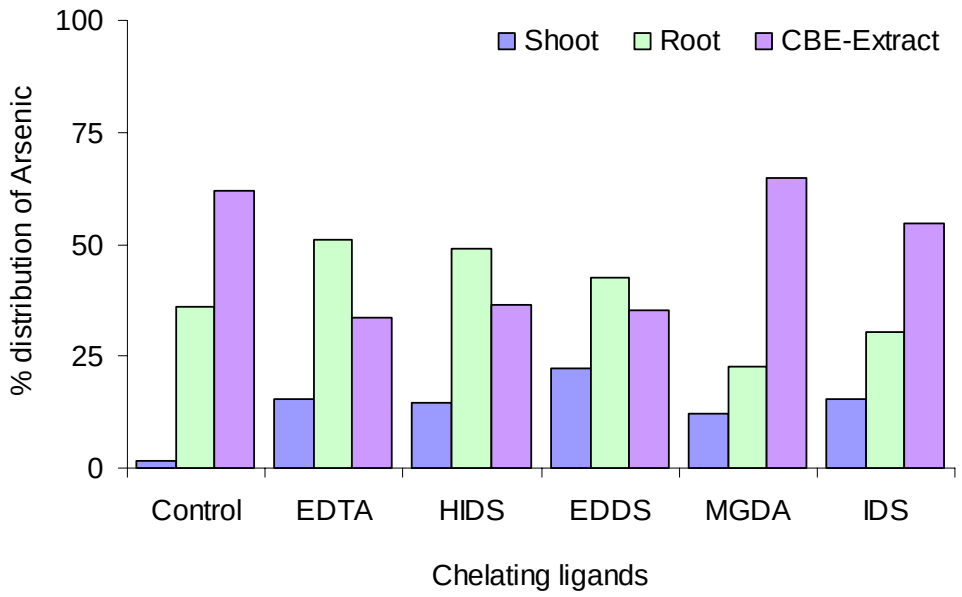

Fig. 4: Percentage uptake of arsenic in deferent parts of rice seedlings (*Oryza sativa* L.) influenced by chelating ligands.

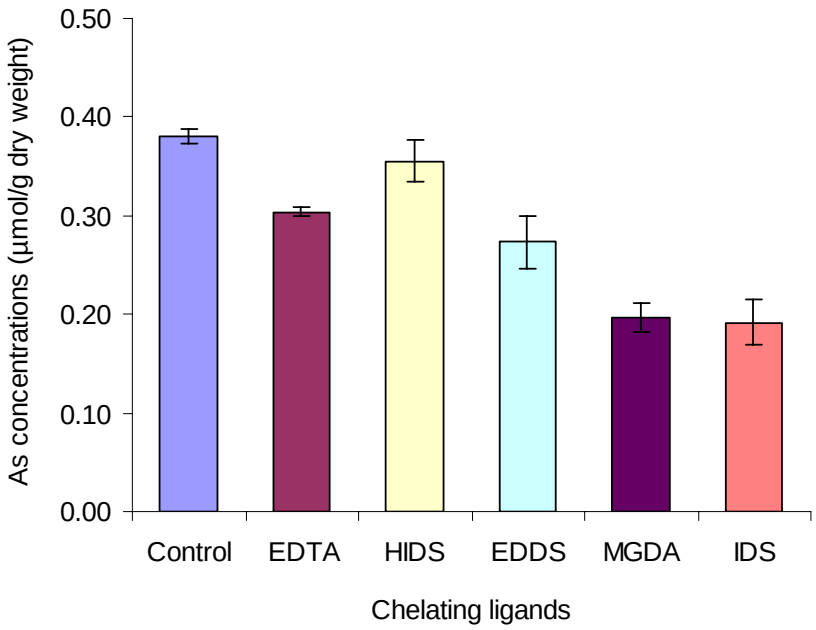

Fig. 5: Influence of chelating ligands on total arsenic uptake in rice seedlings (*Oryza sativa* L.).