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Effects of EDTA Addition on Cu Fractions in Soil to Enhance Cu Absorption by Maize

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The effect of EDTA addition on copper (Cu) fractions in soils and soil solutions were investigated in phytoextraction of Cu polluted soil (about 290 mg Cu kg⁻¹) by maize (*Zea mays* L.). Cu accumulation in the shoots was accelerated by the EDTA addition to the soil. In the non-EDTA added soil, the exchangeable Cu fraction was 0.3% of the total Cu and the organically adsorbed Cu fraction was dominant (43.4%). The exchangeable fraction increased by the EDTA addition was 2.4% of the total Cu, indicating the increase in the plant useful Cu portion in the soil. The total Cu concentrations in the soil solutions were also elevated by the EDTA addition. The increments in the EDTA added soil solutions were attributed to the increase in the EDTA–Cu. Consequently, the EDTA addition would increase the amount of EDTA–Cu in the soil solutions, followed by an acceleration of the Cu accumulation in the maize shoot.

INTRODUCTION

More than 150 metal mines are distributed all over Japan (Kitagishi and Yamane, 1981). About half of them have ore containing copper (Cu). Most of the mines are located in the mountain areas (Taniyama, 1991). Copper contamination has spread through the irrigation system to the down-stream paddy fields that have developed in the low land areas. Although Cu is an essential element for plants, excessive uptake of the element could cause direct toxicity to plants and environmental risk due to biomagnification in food webs (Kabata-Pendias, 2001). The remediation of Cu polluted agricultural soils has been an important need in last decades in Japan.

Conventional techniques for remediation of heavy metal polluted soils are time consuming, expensive, and labor-cost. Phytoextraction is likely to be a novel method that could clean-up metals from soils using conventional crop cultivation. A plant's ability for phytoextraction may be evaluated by the biomass production as well as by the heavy metal absorption capacity. Indian mustard (*Brassica juncea*) has been frequently studied for heavy metal phytoextraction due to its high accumulation capabilities (Ebbs and Kochian, 1998; Quartacci *et al.*, 2001). However, the biomass production of Indian mustard is extremely low, about 0.5 g per plant in dry weight (Quartacci *et al.*, 2001). Therefore, maize (*Zea mays* L.) is often used as an experimental species for phytoextraction because of the high biomass production and its comparatively strong heavy metals-resistance (Huang *et al.*, 1997; Wu *et al.*, 1999; Lombi *et al.*, 2001; Ali *et al.*, 2002; Wenger *et al.*, 2002).

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The addition of chemicals, such as a chelating agents, to soils has been used as a method to enhance the extraction of heavy metals by plants. For example, Huang *et al.* (1997) found that the lead absorption by peas (*Pisum sativum* L.) increased with the addition of EDTA. Similarly, the Pb accumulation increased in Indian mustard and in wheat (*Triticum aestivum* L.) with the addition of EDTA as reported by Blaylock *et al.* (1997) and Begonia *et al.* (2002), respectively. EDTA addition to metal-polluted soils elevated Zn accumulation in Indian mustard, but had no effect on oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) (Ebbs and Kochian, 1998). Translocations of Pb and Cd from the root to shoot of radish (*Raphanus sativus* L.) were enhanced by the addition of citric acid, resulting in reduction of soil toxicity to plants (Chen *et al.*, 2003). Increases of Se absorption in barley and wheat were also observed with the additions of organic acids such as ascorbic acid to soils (Blaylock and James, 1994). These observations showed the efficiency of application of some chelating agents for phytoextraction of metal polluted soils.

An important secondary effect in situ of addition of chelating agents to metal polluted soil may be the leaching of soluble metals into the ground water (Grcman *et al.*, 2001). Good estimation of the amount of metals available to plants induced by application of chelating agents to soils is essential in order to efficiently raise the heavy metals absorption by the plants along with a control of leaching to ground water. Sequential chemical extraction methods are often used to determine the fractions of heavy metals in soils and sediments (McLaren and Crawford, 1973; Tessier *et al.*, 1979; Sadamoto *et al.*, 1994; Saeki *et al.*, 1993a, b). Among these fractions, metals in soil solution are very important for the plant-soil relationships, because almost all terrestrial plants absorb most of their essential elements through soil solution; in other words, only heavy metals dissolved in the soil solution can be absorbed by plants. Therefore, it is also essential for phytoextraction to analyze the amount and the forms of heavy metals present in soil solutions. Several studies reported that heavy metal concentrations in soil solutions are increased by application of chelating agents (Huang *et al.*, 1997; Jiang *et al.*, 2003; Wu *et al.*, 1999, 2003). No research has been carried out to determine the amount of chelated metals in soil solutions after application of chelating agents to soils, such as EDTA-Cu, in relation to phytoextraction.

In a previous study, we compared the efficiency of the EDTA application for Cu removal from contaminated soils by Indian mustard and by maize (Inoue *et al.*, 2003). The purpose of the present study is to investigate the effects of the EDTA addition on the Cu forms in soils and soil solution for phytoextraction by maize.

MATERIALS AND METHODS

Soils and plant cultivation

The present study used a volcanic ash soil (Andosol) collected from a field at the National Agricultural Research Center for the Kyushu Okinawa Region in Nishigoushi, Kumamoto. The basic properties of the soil were pH (H₂O) 5.6; 23.3% organic matter; 31.2 cmol. kg⁻¹ CEC; and 42.2 mg g⁻¹ total Cu. Andosol was not fertilized because of the continuous application of manure before the cultivation. Soils were mixed with anhydrous copper (II) sulfate powder to 250 mg Cu per kg soil. The Cu spiked soil was mixed

with water to 50% of the field water capacity and incubated for 1 week at room temperature (about 293 K), then used for maize cultivation.

Maize (*Zea mays* L. cv. pioneer 33G26) was grown in a 350 mL size pot for 3 weeks inside a phytotron (at room temperature of 298 K and 70% humidity). Distilled water was daily added to the soils to maintain field water capacity ($0.60 \text{ m}^3 \text{ m}^{-3}$) during the cultivation.

Shoots and roots were sampled 3 weeks after the sowing, dried at 343 K and milled. The milled samples were ashed at 773 K, and then dissolved in dilute HCl and HNO_3 solution. The copper concentration of the digested solutions was measured by atomic absorption spectrometry (AAS) (Shimazu Co., Ltd., AA-670).

EDTA addition

Ten mL of a EDTA2Na solution of 0, 0.1, 1, 10, and 100 mmol L^{-1} (mM) were added by syringe to the soil surface one week after sowing. The amount of EDTA2Na addition corresponds to 3.33 mmol kg^{-1} soil (1.12 g kg^{-1} soil) in the EDTA 100 mM treatment.

Sequential chemical extraction

After plant cultivation, the Cu fractions of the soils were analyzed by a method modified by Sadamoto *et al.* (1994). Three g of soil was shaken with 30 mL of 0.05 mol L^{-1} potassium nitrate for 24 h in a 50 mL tube. The supernatant taken from the tube after centrifuging and filtering through 0.22 μm pore size filter was used for the analysis (ion-exchangeable form (EX fraction)). The residue from the above extraction was shaken for 24 h with 30 mL of 25 g L^{-1} acetic acid. The supernatant obtained by centrifuging and filtering was used for the analysis (form specifically adsorbed on clay minerals (AC fraction)). The residue was digested with 30 mL of 6% hydrogen peroxide, dried, then extracted with 30 mL of 25 g L^{-1} acetic acid, with shaking for 24 h. The supernatant obtained by centrifuging and filtrating was used for the analysis (form specifically adsorbed in organic matter (ORG fraction)). One gram of the residue was added to a 30 mL mixture of 0.1 mol L^{-1} oxalic acid and 0.175 mol L^{-1} ammonium oxalate and with 1 g ascorbic acid powder, then shaken for 1 h in a boiling water bath. The supernatant obtained by centrifuging and filtrating was used for the analysis (form occluded in oxides (OX fraction)). The final residue was digested by perchloric acid and the liquid phase obtained by filtrating was used for analysis (residue (LA fraction)). Copper in the solutions from each fraction was determined using AAS.

Analysis of EDTA-Cu in soil solution

Samples of the soil solutions were obtained from a water-saturated extract prepared by the modified method of Rhoades (1982) (Saeki *et al.*, 2002). The water-soil mixture was centrifuged at $3500 \times g$, and then the extract was filtrated through a 0.22 μm pore size filter. The solutions were then measured for total Cu by graphite furnace AAS (Nippon Jarrell-Ash Co. Ltd., SOLAAR AA).

The soil solution was passed through an anion exchange type column (TOA, PCI-201S, $4.6 \times 100 \text{ mm}$) using an HPLC (TOA, ICA-5220). The operational condition for the HPLC is listed as follows; Eluent: a mixed solution of 3 mmol L^{-1} phthalic acid, 2.74 mmol L^{-1} tris (hydroxymethyl) aminomethane and 200 mmol L^{-1} boric acid, Flow rate:

0.9 mL min⁻¹, Injected sample volume: 10 μ L. The eluted liquid after the column passage was divided into 0.9 mL using a fraction collector. The copper in each fraction of the elutes was determined by graphite furnace AAS.

Statistical analysis

Each experiment was performed in triplicate. The variance for each of the results was analyzed and the differences between the averaged values was tested using Tukey's method.

RESULTS AND DISCUSSION

Cu accumulation by maize

Table 1 shows that Cu concentration in maize shoots with the 10 and the 100 mM EDTA treatments were higher than the 0 mM EDTA treatment but without any significant difference. The shoot dry weight in the 10 and the 100 mM EDTA treatments were lower than the 0 mM EDTA treatment. Cu accumulation in maize shoots in the 100 mM EDTA treatment was 3.7 times greater than in the 0 mM EDTA treatment ($p < 0.01$). It confirmed that the addition of EDTA has effectively enhanced Cu accumulation in the maize shoot. Two possible mechanisms are assumed to cause the increase in the Cu accumulation in the shoot by the EDTA addition. One is that the Cu accumulated by the roots combines with EDTA, then transported to the shoot as the form of EDTA-Cu. Another is that Cu adsorbed on the soil particles are desorbed by EDTA, then converted to EDTA-Cu in the soil solution, which could be easily absorbed by the maize. The latter can be confirmed by an investigation of the Cu forms in the soil and the existence of EDTA-Cu in the soil solution.

Table 1. Copper concentration, dry weight of shoot, and Cu accumulation of *Zea mays*.

EDTA treatment (mmol L ⁻¹)	Cu conc. (μ g g ⁻¹)		Dry weight (g plant ⁻¹)		Cu accumulation (μ g plant ⁻¹)	
	avg \pm SE		avg \pm SE		avg \pm SE	
0	8.6 \pm	1.0 NS	0.47 \pm	0.04 NS	3.9 \pm	0.36 a
0.1	9.3 \pm	0.9	0.53 \pm	0.04	4.9 \pm	0.72 a
1	8.8 \pm	0.6	0.52 \pm	0.04	4.6 \pm	0.63 a
10	14.0 \pm	3.1	0.39 \pm	0.10	4.9 \pm	0.12 a
100	71.7 \pm	34.1	0.25 \pm	0.06	14.4 \pm	3.48 b

NS shows no significant difference.

Different letters show 5% significant difference.

Fractionation of soil Cu by sequential chemical extraction

Copper fractionations in soil after cultivation were done in both the 0 mM and 100 mM EDTA treatments in order to investigate the effect of the EDTA addition to the soils on the Cu forms (Table 2). The Ex-Cu concentration increased from 0.9 to 7.4 mg kg⁻¹ by the

Table 2. Copper concentration among different fractions of cultivated Cu-mixed soil.

	(0 mmol L ⁻¹)		(100 mmol L ⁻¹)	
	avg ± SE		avg ± SE	
EX fraction*	0.9 ±	0.0	7.3 ±	0.1
AC fraction	12.1 ±	0.5	11.3 ±	0.3
ORG fraction	132.1 ±	8.9	123.9 ±	6.4
OX fraction	89.1 ±	10.7	85.7 ±	3.7
LA fraction	69.8 ±	1.5	74.9 ±	1.2

* 5% significant difference.

100 mM EDTA treatment. These Ex fractions were only 0.3 and 2.4% of the total Cu in the 0 mM and the 100 mM EDTA treatments, respectively. The ORG-Cu fractions decreased from 132 mg kg⁻¹ (43.4%) to 124 mg kg⁻¹ (40.9%) due to the EDTA addition. The OX-Cu fractions were also slightly reduced from 89.1 mg kg⁻¹ in the 0 mM treatment to 85.7 mg kg⁻¹ in the 100 mM EDTA treatment. There was no change in the LA fraction. Most of the Cu portions in the 24 England soils were in the residue fraction (24–77%), and the exchangeable fraction was less than 0.2% (only a sample, 2.3%) (McLaren and Crawford, 1973). Tessier *et al.* (1979) reported that the residual fraction of Cu was about 40% whereas the exchangeable fraction of Cu was under 1% in the sediments. Even in polluted soils, the plant-available Cu fractions (exchangeable) were several % of the total soil Cu content (Sadamoto *et al.*, 1994). Because the Cu polluted soil was artificially made by adding the reagent CuSO₄ into the study soils, the proportion of the exchangeable Cu to the total Cu content was expected to be higher than in the actual polluted soils. However, the exchangeable fraction in this soil was similar to the reported levels of *in situ* actually polluted soils. These results indicated that the EDTA addition to the soils could efficiently increase the amount of the plant-available Cu portion, although being a small proportion of the total soil Cu.

Analysis of Cu in the soil solutions

It has been reported that the heavy metal (Pb, Cd and Cu) concentrations in the soil solution increased due to EDTA addition to the soils (Huang *et al.*, 1997; Jiang *et al.*, 2003; Wu *et al.*, 1999, 2003). Similar to these reports, Cu concentration in the soil solution rose proportionally with added EDTA to soils (Fig. 1). The copper concentration in the soil solution in the 100 mM treated soil was significantly higher than those in the other soils ($p < 0.05$).

Each chromatograph of Cu ion and EDTA-Cu was checked using CuSO₄ and EDTA2Na solutions (Fig. 2). When injecting only a 2 mmol L⁻¹ CuSO₄ solution to the HPLC, most of the Cu was detected in the 2 min. fraction (meaning that the elute portion was taken from 2 to 3 minutes after passing through the column) with a small peak in the 3 min. fraction. Copper was detected in the 7, 8, 9 min. fractions with a maximum peak for the 8 min. fraction in an injection of a 1:1 (v/v) mixture of the 2 mmol L⁻¹ EDTA2Na and the 2 mmol L⁻¹ CuSO₄ solutions. Therefore, we assumed that the Cu peaks observed in the 7 to the 9 min. fractions corresponded to EDTA-Cu. With a 2:1 (v/v) mixture of the

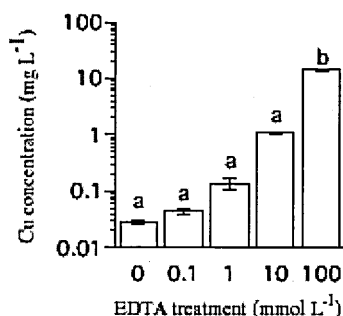


Fig. 1. Copper concentration in soil solution of Cu-spiked soil. Error bars represent standard error range of the mean, $n=3$. Different letters show 5% significant difference.

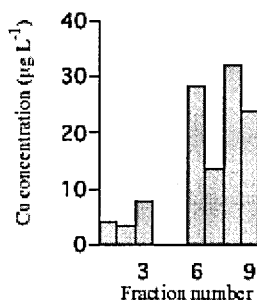


Fig. 3. Copper fractions of the soil solution in the EDTA 100 mmol L⁻¹ treatment soil.

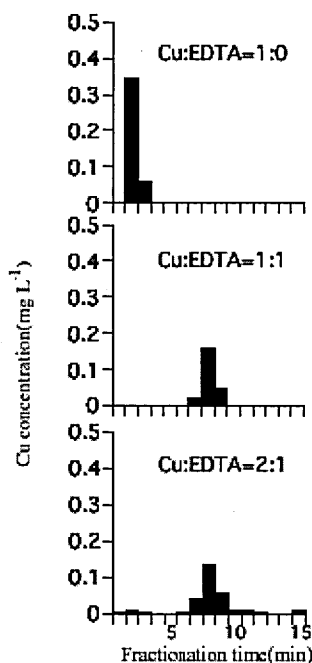


Fig. 2. Copper concentrations of the solution mixed CuSO₄ and EDTA2Na fractionated by the anion exchange column.

EDTA2Na and the CuSO₄ solutions, Cu was detected in the 1, 2, and 3 min. fractions and in the 6, 7, 8, and 9 min. fractions with largest peaks in the 2 min. fraction and the 8 min. fraction. This result means that the Cu ion and EDTA-Cu existed together in the mixture though most of the Cu existed as EDTA-Cu. Probably, the existent ratio of Cu ion: EDTA-Cu was not 1:1 in the 2:1 mixture of EDTA2Na and CuSO₄ solutions. Any Cu compounds eluted in the 6 min. fraction could not be exactly identified, but one can certainly distinguish this portion from the Cu ion.

A chromatograph of the soil solution of the 100 mM EDTA treatment is shown in Fig. 3. Copper in the soil solution was divided into the 1 to 3 min. fractions and into the 6 to 9 min. fractions. When interpreting Cu in the 7 to 9 min. fractions as EDTA-Cu, we calculated that 61.4% of the total Cu in the soil solution existed as EDTA-Cu. The Cu ion was estimated to be only 15.2% of the total Cu.

Because the 2 min. and the 8 min. fractions had the largest peaks in the 1, 2, and 3 min. and in the 6, 7, 8, and 9 min. fractions, respectively, both in Figs. 2 and 3, this study

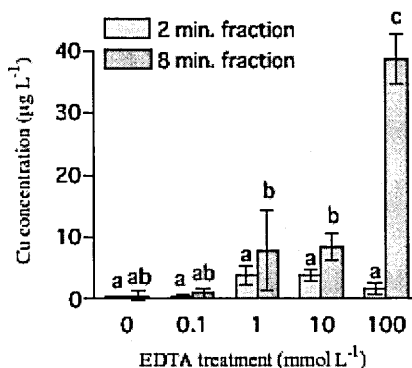


Fig. 4. Copper concentration of soil solution of Cu-mixed soil fractionated by anion exchange column. Error bars: standard error range of the mean, $n=3$. Different letters show 5% significant difference.

regarded the 2 min. fraction and the 8 min. fraction as representatives of the Cu ion and EDTA-Cu in the soil solutions, as shown in Fig. 4. The Cu in the 2 min. fractions did not significantly fluctuate with the concentration of added EDTA, implying that Cu ion in the soil solution was not drastically increased by the EDTA additions to the soil. In contrast, the Cu concentration in the 8 min. fraction with the 100 mM EDTA treatment was higher than those in the other treatments ($p < 0.05$). However, this Cu concentration did not increase in proportion to the added EDTA concentrations.

The Cu amounts were significantly higher in the 8 min. fractions than that in the 2 min. fractions of the 1, 10 and 100 mM EDTA treatments ($p < 0.05$). These results indicated that the EDTA-Cu in the soil solution is drastically increased by the addition of EDTA to the soil, whereas the concentration of Cu ion is not greatly changed by this addition. Consequently, the change in Cu accumulation in the maize by the EDTA addition to the soil follows the increase in the exchangeable fraction in the soil or especially of the EDTA-Cu in the soil solution.

For the EDTA application to soils, one should pay attention to its direct toxicity to plants and its leaching into the ground water systems. Our previous study showed that the maize shoot, which was in contact with the 100 mmol L⁻¹ EDTA solution at 7 days after sowing died, probably because of salt damage induced by the EDTA addition (Inoue *et al.* 2003). In this study, the dry weights of the maize shoot after the 100 mM EDTA treatment also decreased compared to those in no EDTA treatment without significance. The efficiency of phytoextraction generally decreases with the decrease in the plant biomass due to the toxicity of EDTA or EDTA-metals. The countermeasures against the salt damage may be suggested; lowering the EDTA concentration and the addition at a specific growth stage. The EDTA or EDTA-metals leaching into the groundwater systems should be prevented by controlling the amount of added EDTA to have a balance between the plant available metal amount and the soluble metal amount by EDTA, and monitoring the

concentrations of heavy metals in the soil solutions. Any studies for both of these problems would require investigating the Cu speciation in any plant tissues which absorbed the EDTA-Cu from the soil solutions.

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