Effect of Various Biodegradable Chelating Agents on Root Growth of Plants under Copper Stress

Sangman Lee*

Division of Applied Biology and Chemistry, School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, Daegu 702-701, Korea

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Phytoextraction is a method of phytoremediation using plants to clean up metal-contaminated soils. Recently, various chelating agents were used in this method to increase the bioavailability of metals in soils. Even though phytoextraction is an economic and environmentally friendly method, this cannot be applied in highly metal-contaminated areas because plants will not normally grow in such conditions. This research focuses on identifying chelating agents which are biodegradable and applicable to highly metal-contaminated areas. Copper (Cu) as a target metal and cysteine (Cys), histidine (His), citrate, malate, oxalate, succinate, and ethylenediamine (EDA) as biodegradable chelating agents were selected. Ethylenediamine tetraacetic acid (EDTA) was used as a comparative standard. Plants were grown on agar media containing various chelating agents with Cu to analyze the effect on root growth. Cys, His, and citrate strongly diminished the inhibitory effect of Cu on root growth of plants. The effect of oxalate was weak, and malate and succinate did not show significant effects. EDTA diminished and EDA promoted the inhibitory effects of Cu on root growth. These effects of chelating agents are correlated with Cu uptake into the roots. In conclusion, as biodegradable chelating agents, Cys, His, and citrate are good candidates for highly Cu-contaminated areas, while EDA can be useful in phytoextraction for Cu.

Key words : Chelate, copper, heavy metal, phytoextraction, phytoremediation

Introduction

Heavy metal contaminated soils have been serious problems worldwide, threatening human health and the environmental ecology. When metals are present at high concentrations in soils, they inhibit growth of living organisms and sometimes lead to their death. Cu is one of such contaminants in soils, and it is used as an active ingredient in fungicides and as a timber preservative. Cu is usually strongly bound to soil organic matter and thus relatively unavailable to microbes and plants. However if the soil adsorption sites in the soil become saturated with Cu, it then becomes toxic for living organisms, resulting in decreased soil fertility [21].

Conventional methods to remediate heavy metal-contaminated soils can be applicable at highly contaminated sites but not to large areas. Moreover, they are expensive and destroy soil fertility. Due to these disadvantages, an alternative technology, phytoremediation, has been developed. Phytoremediation is a technology using plants to clean-up contaminated soils and waters. One technique of phytoremediation and an economical and environment-friendly method, phytoextraction has been used to extract heavy metals from moderately polluted soils [4] and does not destroy soil fertility [5,23]. Although several factors must be satisfied in order for phytoextraction to be effective, the bioavailability of the target metal in the soil is a critical requirement for plant metal uptake to occur.

To meet the factor of bioavailability, various chelating agents have been used in phytoextraction. Of the tested artificial and natural chelating agents, EDTA has been found to be the most effective for increasing the uptake of various metals from soils in plants [1,6,7,10,12,18]. The complexation of heavy metals with various chelating agents typically follows the order EDTA and related synthetic chelates > nitrilotriacetic acid (NTA) > citric acid > oxalic acid > acetic acid [11,16]. However, EDTA and EDTA-heavy metal complexes can be toxic to plants and soil microorganisms and they can also persist in the environment due to their low biodegradability [2,10]. In recent years, the use of some easily biodegradable chelating agents, such as NTA and EDDS (*S,S*-ethylene diamine disuccinic acid), has been proposed to enhance the uptake of heavy metals in phytoextraction

*Corresponding author
Tel : +82-53-950-7345, Fax : +82-53-953-7233
E-mail : sangman@knu.ac.kr

[13,15,17].

Until recent, researches of phytoextraction in heavy metal-contaminated soils focused on increasing metal bioavailability, and desorbing metals from the soil organic matters into soil solution to facilitate their transport and translocation from the roots to shoots of some fast-growing, high-biomass-producing plants [1,3,12,24,27]. However, these researches are useless in highly metal-contaminated soils as plant cannot grow normally in such conditions. There are some known chelating agents that inhibit uptake of metals into plants as in case of EDTA for zinc. Therefore, this study focuses on identifying various biodegradable chelating agents to reduce the concentrations of heavy metals below the critical levels in plants to avoid either reduced growth or the death of plants.

Materials and Methods

Plant material, growth conditions, chemical treatments, and growth assays

Seeds of *Arabidopsis thaliana* cv. Columbia were germinated and grown for 7 days on an agar medium containing half-strength Murashige and Skoog salts, 2% (w/v) sucrose (pH 5.8), and various combinations of both $CuCl_2$ and chelating agents (Cys, His, malic acid, citric acid, succinic acid, oxalic acid, EDTA, and EDA) in 100×100×15 mm square plates. Plates were maintained in a growth chamber at 23°C under an 18 hr photoperiod provided by cool-white fluorescent tubes. Root lengths were measured by the ruler at the end of the incubation time.

Copper measurement

Roots of 7-d-old seedlings were harvested, rinsed with deionized water, and dried at 70°C to a constant weight. The oven-dried samples were ground, and digested with a mixture of HNO_3 (65%) and H_2O_2 (30%) for 8 hr at 120°C. The digested samples were dried at 140°C, and then resuspended with diluted nitric acid. Total copper concentrations of the digests were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES; Thermo Jarrell Ash, Franklin, MA).

Results and Discussion

Metal ions are highly reactive with S, N, and O. Therefore, some of amino acids such as Cys and His are involved in heavy metal chelation. Cys, as a major component of metal binding proteins (e.g. metallothioneins) and polypeptides (e.g. phytochelatin), is involved in chelation of Cd, Cu, and zinc (Zn) [25]. While, His is involved in chelation of nickel (Ni) [14]. Therefore, it was analyzed whether Cys and His can promote or inhibit the uptake of Cu into the roots of plants growing on agar media. Both Cys and His significantly decreased the inhibitory effect of Cu on plant root growth (Fig. 1). At 100 μM Cu concentration, plant root lengths were approximately 10%, 50%, and 95% of that of non-treated plant in the control plant, the 200 μM Cys treated plant, and the 200 μM His treated plants respectively. This result implies that Cys and His are strong chelating agents for Cu. And chelated Cu is not easily taken into the roots compared with Cu alone. Moreover, His inhibits uptake of Cu into the roots more than Cys.

Several low molecular weight organic acids, such as citrate, malate, oxalate, and succinate, are involved in metal tolerance through their capacity of chelation with various metals [9,20]. For example, citrate is involved in tolerance to Cd, Ni, and zinc (Zn) [8,22,26]. Similarly, malate is involved in Zn tolerance [19]. Both citrate and oxalate decreased the inhibitory effect of Cu on root growth of plants in this experimental conditions, while, malate and succinate did not (Fig. 2). The inhibitory effect of citrate is stronger on Cu-induced retardation of plant root growth than that of other tested organic acids. This result implies that the citrate-Cu complex is not easily taken into the roots of plant.

Fig. 1. Effects of cysteine and histidine on growth of *Arabidopsis* roots under copper stress. *Arabidopsis* seeds were germinated and grown for 7 days on an agar medium containing various concentrations of $CuCl_2$ (control, open circle) with either 200 μM cysteine (closed square) or 200 μM histidine (closed circle). Afterward, root lengths were measured. Values are means±SE of 30 seedlings.

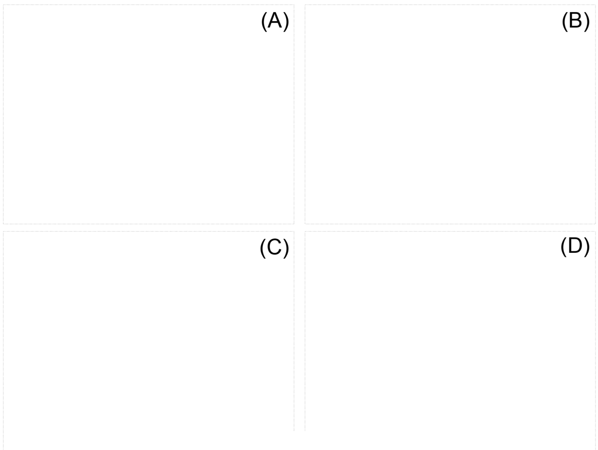

Fig. 2. Effects of citrate, malate, oxalate, and succinate on growth of *Arabidopsis* roots under copper stress. *Arabidopsis* seeds were germinated and grown for 7 days on an agar medium containing various combinations of both $CuCl_2$ and chelating agents, A, citrate; B, malate; C, oxalate and D, succinate. Afterward, root lengths were measured. Values are means±SE of 30 seedlings.

EDTA is a polyamino carboxylic acid with the formula $[CH_2N(CH_2CO_2H)_2]_2$ and known as a chelating agent to sequester metal ions such as Ca^{2+} and Fe^{3+}. EDA (ethylenediamine) is the organic compound with the formula $C_2H_4(NH_2)_2$ and used in large quantities for production of many industrial chemicals. EDA is the prototypical chelating ligand for coordination compounds, such as $[Co(EDA)_3]^{3+}$. The effect of EDTA and EDA on growth of plant roots under Cu stress was tested. EDTA strongly diminished the inhibitory effect of Cu on root growth, while, EDA significantly promote the effect of Cu (Fig. 3). The retardation of root growth by Cu may due to uptake of Cu into the roots over supra-optimal concentrations. To explore this, the Cu concentrations in the roots were analyzed. EDTA inhibited the Cu uptake,

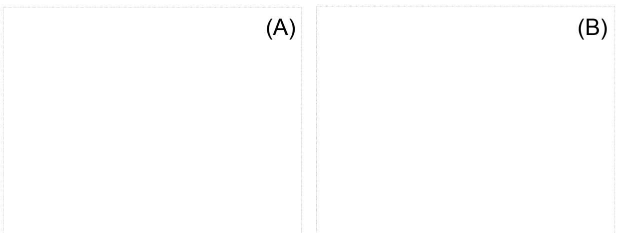

Fig. 3. Effects of EDTA and EDA on growth of *Arabidopsis* roots under copper stress. *Arabidopsis* seeds were germinated and grown for 7 days on an agar medium containing various concentrations of $CuCl_2$ (control, open circle) with either 200 μM EDTA (A) or 200 μM EDA (B). Afterward, root lengths were measured. Values are means±SE of 30 seedlings.

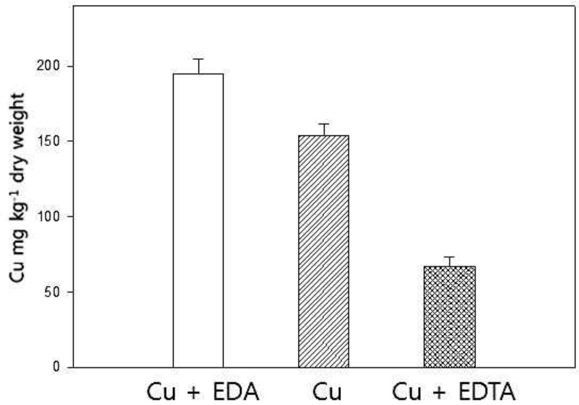

Fig. 4. Effects of EDA and EDTA on copper concentrations in *Arabidopsis* seedlings. *Arabidopsis* seeds were germinated and grown for 7 days on an agar medium containing 75 μM $CuCl_2$ with either 200 μM EDA or 200 μM EDTA. Afterward, copper concentrations were measured by ICP-AES. Values are means±SE of 3 replicates.

while, EDA promoted the Cu uptake into the roots of the plants (Fig. 4). Therefore, chelating agents can promote or inhibit Cu uptake into the plants, and these resulted in either increased or decreased Cu concentration.

In conclusion, His, Cys, citrate, and oxalate can be used in highly Cu-contaminated areas, while, EDA can be used in low Cu-contaminated areas for phytoremediation as environment-friendly chelating agents.

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초록 : 생분해 되는 다양한 킬레이트가 구리에 노출된 식물의 뿌리성장에 미치는 영향

이 상 만*
(경북대학교 응용생명과학부)

Phytoextraction은 식물을 이용하여 환경 정화하는 phytoremediation의 일부분으로서 금속으로 오염된 토양을 정화하는 것이다. 토양에 존재하는 금속의 추출을 용이 하기 위해서 현재 다양한 킬레이트가 사용되고 있다. Phytoextraction이 경제적이고 친환경적인 장점이 있지만 고농도로 오염된 지역에서는 적용이 어려운데 이는 식물이 이러한 지역에서 살아남기 어렵기 때문이며 이러한 문제점을 해결하는 것이 본 연구의 목적이다. 연구 대상의 금속으로서 구리를 선택하였고, 킬레이트는 아미노산인 시스테인과 히스티딘, 작은 크기의 유기산으로서 citric acid, malic acid, succinic acid, oxalic acid, 그리고 ethylenediamine (EDA)를 선택하였으며, EDTA는 비교대상으로 본 연구에 사용되었다. 다양한 농도의 구리를 포함하는 배지에 식물을 키우면서 여러 킬레이트가 식물의 뿌리 성장에 미치는 영향을 분석하였다. 시스테인, 히스티딘, 그리고 citric acid은 식물의 성장을 억제하는 구리의 영향을 완화시켜 주었지만 EDA는 오히려 더 강화시켜 주었다. 또한 킬레이트의 식물성장에 대한 영향은 구리의 식물 내 흡수를 억제 또는 촉진에 의한 것이다. 따라서 구리의 식물성장억제를 완화시켜주는 시스테인, 히스티딘, 그리고 citric acid는 고농도의 구리로 오염된 지역에 식물의 성장이 가능하도록 사용될 수 있으며 EDA는 기존의 phytoextraction에 유용하게 사용될 수 있을 것이다.