AMENDMENTS FOR ENHANCING COPPER UPTAKE
BY BRASSICA JUNCEA AND LOLIUM PERENNE
FROM SOLUTION

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Phytoextraction of metals is frequently limited by contaminant bioavailability and plant uptake rates. Chemical amendments can be added to increase the uptake and translocation of metals to aerial biomass. A range of amendments of various types was tested for increasing the copper uptake with the test species Indian mustard and ryegrass. These included citric acid (an organic acid); histidine (an amino acid); ethylenediaminetriacetic acid (EDTA), nitrilotriacetic acid (NTA), and ethelynediaminedisuccinic acid (EDDS) (aminopolycarboxylic acids); rhamnolipid (a biosurfactant); and Triton X-100 (a synthetic surfactant). EDTA was the most effective amendment for enhancing copper uptake and translocation into the shoots of Indian mustard and ryegrass, with respective shoot tissue copper levels of 1230 and 1360 µg-Cu/g-dry weight after 10 d compared to 90 and 220 µg-Cu/g-dry weight, respectively, in the unamended treatments. However, the EDTA application resulted in symptoms of toxicity in both Indian mustard and ryegrass, leading to drastic decreases in biomass yield. The application of high levels (300 mg/L) of the biodegradable chelator EDDS was found to be effective for improving translocation of copper in both species. The NTA addition provided benefits to root and shoot growth, with increased copper translocation to shoot tissue. Tests with biosurfactants and synthetic surfactants indicated detrimental effects on copper uptake, biomass yield, and the translocation of copper from roots to shoots in both plant species.

KEY WORDS phytoextraction, metal, chelate, organic acid, amino acid, surfactant

INTRODUCTION

Phytoextraction is the use of plants to remove metals and other contaminants from contaminated media (Romkens et al., 2002; Jiang, Yang and He, 2004). This technique relies on the ability of plants to translocate contaminants from their roots to the aboveground biomass for storage (Morikawa and Erkin, 2003; Robinson et al., 2003) and involves the fundamental processes of mobilization, sorption, uptake, translocation, and sequestration. The idea of using plants to extract heavy metals from soils emerged with the discovery of various wild plants that accumulate high concentrations of metals in their foliage (Lasat, 2000; Garbisu and Alkorta, 2001; do Nascimento and Xing, 2006). While some metals are essential (i.e., potassium, calcium, magnesium, iron, manganese, zinc, copper,
molybdenum, and nickel) or beneficial (i.e., sodium and cobalt) for plant growth and development, at high concentrations they can be toxic. The heavy metals cadmium, nickel, zinc, and copper are known to be taken up by plants (Garbisu and Alkorta, 2001; do Nascimento and Xing, 2006) and are specified as constituents that are amenable to phytoextraction (USEPA, 1999).

Two major approaches have been proposed for the application of phytoextraction: natural phytoextraction and chemically enhanced phytoextraction (Salt et al., 1995a; Lasat, 2000; Garbisu and Alkorta, 2001; do Nascimento and Xing, 2006). The first concept is based on the use of hyperaccumulators, metal-tolerant plants with exceptional metal-accumulating capacities (Lombi et al., 2001). However, such hyperaccumulating plants are usually found to be slow growing with low biomass yields (Chiu, Ye, and Wong, 2005). Results from a study conducted using field pennycress (Thlaspi caerulescens) have been used to estimate that 28 years of growth on a site would be required to treat soil containing 2100 mg-Zn/kg soil (Brown et al., 1995). These extended treatment times are one of the current limitations preventing the widespread uptake of phytoremediation as an alternative to physical and chemical treatment methods. As an alternative, during the last decade, researchers have developed the concept of enhancing metal accumulation in normal plants that are exposed to excess metal concentrations (Huang et al., 1997; Romkens et al., 2002; Anderson, 2005). This approach makes use of species with high biomass and metal tolerance, and/or chemical treatments that increase metal mobility and bioavailability in soil and water. The effectiveness of phytoextraction for the remediation of heavy-metal–contaminated soils is highly dependent on metal bioavailability, a factor that limits heavy metal uptake by plant roots (Kamnev and van der Lelie, 2000; Lasat, 2000). Researchers have shown that for effective uptake to occur, metals need to be solubilized in the rhizosphere and then moved across the root–cell plasma membrane for subsequent transport into the xylem (Robinson et al., 2003). Chemical amendments are known to change metal bioavailability and speciation in the growing medium, contributing to improved metal uptake by plant roots (Chiu et al., 2005). Thus, chemical-enhanced phytoremediation has been proposed as an effective method that may be applied as an alternative to natural phytoextraction for the increased extraction of heavy metals from soils by plants (Lombi et al., 2001; do Nascimento and Xing, 2006).

Various compounds have been studied for their ability to mobilize metals and enhance metal accumulation in plants, including chelating agents (Kulli et al., 1999; Chen and Cutright, 2001; Grčman et al., 2003), organic acids (Chen et al., 2003; Evangelou, Ebel, and Schaeffer, 2006), and amino acids (Kerkeb and Kramer, 2003; Singer et al., 2007). Potential ligands for increasing contaminant uptake include organic and amino acids, such as citric acid and histidine, and a wide range of aminopolycarboxylic chelating agents such as trans-1,2-cyclohexylenedinitrilotetraacetic acid (CDTA), diethylenetriaminepentacetic acid (DTPA), ethylenediaminetriacetic acid (EDTA), hydroxyethylenediaminetriacetic acid (HEDTA), ethylenebis(oxyethylenetri)tetraacetic acid (EGTA), nitrilotriacetic acid (NTA), and ethelynediaminedisuccinic acid (EDDS). Research has shown that the metal–ligand compounds formed can improve metal translocation from root to shoots, achieving the objective of chemically enhanced phytoextraction (Kramer and Chardonnens, 2001; Kerkeb and Kramer, 2003). However, some ligands cause toxic symptoms in plants. Furthermore, metal uptake and translocation is plant specific and governed by the complexes present in the solution (Callahan et al., 2005). The ability of a chelate to enhance metal uptake is known to be related to its affinity for metals (Lasat, 2000), with the critical stability constant a measure of this chelating strength. The chelate itself must be highly
water soluble and sufficiently stable to facilitate uptake and translocation within the plant. Once inside tissue, the complex should remain intact to reduce the potentially toxic effects of free heavy metals. It should have no effect on the ability of a plant to continue to actively transpire and, if possible, continue growing (Wu, Hsu, and Cunningham, 1999). Although plant responses correlate better with the concentration of free metal ions present in the solution (Chiu et al., 2005), intact EDTA, EDDS, and histidine complexes detected inside plants indicate that both metals and the chelating agent can be taken up by the plant and translocated to the aboveground biomass (Vassil et al., 1998; Tandy, Schulin and Nowack, 2006a).

Previous work has demonstrated that plants that are effective for phytoextraction display characteristics such as a high tolerance to elevated metal concentrations, an ability to accumulate high levels of metal in harvestable parts, a rapid growth rate, a high biomass production in field conditions, and a prolific root system (Garbisu and Alkorta, 2001; Alkorta et al., 2004). Indian mustard (Brassica juncea), a species known to accumulate metals (Ebbs et al., 1997), and perennial ryegrass (Lolium perenne), a widely used test species, were selected for the present study to investigate the effect of different amendments on metal toxicity and uptake. Indian mustard has been investigated in a number of studies, demonstrating its potential for metal uptake (Huang and Cunningham, 1996; Blaylock et al., 1997; Wu et al., 2004; Quartacci et al., 2006) and it has been identified as a species that is able to take up and accumulate into its aboveground parts metals such as cadmium, copper, nickel, lead, and zinc (Quartacci et al., 2006). Perennial ryegrass has been used as a test species (Sauve et al., 1996; Arienzo, Adamo, and Cozzolino, 2004) and a limited number of studies have been carried out to study heavy metal uptake in the presence of chemical amendments (Kulli et al., 1999).

To date, studies on the phytoextraction of copper by Indian mustard and ryegrass as affected by various types of amendments are limited. This article presents a comparison of the toxicity and effectiveness of potential amendments for enhancing phytoextraction with these plant species. Seven amendments encompassing a range of ligand types were selected for this study, to investigate their influence on the uptake of copper from solution by a known hyperaccumulator species (B. juncea) and an indicator species (L. perenne). An organic acid, an amino acid, three aminopolycarboxylic acids, and two surfactants were trialled.

CHEMICAL AMENDMENTS

Amendments such as the organic and amino acids were selected for their similarity to naturally produced plant root exudates (Marschner, 1995), while others such as aminopolycarboxylic acids were chosen for their high complex stability (Martell and Smith, 1974). Biosurfactants have been used to solubilize metals in soil-washing applications (Mulligan, Yong, and Gibbs, 2001a) and so were also included in this study to investigate how the presence of the surfactant might influence a plant’s metal uptake.

Organic and Amino Acids

Organic and amino acids have potential in phytoextraction applications as they not only have the ability to complex metals, but they are naturally produced by bacteria and plants, and are released into soil. In some cases, these compounds play a key role in natural plant-uptake mechanisms. Additionally, they are generally easily biodegraded and nontoxic
to soil biota. For this study, citric acid was chosen as a representative organic acid that is naturally produced by plants and microorganisms (Romkens et al., 2002). It is released by plant roots for the mobilization of metals from soil and forms complexes with metal ions (Marschner, 1995). Researchers have demonstrated that citric acid can be successfully used as a chelating agent to mobilize lead, cadmium, copper, nickel, and zinc from soil (Blaylock et al., 1997). Huang et al. (1998) found that citric acid was the most effective organic acid for increasing the availability and plant uptake of uranium. In a similar study, Chang et al. (2005) found that five additions of 30 mM citric acid solution resulted in a nearly six-fold increase in uranium stored in the leaves of B. juncea, 1 week after the 10-day exposure period.

Histidine was selected as an amino acid to be trialled in this study. It is one of the natural amino acids known to play an important role in various biological processes (Martusevicius et al., 1995) including metal hyperaccumulation by virtue of its ability to act as a tridentate ligand via its carboxylate, amino, and imidazole functional groups (Sigel and McCormick, 1971; Callahan et al., 2005). In nature, it is present as the stereoisomers D-histidine and L-histidine (Abramenko, Bolotin, and Nikolaienko, 2001; Venelinov et al., 2006). The apparent importance of histidine in metal hyperaccumulation has led to studies that investigate its ability to enhance metal uptake and translocation in various plant species. A study was conducted by Kerkeb and Kramer (2003) to explore the role of free histidine in xylem loading of nickel in Alyssum lesbiacum (a hyperaccumulator) and Brassica juncea L. cv Vitasso (a nonaccumulator species). The equimolar addition of L-histidine to the root medium containing 300 µM of nickel increased the translocation of nickel from root to shoot by around 75% in Brassica juncea. Kramer et al. (1996) also found that the addition of histidine in the root medium reduced the adverse effect of nickel in biomass production, root elongation, and xylem exudation, while greatly increasing nickel flux through the xylem of A. montanum.

Aminopolycarboxylic Acids

Aminopolycarboxylic acids are naturally and synthetically produced chelating agents that form high stability complexes with a range of cations. This property, combined with their apparent mobility in plant tissues, has led to impressive improvements in metal uptake by plants in the presence of these compounds. However, in some cases, aminocarboxylic acids have been recognized as causing a reduction in growth or toxic effects when applied to plants.

EDTA is a multi-dentate chelator that forms very stable complexes with divalent cations and has been popularly considered for phytoremediation applications (Hong et al., 1999; Wu et al., 2004). For example, EDTA has been shown to increase the uptake of cadmium in the shoots of B. juncea from 164 to 875 gCd/g dry weight when applied at 1 mmol/kg soil (Salt et al., 1995b). The potential for EDTA to cause toxic effects in plants and soil biota has prompted several studies. The dosage of 1.7 mmolEDTA/kg soil applied to barley (Hordeum vulgare L. ‘Weskan’) caused wilting in plant shoots and immediate cessation of root and shoot growth (Madrid, Liphadzi, and Kirkham, 2003). Soil column experiments by Kos and Lestan (2004) into the effect of EDTA on the uptake of copper from contaminated soil by Chinese cabbage (B. rapa var. pekinensis) found that a 5-mmol/kg chelator addition increased the shoot copper concentration by 190%. No reduction in biomass was measured after 42 days; however, necrotic lesions were observed on the leaves of the plant after the EDTA addition. Interestingly, de la Rosa et al. (2004)
found that the EDTA addition at levels between 17 and 428 µM did not affect the biomass accumulation or elongation of either roots or shoots of tumbleweed (*Salsola kali*) in agar studies investigating lead uptake.

NTA is an aminotricarboxylic substance known to be an easily biodegradable synthetic chelating agent for assisting phytoextraction by forming strong water-soluble complexes with a wide range of metal ions and radionuclides (Kulli *et al.*, 1999; Kayser *et al.*, 2000; Wenger *et al.*, 2002). It is more biodegradable than EDTA (Pitter and Šykora, 2001; Wenger *et al.*, 2002) and degrades as rapidly as glucose and citric acid in soils (Alkorta *et al.*, 2004; Quartacci, Baker, and Navari-Izzo, 2004). Few studies have been performed to investigate the effect of using NTA as a synthetic chelating agent to enhance metal uptake by plants from soil. Wenger *et al.* (2003) found that the application of 500 µM NTA increased the copper uptake into shoots of *Zea mays* by a factor of 26 while decreasing the root copper concentration by a factor of 3.6. Experiments by Kulli *et al.* (1999) found that high doses of NTA (5.3 mol/m²) enhanced metal uptake by lettuce and ryegrass, with concentrations in the aboveground biomass increasing by a factor of between 4 and 24 times that of control plants. Although high levels of NTA addition resulted in severe visual symptoms of toxicity and reduced biomass yield, NTA by itself was not found to be phytoxic (Shuman, Wilson, and Ramseur, 1991; Kulli *et al.*, 1999); therefore, the toxicity effects were attributed to the increased phytoavailability of the heavy metals.

EDDS is found as a naturally occurring substance (for example, it has been isolated from a culture filtrate of the actinomycete *Amycolatopsis orientalis* [Alkorta *et al.*, 2004](Alkorta *et al.*, 2004)). Among the stereo-isomers of EDDS that exist (such as SS-, RR-, RS-, and SR-arrangements), S,S-ethylenediamine-N,N′-disuccinic acid has been identified as an easily biodegradable, low-toxic chelator with a strong chemical affinity for heavy metals such as Cu and Zn to form biodegradable complexes (Takahashi *et al.*, 1997; Whitburn, Wilkinson, and Williams, 1999; Luo *et al.*, 2006). Thus, the use of this chelant in remediation processes has received much attention in the past few years (Vandevivere *et al.*, 2001; Lestan and Kos, 2003; Luo, Shen, and Lia, 2005; Meers *et al.*, 2005; Tandy *et al.*, 2006a). Soil column experiments have shown the ability of EDDS to greatly increase heavy metal uptake by plants (Grčman *et al.*, 2003). Furthermore, the same study showed that EDDS was less toxic to soil fungi and caused less stress to soil microorganisms than EDTA. Thus, the literature shows that special attention is currently needed to determine whether this new agent, EDDS⁴⁻, is capable of replacing the older generation of chelating agents such as EDTA⁴⁻ and NTA³⁻ (Jones and Williams, 2001; Kos and Lestan, 2004; Tandy *et al.*, 2006a).

**Surfactants**

Surfactants have the ability to solubilize a range of contaminants including heavy metals that are sorbed to soil particles or soil organic matter (Mulligan, Yong, and Gibbs, 2001b; Hong, Tokunaga and Kajuuchi, 2002), forming complexes with attached metal ions (Mulligan *et al.*, 2001a). Surfactants are included as potential amendments in this study in order to assess their toxicity to plants and their influence on metal uptake by roots.

Biosurfactants have been successfully used in a number of soil-washing applications for the removal of both organic (Zheng and Obbard, 2000; McCray *et al.*, 2001; Ron and Rosenberg, 2002; Urum and Pekdemir, 2004) and heavy metal contaminants (Mulligan, Yong, and Gibbs, 1999) from soil. It has been found that the heavy metals cadmium and lead preferentially associate with rhamnolipid biosurfactants over soil constituents to which they
typically bind (Frazer, 2000). Rhamnolipids can be produced by the bacteria *Pseudomonas aeruginosa*, which has been isolated from soil (USEPA, 2004). Research by Ochoa-Loza, Artiola, and Maier (2001) using anionic rhamnolipid biosurfactants demonstrated that the conditional stability constants for metal-surfactant complexes compared favorably with those for metal–organic acid complexes. They found that heavy metals such as cadmium, lead, and mercury were preferentially complexed above the metal cations naturally present in soils such as calcium, magnesium, and potassium, which would allow the removal of contaminants without stripping the soil of nutrient ions.

Synthetic surfactants such as Triton X-100 may also be used to mobilize metals from soil. Triton X-100 is a nonionic surfactant that is frequently used in biochemical applications (Sigma Chemical Company, 1993; Rajakumari, Srinivasan, and Rajasekharan, 2006). It is included in the trial as a comparison with the anionic rhamnolipid biosurfactant.

**EXPERIMENTAL METHODOLOGY**

A hydroponic experiment was carried out using 30-day-old Indian mustard (*Brassica juncea*) and perennial ryegrass (*Lolium perenne* SR4500) plants to assess their copper tolerance and uptake capacity over a 10-day study period. The seeds of both species were first germinated in a solid substrate of glass beads (ABR T-170, Syntech Distributors, Auckland, New Zealand) and supplemented with full-strength Hoagland’s nutrient solution, modified with iron supplied as ferric ammonium citrate instead of ferric tartrate. The water level of the glass-beads containers was maintained at the surface using distilled water until seeds germinated. Germination periods of 2–3 and 5–6 days were observed for Indian mustard and perennial ryegrass, respectively. After germination, the water level of the containers was maintained at approximately 1 cm below the surface of the beads. After 30 days of growth, plants were transplanted into vials containing the treatment solutions. Using PTFE tape, 10 plants were suspended in each of the vials containing 10 mg/L copper and one of the seven amendments at low, equimolar, or high concentrations (Table 1). Seven amendments were used: citric acid (Baker, Phillipsburg, NJ, USA), histidine (Ajax Finechem), NTA (Sigma), EDDS (Fluka, UK), EDTA (Sigma, St. Louis, MO, USA), synthetic nonionic surfactant Triton X-100 (Scharlau Chemie S.A., Barcelona, Spain), and an anionic rhamnolipid biosurfactant JBR425 (Jeneil Biosurfactant Company, Saukville, WI, USA).

The experiments were carried out in triplicate. Tables 1 and 3 summarize the types of chemicals and composition of different treatment solutions, while Table 2 reports copper accumulation correlations for the two plants. The control treatment included 10 mg/L copper as Cu(NO₃)₂·2.5 H₂O added to the nutrient solution. The solutions were initially adjusted to pH 6, a level found to be beneficial for both plant growth and metal solubility. Experiments were conducted with a 12-hour photoperiod at ambient an temperature ranging from 20 ± 2°C (day) to 16 ± 2°C (night). After 10 days, the plants were removed from the solution for analysis. Roots of intact plants were rinsed briefly with deionized water to remove any metals loosely adhering to root surfaces. Roots and shoots (stem and leaves) were separated and oven dried at 104°C for 24 hours. Dry weights of different plant parts were then determined. The dried plant materials were ashed at 550°C for 4 hours in a muffle furnace. Ash samples were dissolved using 2 mL of 2 M nitric acid. Acid-digested samples were diluted individually with deionized water for copper analysis by graphite furnace atomic absorption spectrophotometry (GTA-110/SpectrAA50, Varian, Inc., Palo Alto, CA, USA).
**Table 1** Concentrations of amendments trialled

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/L)</th>
<th>Treatment Level</th>
<th>Ratio (Amendment:Cu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>10.0</td>
<td>Low</td>
<td>0.3</td>
</tr>
<tr>
<td>Citric acid</td>
<td>10.0</td>
<td>Low</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>High</td>
<td>9.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>10.0</td>
<td>Low</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>33.0</td>
<td>Equimolar</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>High</td>
<td>9.1</td>
</tr>
<tr>
<td>NTA</td>
<td>10.0</td>
<td>Low</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>40.5</td>
<td>Equimolar</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>High</td>
<td>7.4</td>
</tr>
<tr>
<td>EDDS</td>
<td>10.0</td>
<td>Low</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>56.4</td>
<td>Equimolar</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>High</td>
<td>5.3</td>
</tr>
<tr>
<td>EDTA</td>
<td>10.0</td>
<td>Low</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>300.0</td>
<td>High</td>
<td>6.5</td>
</tr>
<tr>
<td>JBR 425</td>
<td>25</td>
<td>Low</td>
<td>0.5 × CMC</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>High</td>
<td>4 × CMC</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>200</td>
<td>Low</td>
<td>0.5 × CMC*</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>High</td>
<td>4 × CMC</td>
</tr>
</tbody>
</table>

*CMC represents the critical micelle concentration, which for JBR425 is 30 mg/L (Mulligan et al., 2001a) and for Triton X-100 is 400 mg/L (Wang and Mulligan, 2004a).

**RESULTS AND DISCUSSION**

The amendments can be compared on the basis of their toxicity to plants, influence on metal uptake by roots, and ability to manipulate the translocation of metals from roots to shoot tissue. It is desirable to select an amendment that facilitates a high rate of root and shoot growth, with low toxicity, while increasing the rate of metal translocation to the shoots in order to extract metals rapidly from the soil. Data have been presented relative to the control samples for each species in order to facilitate a comparison of amendment effects.

**Toxicity**

The potential toxic effects of the amendments were assessed by visual observations of the root and shoot tissue during the growth period, in addition to measurements of the root and shoot biomass production after amendment exposure. Observations were made of toxicity symptoms such as wilting, discoloration, and leaf necrosis. Dry masses of root

**Table 2** Correlation coefficients for total copper accumulation

<table>
<thead>
<tr>
<th>Total copper accumulation correlation parameters</th>
<th>Ryegrass</th>
<th>Indian Mustard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root biomass</td>
<td>0.36</td>
<td>−0.27</td>
</tr>
<tr>
<td>Shoot biomass</td>
<td>−0.015</td>
<td>−0.12</td>
</tr>
<tr>
<td>Root copper concentration</td>
<td>0.39</td>
<td>0.96</td>
</tr>
<tr>
<td>Shoot copper concentration</td>
<td>0.96</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Table 3 Speciation of copper in amendment solutions

<table>
<thead>
<tr>
<th>Amendment</th>
<th>Copper Species</th>
<th>Low (%)</th>
<th>Equimolar (%)</th>
<th>High (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTA</td>
<td>Cu^{2+}</td>
<td>17</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cu-Citrate^-</td>
<td>54</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cu-NTA^-</td>
<td>25</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>EDDS</td>
<td>Cu^{2+}</td>
<td>28</td>
<td>27</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Cu-Citrate^-</td>
<td>65</td>
<td>64</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Cu-Succinate(aq)</td>
<td>0.6</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>EDTA</td>
<td>Cu^{2+}</td>
<td>20</td>
<td>Not included</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Cu-Citrate^-</td>
<td>52</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cu-EDTA^{2-}</td>
<td>22</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>Cu^{2+}</td>
<td>17</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cu-Citrate^-</td>
<td>77</td>
<td>97</td>
<td></td>
</tr>
</tbody>
</table>

and shoot tissues were measured at the end of the growth period and compared to that of the control. Observations of the growing plants were made to assess the possible effects of toxicity caused by the amendments. From visual inspections, ryegrass seemed to generally show greater tolerance to copper and amendment treatments than Indian mustard. Citric acid did not appear to affect the health of Indian mustard stems or leaves at either the low (10-mg/L) or high (300-mg/L) level. High levels of histidine addition resulted in better growth of Indian mustard; however, at all treatment levels the edges of Indian mustard leaves took on a pink coloration after 10 days of exposure. Ryegrass showed similar growth at both high and low levels of histidine treatment, but growth was stunted at equimolar levels. Indian mustard plants in the high-level NTA treatment showed generally improved growth compared to the control and low-level treatments, whereas ryegrass was less sensitive to the treatment level, showing steady shoot growth at all treatment levels. EDDS did not have a noticeable effect on the growth of either species across the treatment levels, but ryegrass showed more vigorous growth than Indian mustard with the EDDS application. The EDTA addition resulted in wilting and necrosis of Indian mustard leaves in both high- and low-level treatments, with low-level treatments more noticeably affected. High levels of the rhamnolipid surfactant caused wilting in the stems of Indian mustard, whereas plants in the low-level treatment showed healthy growth. Both high and low levels of the synthetic surfactant Triton X-100 caused noticeable wilting of the leaves of both species after 5 days and the leaves had begun to turn brown after 10 days.

As shown in Figures 1 and 2, the species showed different responses to the amendments applied, with ryegrass generally showing increased biomass and Indian mustard decreased biomass. Ryegrass responded favorably to treatments of histidine, NTA, and EDDS, with high levels of the NTA addition encouraging the highest root growth. Indian mustard root mass significantly decreased with treatments of citric acid, EDDS, EDTA, and both surfactants, likely due to the toxic effects of these amendments, corresponding to observations during growth. The shoot mass of ryegrass increased after the amendment with histidine and EDDS, but decreased due to citric acid and Triton X-100. Both EDTA and Triton X-100 impeded the shoot growth of Indian mustard, as did histidine and EDDS, which had caused the
opposite effect in ryegrass. Histidine increased shoot biomass in ryegrass, an effect that has been previously observed in a nonhyperaccumulating species growing in the presence of toxic metals (Kramer et al., 1996). Indian mustard shoot growth was more sensitive to the amendments than ryegrass, with nearly all amendment applications resulting in decreased dry matter accumulation. From Figures 1 and 2 it can be seen that root growth is more significantly affected than shoot growth due to the addition of chemical amendments.
Copper Concentration

Histidine, NTA, and moderate treatments of EDDS significantly increased the copper accumulation in ryegrass root tissue, whereas citric acid caused the most significant increases for Indian mustard, more than 15-fold for the low-level treatment and nearly four-fold for the high-level treatment. From the results shown in Figure 3 it can be seen that low (10-mg/L) and high (300-mg/L) levels of citric acid increased the root concentrations of copper in ryegrass by 5.7 and 6.4 times, respectively. As the highest increases for any amendments, this shows the effectiveness of citric acid for increasing the concentration of copper in roots.

While copper levels in roots of Indian mustard were significantly increased by citric acid and histidine, they were decreased by high levels of the aminopolycarboxylic acids NTA, EDTA, and EDDS, although lower levels of these amendments tended to increase root copper concentrations. Increases in the level of NTA resulted in a consistent decrease in Indian mustard root copper mass of up to 87% at the highest treatment level. High levels of EDDS were sufficient to dramatically reduce the mass of copper accumulated in the root tissue by 70% and 90% for Indian mustard and ryegrass, respectively. Similarly, high levels of EDTA reduced the total root copper by around 90% for each species. These results are not unexpected, as these strong chelating agents would tend to increase the mobility and translocation of copper to shoot tissue, reducing the accumulation in roots. In comparison, the surfactants reduced copper accumulation in the roots of both species, but did not achieve this through increased copper translocation to the shoots. The surfactant molecules are large and intact micelles would not be likely to traverse cell membranes or enter cortical tissues.

Citric acid greatly increased copper levels in the shoots of both species (Figure 4). At an addition rate of 10 mg/L, citric acid increased copper levels in the shoots of ryegrass by a factor of 2.5 and more than five-fold in Indian mustard. Increasing the rate of application of citric acid from 10 mg/L (low) to 300 mg/L (high) decreased the total copper mass of Indian mustard shoot tissues by 70% and that of ryegrass by 38%. As it is a low molecular weight organic acid and produced naturally by plants as part of the tricarboxylic acid cycle.

![Figure 3: Effect of chemical amendments on concentration of copper in root tissue.](image-url)
AMENDMENTS FOR ENHANCING COPPER UPTAKE

Figure 4 Effect of chemical amendments on concentration of copper in shoot tissue.

(Nobel, 2005), citric acid would be expected to be mobile in plant tissues and participate in biochemical reactions.

The addition of histidine and EDDS did not cause any significant variation of shoot copper levels in Indian mustard; however, in ryegrass the high rate of the addition of histidine and EDDS consistently enhanced the shoot copper levels, showing approximately 2.9- and two-fold increases, respectively. The total copper mass in ryegrass shoots was noticeably improved with higher histidine doses, with a 300% increase observed as histidine levels were raised 30-fold. NTA treatment did not cause a consistent influence on ryegrass and Indian mustard shoot copper mass, with only the equimolar treatment of NTA having a significant effect (increases in copper concentration of 80% in ryegrass and 200% in Indian mustard). For both plant species, the most significant effect on copper levels in shoot tissue was observed with high levels of EDTA addition. EDTA consistently increased the concentration of copper in plant shoots. At low levels (10 mg/L) it affected a three-fold increase in shoot copper levels in ryegrass and a nearly nine-fold increase in Indian mustard. The effectiveness was even greater at high levels, with levels of copper in shoots of Indian mustard increasing 14-fold to more than 1200 µg/g and ryegrass shoot copper concentrations reaching nearly 1400 µg/g—greater than 0.1% copper by dry weight of tissue after 10 days of exposure. The effectiveness of these amendments may be further realized in soil, where complex-formation results in increased solubilization of metals from soil and higher concentrations in the soil solution (Tandy, Schulin, and Nowack, 2006b).

The use of surfactants as amendments did not result in any improvement in total shoot copper mass in either species, but instead led to a 70% reduction in Indian mustard shoots with high (300 mg/L) treatment of Triton X. Low levels of both surfactants significantly decreased shoot copper concentrations in ryegrass to around half the level in control treatments.
Figure 5  Correlation between copper concentration and total mass of copper in shoot tissue.

Mass Accumulation

Analysis of the total mass of copper accumulated in root and shoot tissue provides an indication of the overall effectiveness of the amendment treatment. The quantity of metal extracted from soil by plant tissue has direct bearing on the time required for phytoextraction to achieve target contaminant levels. The total mass of copper in plant tissues is due to the influence of amendments on both biomass production and metal accumulation in tissue. The results (see Table 2) show that mass removal patterns are more closely correlated with tissue metal concentrations than the tissue mass. This is also reflected in Figure 5, which shows a strong correlation (0.96–0.98) between the mass and concentration of copper in shoot tissue for both species. This indicates that mass accumulation is less dependent on shoot growth than on the sequestration of copper in shoot tissue.

Translocation

The translocation coefficient is expressed as the ratio of the content of copper in the shoots to that in the roots on either a concentration or total mass basis. The coefficient is a useful indication of the ability of amendments to effect the translocation of metals to shoot tissue.

Copper translocation from root to shoot in ryegrass and Indian mustard differed widely by amendment (Figure 6). The addition of citric acid (10 and 300 mg/L) caused the most noticeable reduction of copper translocation to shoots in both ryegrass and Indian mustard. Mass-based translocation was not as greatly affected, due to the reduction of root biomass in citric acid treatments, which kept the total copper mass low despite the high concentrations of copper present in the root tissue. Histidine did not enhance copper translocation in Indian mustard at any treatment level; however, in ryegrass higher histidine levels brought about an increase in the mass- and concentration-based translocation coefficients. Compared to low and high treatments, the application of NTA at equimolar concentration proved to be effective for enhancing copper translocation to ryegrass shoots, with an increase of 130%
over unamended plants. The addition of NTA also improved copper translocation in Indian mustard, with high-dose (300 mg/L) NTA resulting in an 1100% increase compared to the low-level (10-mg/L) NTA treatment.

The treatments that caused the greatest increase in translocation of copper were the highest levels of EDTA and EDDS, which respectively resulted in mass translocation rates in ryegrass that were 74 and 35 times greater than the control treatment. In Indian mustard, however, the effects of EDDS on translocation were not as great, with only a 3-fold increase in the translocation coefficient. Speciation analysis of the nutrient solution was carried out for citric acid, NTA, EDDS, and EDTA using Visual MINTEQ version 2.52 (obtained from www.lwr.kth.se/english/oursoftware/vminteq/), which was used to estimate the fraction of copper present as different species in the exposure solutions. The results of the speciation modeling (Table 3) indicate that Cu-EDDS would have only been present in the bulk solution at low concentrations (14% of total copper). The effects of EDTA were far greater, with the translocation coefficient increasing to nearly 67 times that of untreated plants on a concentration basis (110 times greater on a mass basis). EDTA and EDDS had been previously found by Kos and Lestan (2003) to increase lead concentrations in *B. rapa* by 158 and 89 times control levels, respectively. The NTA addition at equimolar and high (300-mg/L) levels resulted in significantly increased translocation of copper in Indian mustard, with translocation rates increasing to 5.6 and 13.6 times, respectively, that of the control. Mass-based translocation in ryegrass, however, was not significantly affected.

For both species, influences of the surfactants on copper translocation were not significant. This is not surprising, as the large size of the surfactant molecules (Table 4), particularly when aggregated into micelles, would prevent their entry into the small pores in the root tissue (Marschner, 1995). Additionally, these molecules would be unlikely to traverse the cell membranes of the root tissue for transport into and within the vascular tissue. There was no correlation between the copper-amendment stability constants (shown

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**Figure 6** Effect of chemical amendments on translocation of copper from roots into shoot tissue.
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Molar Mass</th>
<th>Molecular Formula</th>
<th>Cu:L ratio</th>
<th>Log K</th>
<th>Relative Biodegradability</th>
<th>Ref$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>292.24</td>
<td>C_{10}H_{16}N_{2}O_{8}</td>
<td>1:1</td>
<td>18.7</td>
<td>Low: Requires specific strains and conditions</td>
<td>1, 2</td>
</tr>
<tr>
<td>EDDS</td>
<td>292.22</td>
<td>C_{10}H_{13}N_{2}O_{8}</td>
<td>1:1</td>
<td>18.36</td>
<td>High: Biodegradable as free ligand, but not as Cu complex</td>
<td>1, 2</td>
</tr>
<tr>
<td>NTA</td>
<td>191.14</td>
<td>C_{6}H_{6}NO_{6}</td>
<td>1:2</td>
<td>17.42</td>
<td>Relatively high</td>
<td>1, 2</td>
</tr>
<tr>
<td>Citric Acid</td>
<td>192.12</td>
<td>C_{6}H_{8}O_{7}</td>
<td>1:1</td>
<td>5.9</td>
<td>High: Biodegradable as free ligand, but not as Cu complex</td>
<td>1, 3, 4, 5</td>
</tr>
<tr>
<td>Histidine</td>
<td>155.15</td>
<td>C_{6}H_{9}N_{3}O_{2}</td>
<td>1:2</td>
<td>18.1</td>
<td>High</td>
<td>1, 3, 6</td>
</tr>
<tr>
<td>JBR 425</td>
<td>504–650</td>
<td>C_{26}H_{48}O_{8}, C_{12}H_{26}O_{13}</td>
<td>9</td>
<td>9.27</td>
<td>High</td>
<td>7, 8</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>625</td>
<td>C_{8}H_{17}C_{6}(OC_{2}H_{4})<em>{10}OH, C</em>{8}H_{17}C_{6}(OC_{2}H_{4})_{10}OH</td>
<td></td>
<td></td>
<td>Partial</td>
<td>7, 8, 9</td>
</tr>
</tbody>
</table>


$^*$Stability constants are given for the most stable form with copper at an ionic strength of 0.1 and a temperature of 25°C (with the exception of citric acid at 20°C).
in Table 4) and the mass of copper in shoot tissue, with only a weak correlation (around 0.5) between stability constants and translocation rates for both species.

There are clear differences between the two species tested in terms of tolerance and translocation. Observations and measurements of toxicity and biomass production revealed that ryegrass shows greater tolerance to the amendments trialled, potentially making ryegrass a more suitable species for the phytoremediation of copper. However, for Indian mustard, translocation coefficients for the control plants were double that of ryegrass, on both a mass and a concentration basis. This trend continued across all amendment treatments, indicating that Indian mustard has a naturally higher rate of solute translocation. This may be due to differences in the transport or uptake mechanisms, such as the generally higher cation exchange capacity of dicotyledons (including Indian mustard) over monocotyledons (such as ryegrass) (Crooke, 1964), higher transpiration rate, or increased solute transfer from solution to the vascular system.

A summary of the effects of amendments on the key growth and uptake parameters for each species is shown in Table 5.

CONCLUSIONS

Chemically enhanced phytoextraction has been identified as a promising technology to remediate contaminated soil. Over the past few decades, trials have been conducted with a variety of plants and chemicals to study their effectiveness for increasing the uptake and translocation of metals to clean contaminated soils and water. The results of the present study have shown the potential of several types of amendments (organic and amino acids, aminopolycarboxylic acids, and surfactants) for enhancing copper accumulation by roots and translocation to the shoots in ryegrass and Indian mustard while improving biomass yield.

From the current study with *B. juncea* and *L. perenne*, NTA has been identified as one of the most promising amendments for use in phytoextraction. NTA addition was beneficial to root and shoot growth of both plant species, with no visible toxic effects. Additionally, NTA significantly increased root growth in ryegrass, which is an important benefit for field applications as roots can explore a larger volume of soil for contaminant interception. The NTA addition also resulted in higher rates of copper translocation to shoot tissue, with the equimolar application resulting in the highest mass uptake. EDTA was responsible for the greatest increases in copper accumulation and translocation, although root and shoot growth were detrimentally affected at both levels. Although citric acid did not increase translocation rates due to the large increases in root copper concentrations, shoot copper levels were greatly improved for both species. EDDS was only effective for increasing translocation rates at high doses and affected biomass production differently for each species—enhancing ryegrass growth but depressing the growth of Indian mustard. However, the reduced influence of EDDS is thought to be a function of the low proportion of the Cu-EDDS species in solution and it is expected that the effectiveness of EDDS in increasing metal translocation would be far greater if competing ligands were not present. Although both surfactants proved to be ineffective for enhancing metal accumulation by the plants, these may still be useful for enhancing metal mobilization from soil particles. In general, ryegrass showed greater tolerance than Indian mustard to the application of amendments. When applied to soils, amendments would have the additional influence of increasing metal solubility and availability in soil solution.
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Toxicity</th>
<th>Mass Uptake</th>
<th>Translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Biomass</td>
<td>Root</td>
</tr>
<tr>
<td></td>
<td>IM</td>
<td>RG</td>
<td>IM</td>
</tr>
<tr>
<td>Citric L</td>
<td>na</td>
<td>na</td>
<td>–</td>
</tr>
<tr>
<td>Citric H</td>
<td>na</td>
<td>na</td>
<td>+</td>
</tr>
<tr>
<td>Histidine L</td>
<td>+</td>
<td>na</td>
<td>–</td>
</tr>
<tr>
<td>Histidine E</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Histidine H</td>
<td>++</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>NTA L</td>
<td>na</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>NTA E</td>
<td>na</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>NTA H</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EDDS L</td>
<td>na</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EDDS E</td>
<td>na</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EDDS H</td>
<td>na</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EDTA L</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>EDTA H</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>JBR425 L</td>
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<td>na</td>
<td>na</td>
</tr>
<tr>
<td>JBR425 H</td>
<td>–</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>TritonX-100 L</td>
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<td>–</td>
</tr>
<tr>
<td>TritonX-100 H</td>
<td>–</td>
<td>–</td>
<td>na</td>
</tr>
</tbody>
</table>

Legend:

- % change
- 0–10% na
- 10–50% –
- 50–100% ––
- >100% –––

- Detriment
- 0–10% na
- 10–50% –
- 50–100% ––
- >100% –––

- Improvement
- na
- +
- ++
- +++

Table 5: Effects of amendments on key parameters for Indian mustard (IM) and perennial ryegrass (RG)
REFERENCES


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