EDDS-enhanced metal phytoextraction by *Brassicaceae* species. Uptake and translocation of CuEDDS complexes by *Brassica carinata* and responses at transcriptional and metabolic levels

Benedetta Cestone

in a joint supervision of

The University of Pisa

Doctor of Philosophy
in *Crop Production*

Hasselt University

Doctor of Philosophy
in *Science*

Supervisors

Prof.dr. Flavia Navari-Izzo

Prof.dr. Jaco Vangronsveld

Dr. Mike Frank Quartacci

PhD Student: Dr. Benedetta Cestone

From January 2008 to December 2010

Supervisors

Prof.dr. **Flavia Navari** and Dr. **Mike Frank Quartacci**
Department of Crop Plant Biology
University of Pisa
Italy

Prof.dr. **Jaco Vangronsveld**
Centre for Environmental Sciences
Hasselt University
Belgium

DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

Pisa, October 2011

Dr. Benedetta Cestone

TABLE OF CONTENTS

SUMMARY

The present research deals with the phytoremediation of a metal-contaminated, markedly degraded area located at Torviscosa (Udine, North-East Italy), used since the Thirties as a dump for pyrite cinders derived from ore roasting for sulphur extraction. Currently, this site is included within the perimeter of the polluted area named "Grado and Marano lagoon and neighbouring water-courses" identified as a site of national interest for restoration by the Italian Cabinet Decree 468/2001 "National Programme for Environmental Restoration of Polluted Sites". In accordance with the Italian legal thresholds for the agricultural uses (Ministerial Decree 152/2006), As, Co, Cu, Pb and Zn resulted the metals present at toxic levels. The pyrite cinders extends for a depth of 0.7 m over a deep horizon of impermeable clay and has been covered with a less polluted gravelly soil 0.15-0.2 m in depth. Because of the substrate composition, poor physical structure, nutrient deficiency, and extreme hydrological conditions, the site is colonized only by a rare and inadequate vegetation cover.

The cleaning up of this dismissed dump has required the selection of the right dosage and time application of suitable chelants as well as the selection of high biomass species able to enhance metal accumulation capacities and to prevent the possible movement of metal complexes into groundwater without any growth depression induced by metal toxicity. (S,S)-N,N'-ethylenediaminedisuccinic acid (EDDS), an EDTA structural isomer, has been considered for a chelant-enhanced phytoextraction programme because it is a strong chelant and unlike EDTA it is easily biodegradable. The phytoextraction effectiveness was investigated using the two accumulator crops *Raphanus sativus* L. var. *oleiformis* cv Siletta nova and *Brassica carinata* A. Braun cv 180. In order to reclaim the asphyxial soil conditions and allow the plant growth, these crops were grown in opaque PVC pots filled with the multiple polluted cinders

mixed with sand (1:1, w/w) and amended with single (2.5 or 5 mmol kg^{-1}) or repeated doses (1 mmol kg^{-1}) of EDDS distributed in different growth stages. The lower single application, one week before harvest, and *B. carinata* resulted more effective in metal phytoextraction (+31% compared to not amended plants) because no metal leaching and dry biomass reduction occurred. Among the metals present in the cinders, Cu – which exceeded 14-fold the national legal limits – showed the highest rate of complexation with EDDS (log K 18.4). Therefore, to improve metal phytoextraction, the key mechanisms of chelant-stimulated metal acquisition, uptake and translocation of CuEDDS complexes by *B. carinata* were studied both in excised and intact roots. Cu influx into the plants is represented by an active biphasic mechanism and to better illustrate the two influx mechanisms 30 and 150 µM copper concentrations were chosen. Then, Cu, EDDS and CuEDDS uptake kinetics of *B. carinata* excised roots incubated in solutions containing 30 or 150 µM of either the metal, the chelant, and the complex were determined in presence or not of the ATPase inhibitor vanadate. Following both Cu and CuEDDS incubations, metal uptake was negatively influenced by vanadate, while EDDS uptake did not, suggesting that Cu and EDDS did not enter the roots in their complexed form but by two different pathways. In fact, CuEDDS treatment determined a lower Cu uptake compared to the metal alone because the complex had probably to be split before Cu uptake. The treatments in the same solutions of *B. carinata* intact plants showed that the CuEDDS treatment resulted in a lower Cu concentration in comparison with the metal alone, but in a higher metal translocation (3- and 10-fold, respectively, at 30 and 150 µM), confirming that the uptake routes of Cu and EDDS were different. To gain a better understanding of Cu uptake, distribution and tolerance in *B. carinata* roots and leaves, spatial distribution of Cu was studied by micro-PIXE technique. EDDS-assisted Cu uptake and transport resulted in preserved root endodermal barrier indicating that CuEDDS complexes were less toxic to the plant than free Cu ions. At the same time, EDDS increased

Cu translocation from roots to shoots at the highest concentration. In addition, in *B. carinata* leaves Cu accumulated preferentially in vascular tissues, inhibiting metal translocation to photosynthetically active mesophyll cells and conferring, therefore, tolerance of to Cu. Finally, the metabolic and transcriptional responses in *B. carinata* exposed to 30 μM $CuSO_4$ or CuEDDS were studied. Antioxidants together with transcriptional levels and activities of antioxidative enzymes were investigated for their role in reducing the highly harmful potential of reactive oxygen species (ROS). Free Cu ions resulted more cytotoxic than CuEDDS and the plants reacted positively to CuEDDS exposure, in contrast with $CuSO_4$, developing adaptive mechanisms by adjusting gene expression and showing tolerance to oxidative stress.

INTRODUCTION

As a result of human activities, the development of the modern industry and agriculture has promoted the release of metals in soils causing environmental pollution and crop yield reduction. At present, the legislation on environmental protection and management of many countries is focusing increasing attention on the less expensive and more environmentally-friendly biological methods of restoration, compared to the more expensive and environmentally invasive conventional ones. Cleaning up of the environment by removing these contaminants needs effective approaches that allow for a precise restoration of polluted sites. In particular, the use of biodegradable chelants in improving metal uptake by plants and in limiting metal leaching from soil has been proposed as an effective tool to remediate metal-polluted soils.

Metal pollution and remediation

Pollution of the biosphere with trace elements (toxic metals and metalloids) has accelerated dramatically since the beginning of the Industrial Revolution (Padmavathiamma and Li, 2007; Kidd *et al*., 2009). The primary sources of these contaminants are the aerial deposition from burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, and sewage (Wei and Zhou, 2008). In addition to sites contaminated by human activity, natural mineral deposits containing particularly large quantities of toxic metals are present in many regions of the globe (Memon and Schröder, 2009). These polluted areas often support characteristic plant species that thrive in these metal-enriched environments. Whereas many species avoid the uptake of metals from these soils, some of these species can accumulate significantly high concentrations of toxic metals, to levels which by far exceed the soil levels (Baker and Brooks, 1989). Fe, Mn, Zn, Cu, Mo, and Ni are essential micronutrients for normal plant growth and development, although they can

become toxic when present in excess (Hall and Williams, 2003). Certain plants also have the ability to accumulate nonessential elements which have not known biological functions, such as As, Cd, Cr, Pb, Co, Ag, Se and Hg, (Baker and Brooks, 1989; Memon and Schröder, 2009; Navari-Izzo and Rascio, 2010). These metals and metalloids become an environmental concern when their concentrations in soil, sediment, or water begin to affect human health as well as plant and animal ones. In the more heavily contaminated sites there is often a significant risk of *off-site* migration of the contaminants due to erosion and leaching of pollutants into groundwater (Vangronsveld and Cunningham, 1998). Remediation of metals presents different problems when compared to organics. In contrast to many organic pollutants, trace elements present a long residence time in the soil system (Kidd *et al*., 2009) and can be eliminated only by removing or immobilizing them (Navari-Izzo and Quartacci, 2001). Environmental restoration of contaminated soils with conventional, civil-engineering methods (based on technique such as leaching of pollutant, solidification/stabilization, size selection and pyrometallurgical processes, electrokinetical treatment, chemical oxidation/reduction of pollutant, excavation) is expensive and environmentally invasive, and demands extreme investments of economic and technological resources (Quartacci *et al*., 2003). A remediation technique that is of low cost, environmentally sound, and equally protective of human health and the environment would be a valuable addition to current remediation methods. The idea that plants can be used for remediation of contaminated soils is an attractive and promising approach. In fact, plants have acquired through constitutive and/or adaptive mechanisms the ability to tolerate and accumulate very high concentrations of contaminants. Phytoremediation is thus based on the use of plants and their associated microorganisms to remove, stabilize, or detoxify pollutants. According to several authors the cost of remediation per contaminated hectare of soil using conventional techniques ranges between 0.27 and 1.6 million $, while phytoremediation costs from about 100-1000 times

less (Kidd *et al*., 2009). The term phytoremediation includes several subsets (rhizofiltration, phytostabilisation, phytovolatilisation, phytodegradation, and phytoextraction): this research is focused on phytoextraction.

Phytoextraction and EDDS

Phytoextraction seems to be a simple and economic technique for the remediation of metal polluted soils. Phytoextraction (Figure 1) is a phytoremediation technique that uses metal-accumulating plants to remove metals and other contaminants from soils, sediments or water, followed by translocation to aboveground plant tissues, which are subsequently harvested.

Harvested plant material may be used to recover valuable metals or can be burnt and the ashes disposed of under controlled conditions or in some cases recycled as a fertilizer (Keller *et al*., 2005). This technique is only effective if the plants accumulate large concentrations of metals/metalloids in shoots and have a reasonable biomass production (McGrath and Zhao, 2003). Phytoextraction is best suited for the remediation of diffusely polluted sites, where contaminants occur superficially and at a relatively low concentration (Kidd *et al*., 2009). In addition, successful phytoextraction requires that the polluted medium is cleaned to a level that complies with environmental regulations, and from an economic viewpoint, this should be achieved at a lower cost than an alternate technology or the cost of inaction (Robinson *et al*., 2003). Nevertheless, ongoing research reveals that the

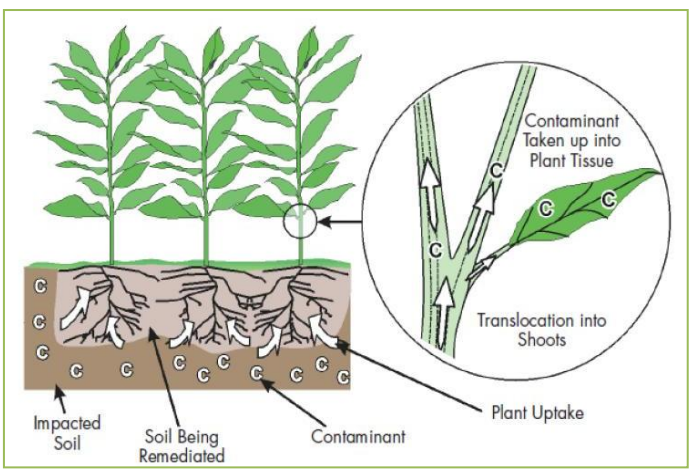


FIGURE 1. Phytoextraction technique.

applicability of the technique might be limited and that the practical implications might not be so evident as first thought. At present, the technology is limited by the long period required for cleanup, the restricted number of target metals that can be extracted, the limited depth that can be assessed by the roots, the difficulty of producing a high-biomass crop of the desired species and the lack of knowledge on the agronomic practices and management of accumulating plants (McGrath *et al*., 2006; Robinson *et al*., 2006). The two strategies of phytoextraction application are: long-term continuous and induced or chelant-assisted phytoextraction. The first one (Figure 2a) uses plants that accumulate high levels of pollutants over their entire lifetime, whereas the second one (Figure 2b) enhances pollutant accumulation towards the end of the plant's lifetime, when they attain their maximal biomass, by adding chelators to the soil that reversibly bind the pollutant (usually a metal), releasing it from the soil and making it available for plant uptake.

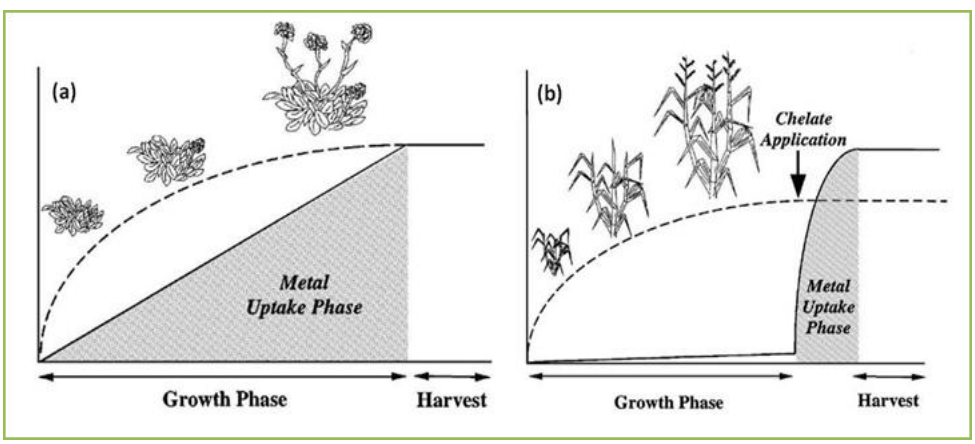


FIGURE 2. Long-term continuous (**a**) and chelant-assisted (**b**) phytoextraction. From Salt *et al*., 1998.

Research in long-term continuous phytoextraction has largely focused on the use of hyperaccumulator plants. Hyperaccumulators are excellent candidates for phytoextraction since they accumulate huge amounts of trace metals in their aboveground biomass when growing in metal-enriched habitats (mg kg^{-1}: > 10,000 (Mn or Zn), > 1000 (Cu, Co, Cr, Ni, Pb) or > 100 (Cd)) (Baker and Brooks,

1989; Kidd *et al*., 2009). To date, about 400 plant species have been identified as metal hyperaccumulators (McGrath and Zhao, 2003; Navari-Izzo and Rascio, 2010), but their potential use in phytoextraction presents several limitations. Factors such as a slow growth rate, shallow root system, small biomass production together with element selectivity in hyperaccumulation, little knowledge about their agronomic features, pest management, genetics and breeding potential, biochemical and physiological processes, limit the use of these species (Navari-Izzo and Quartacci, 2001; Kidd *et al*., 2009). A further limitation is the potential contamination of the food chain if animals graze on the metal-contaminated plants. Hyperaccumulator plants with a very low biomass (0.6 g plant^{-1}) such as *Thlaspi caerulescens* (a Zn/Cd hyperaccumulator with a 3% metal content) could never be used for phytoextraction unless researches will find agronomic or genetic inputs to increase their biomass. *Thlaspi caerulescens* can only represent a model to study the mechanisms of metal accumulation, tolerance and detoxification in higher plants (Navari-Izzo and Quartacci, 2001). More recently, some genotypes of high biomass crop species (such as *Nicotiana*, *Salix*, *Populus* or *Brassica*) which tolerate high concentrations of more trace metals and show sufficient metal accumulation have been proposed as a viable alternatives to hyperaccumulators in phytoextraction technologies (Keller *et al*., 2005; Meers *et al*., 2007; Quartacci *et al*., 2007; Memon and Schröder, 2009). Enhancing metal accumulations without reducing the yield is a potential strategy in the development of phytoextraction. In addition, the frequent occurrence of metals in non-labile forms can severely limit phytoextraction. Since metal solubility and availability are both dependent on soil characteristics and are strongly influenced by pH and the degree of complexation with soluble ligands (Nascimento and Xing, 2006), soil acidification (Dominquez *et al*., 2009; Quartacci *et al*., 2009) and amendment with chelators (Grčman *et al*., 2001; Quartacci *et al*., 2007) could potentially improve the effectiveness of conventional metal phytoextraction. Chelating agents, already employed in

agriculture, such as ethylenediaminetetraacetic acid (EDTA), have been used for a long time in the so-called assisted phytoextraction processes. Although EDTA is widely recognized as the most efficient chelant to increase metal uptake by plants (Leštan *et al*., 2008), limited plant metal uptake, high persistence (poor photo-, chemo- and biodegradability) and potential leaching to groundwater pose severe environmental concerns connected with the use of EDTA and its derivates (Wu *et al*., 2004; Quartacci *et al*., 2006, 2007; Tandy *et al*., 2006; Meers *et al*., 2008). In recent years, aminopolycarboxylic acids such as (S,S)-ethylenediaminedisuccinic acid (EDDS) has been proposed as alternative to EDTA and other persistent synthetic chelants (Quartacci *et al*., 2006, 2007; Tandy *et al*., 2006; Evangelou *et al*., 2007; Luo *et al*., 2008; Meers *et al*., 2008). EDDS is a structural isomer of EDTA and has two chiral carbon atoms and three stereoisomers, but among them, only the (S,S) isomer is readily biodegradable (Irtelli, 2007; Quartacci *et al*., 2007). However, EDDS has the potential to be a substitute of EDTA as it is a strong chelant and unlike EDTA it is easily biodegradable (Quartacci *et al*., 2007, 2009), showing a half-life of 2.5 d in a field experiment (Meers *et al*., 2008). Even if the use of EDDS increases phytoavailable metal concentrations in the soil, its application should be kept to a minimum since the plants are only able to take up a small amount of metals. In order to minimize the main drawbacks and environmental concerns related to risk of leaching and plant toxicity, right time and dosage of application of chelant have to be taken into consideration with the purpose of decreasing the amounts of EDDS in the soil without decreasing the phytoextraction effectiveness. Strategies to control leaching have already been suggested. Kos and Leštan (2004) successfully tested permeable barriers with reactive materials, nutrient enriched vermiculite, peat or agricultural hydrogel and apatite. Other authors proposed to collect the metal-enriched drainage water using a dual-pipe subirrigation–drainage system, and recycle for further phytoremediation (Madrid *et al*., 2003). These techniques, however, are very expensive and would

diminish the highly economical efficiency of phytoextraction, one of its major advantages. Since few field studies, partially with antithetic results, have been performed over several years, many long-term questions, such as the influence of chelates on the crop growth, remain (Evangelou *et al*., 2007). Additionally, studies so far have shown that not all plants react in the same way to the chelating agents applied, because the added chelants might vary their effectiveness depending on their toxicity to plants

Metal tolerance

Several studies have been directed to understand the mechanisms of tolerance of plants to an elevated metal concentration without causing any metabolic alteration of the plants. In order to maintain the concentration of essential metals within physiological limits and to minimize the detrimental effects of nonessential metals, plants have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detossification of metals (Clemens, 2001). Only certain plant species and genotypes posses a naturally selected hyper tolerance toward particular metals different from the basal tolerance common to all plant species and varieties, thanks to that they can survive and thrive in metal-rich soils (Hall, 2002; Navari-Izzo and Rascio, 2010). Furthermore, some plants can grow on soils enriched in combinations of different metals and this tolerance could result from a less specific mechanism that confers a broad resistance to several different metals (co-tolerance) or may involve a series of independent metal-specific mechanisms (multiple tolerance) (Hall, 2002). Basic strategies include exclusion, efflux of toxic metal ions, immobilization, compartmentalization and metal chelation, reduction of metal transport, and expression of other general stress response mechanisms. In addition, plants produce two classes of metal binding proteins, metallothioneins (MT_s) and phytochelatins (PC_s), which play a significant role in tolerance (Robinson *et al*., 2009; Navari-Izzo and Rascio, 2010).

In general, the tolerance mechanisms are essentially metal-specific due to the distinct chemical properties of the various metals. Higher plants employ two basic strategies to tolerate metals: (i) avoidance or exclusion, which restricts the uptake and/or root to shoot transport; and (ii) accumulation and sequestration, which allow plants to survive accumulation and detoxify metals in the shoots by compartmentation of metals in the vacuole, by complexation of metals by organic ligands such as organic acids, amino acids, and metal-binding peptides (Clemens, 2001; Hall, 2002).

Metal toxicity

Excessive concentrations of both essential and nonessential elements in the soil can lead to toxicity symptoms and the inhibition of growth and nutrient uptake of most plants due to a range of interactions at the cellular/molecular levels, besides metabolic disorders induced by metal toxicity (Hall, 2002). At low metal concentrations, the plant cell can resort to a number of avoidance mechanisms such as metal exclusion, translocation and complexation in the cytoplasm (Vangronsveld and Clijsters, 1994). At high concentrations, when primary barriers are broken down, avoidance is insufficient, free metal concentration increases and both redox and nonredox metals can stimulate production of reactive oxygen species (ROS) imposing oxidative stress (Navari-Izzo and Rascio, 2010). The accumulation of ROS, such as $^{1}O_2$, $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$, during abiotic stresses was long considered to be a by-product of stress metabolism as well as an overall unwelcome of by-product of aerobic metabolism (Miller *et al*., 2008). A growing body of evidence indicates that various toxic metals act as catalyst in the oxidative deterioration of biological macromolecules, and therefore the toxicity associated with these metals may be due, at least in part, to oxidative damage to the plant tissues (Navari-Izzo and Rascio, 2010). Some studies have shown that metals such as Cr, Cu, Fe, Hg, Ni, Pb and V exhibit the capacity to increase the production of ROS, resulting in lipid

peroxidation (Quartacci *et al*., 2001), DNA damage, depletion of sulphydryl groups and altered calcium homeostasis (Navari-Izzo and Quartacci, 2001).

Metal tolerance in *Brassicaceae* species

One of the problems in the application of phytoextraction is the selection of effective plants with a high capacity for multiple metal bioaccumulation. The concomitant presence of different metals has a negative influence on the plant physiology (assimilation and growth) and also on the phytoextraction efficiency, probably due to the additive effects on plant metabolism (Marchiol *et al*., 2004). A lot of attention has been focused on the selection of plants able to tolerate high amounts of different metals as in multiple metal-polluted soils without any reduction of their biomass and metal uptake. In the last years, the potential use of accumulator crops as alternatives to hyperaccumulators, which generally fails in multiple metal-contaminated sites, is currently an option under study in the phytoextraction programmes. Then, features as metal uptake efficiency and translocation rate from the soil to the harvestable biomass are directly inherited from some *Brassicaceae* ancestors in accordance with the large number of hyperaccumulators belonging to this family, such as *Thlaspi caerulescens* (Marchiol *et al*., 2004; Vamerali *et al*., 2011). The *Brassicaceae* family is interesting also for the high content of thyocianates in their aboveground tissues, which makes species non-palatable to animals, characteristic that is likely to reduce the chances of metal bioaccumulation and biomagnitudo in the food chain transfer during phytoextraction (Navari-Izzo and Quartacci, 2001). In particular, in this research two close species have been studied and compared, ethiopian mustard (*Brassica carinata*) and fodder radish (*Raphanus sativus*) (Figure 3). The two metal accumulator species both belonging to the botanical family of *Brassicaceae* adapt well to Mediterranean climate and have been almost suggested for the remediation of multiple metal-polluted sites, accumulating moderate levels of several metals in their shoots (Marchiol *et al*.,

2004; Mosca *et al*., 2004; Quartacci *et al*., 2007; 2009; Bandiera *et al*., 2010; Vamerali *et al*., 2011).

FIGURE 3. Ethiopian mustard (*Brassica carinata*) (**a**) and fodder radish (*Raphanus sativus*) (**b**).

The case of copper

Plants require Cu as a redox-active transition metal essential to maintain normal growth and development, and to ensure the completion of their life cycles. Copper is required for plant nutrition only in trace amounts and at high concentration can become strongly phytotoxic to cells due to its redox properties (Sgherri *et al*., 2001, 2007; Smeets *et al*., 2009; Yruela, 2009; Cuypers *et al*., 2011). Typically, critical deficiency levels are in the range of 1-5 μg g^{-1} dry weight and the threshold for toxicity is above 20-30 μg g^{-1} dry weight (Marschner, 1995). Because of its redox potential, Cu can exist in both the Cu^{2+} and Cu^{+} form in living organisms: Cu^{2+} is often linked to nitrogen in histidine side chains, while Cu^{+} interacts with the sulfur present in cysteine or methionine (Yruela, 2009). Toxic level of Cu occurs naturally in some soils, whereas other soils may contain toxic levels as a result of anthropogenic release of metals into

environment through application of pig and poultry slurries, fertilizers, fungicides, industrial and urban activities, metalliferous mining or metal processing, and waste disposal technologies (Yruela, 2009). At the same time, the Cu reversible oxidation–reduction makes it very useful in the cells. Cu can act as a structural constituent in regulatory metalloproteins playing a role in photosynthetic electron transport chains, mitochondrial respiration, oxidative stress responses, chlorophyll biosynthesis, carbohydrate and cell wall metabolisms and hormone signaling. Cu ions can also be cofactor and/or part of prosthetic groups of many enzymes, such as Cu/Zn superoxide dismutase, cytochrome *c* oxidase, ascorbate oxidase, amino oxidase, laccase, plastocyanin and polyphenol oxidase. At cellular level, Cu plays a role in signaling to the transcription protein trafficking machinery, oxidative phosphorylation, iron mobilization and biogenesis of molybdenum cofactor (Sgherri *et al*., 2001; Yruela, 2009). Despite being an essential micronutrient, redox cycling between Cu^{2+} and Cu^{+} can catalyze a high production of ROS through the Fenton and Haber-Weiss reactions, inducing oxidative stress (Sgherri *et al*., 2001, 2007; Navari-Izzo *et al*., 2006). Free radicals so produced may initiate peroxidation of polyunsatured fatty acids, damaging cells at level of lipids, nucleic acids, proteins and other biomolecules (Quartacci *et al*., 2001; Navari-Izzo *et al*., 2006). It has been seen that although Cu exposition could not cause visible symptom of toxicity, when in excess it could lead to chlorosis and necrosis, stunting, plant growth inhibition or, in some cases, even death (Navari-Izzo and Quartacci, 2001; Quartacci *et al*., 2001). Cu excess levels are well known to act on photosynthetic membranes, but Cu causes injure first of all to plasma membrane (PM) level and its lipid composition. PM is the first functional structure in contact with Cu and it is thought to play a critical role in plant metal tolerance (Quartacci *et al*., 2001). In fact, PM lipid composition changes its fluidity with environmental fluctuations controlling, in this way, membrane permeability and its efficiency as a semi-permeable membrane (Meharg, 1993). Cu ions can damage PM with oxidation

and binding to the sulphydryl groups of membrane proteins, because it is not protected by intracellular defence mechanisms such as those present on the cell inside (metal exclusion; translocation to the vacuole; complexation in the cytoplasm with organic acids, peptides like phytochelatins). Cu-stimulated ROS can be generated by the action of several enzymes bound or associated with the cell PM, besides those connected to electron transport system. Among these, we have lipoxygenases (LOX) and NAD(P)H oxidases (Quartacci *et al*., 2001; Smeets *et al*., 2009).

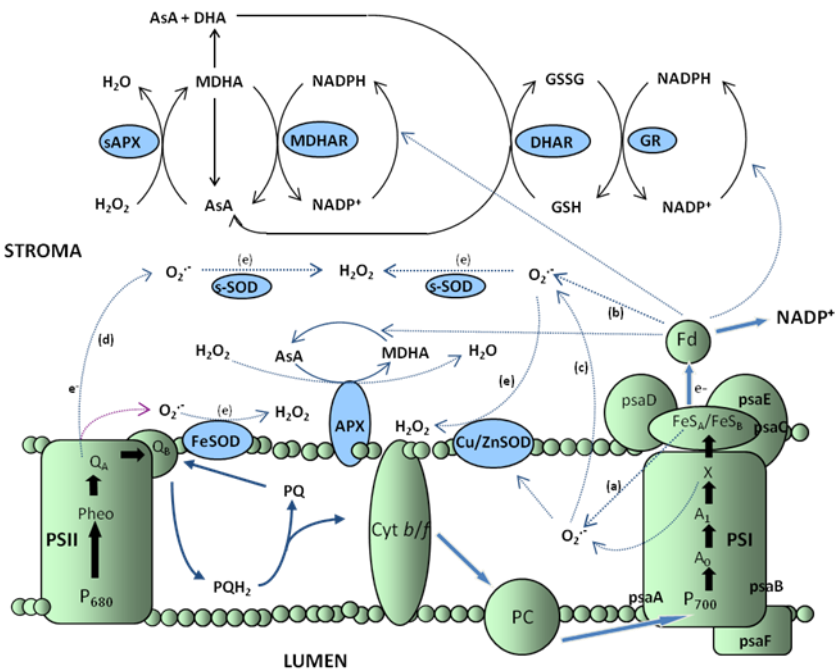


FIGURE 4. Proposed molecular oxygen reduction in thylakoids, and thylakoid and stromal scavenging system in chloroplast. (**a**) Univalent oxygen reduction by FeS_A/FeS_B and X centres; (**b**) univalent oxygen reduction by reduced ferredoxin (Fd); (**c**) diffusion of superoxide into the stroma; (**d**) univalent oxygen reduction by P_{680}, pheophytin (Pheo) and Q_A; (**e**) spontaneous and/or SOD-catalysed disproportionation of superoxide. From Navari-Izzo and Quartacci, 2001.

The cells protect themselves from active oxygen by an efficient defense systems, composed of both enzymatic and non enzymatic cell constituents, which can be summarized as in Figure 4. These defence mechanisms maintain the cellular redox homeostasis within certain limits (Cuypers *et al*., 2011).

Copper acquisition and transport

Copper acquisition and transport into and within cells is relatively little known in plants, but in the last decade, rapid progress has been made to understand these processes. Plants require mechanisms to regulate Cu uptake and distribution because of the variable availability of Cu in the environment. The presence of large families of metal transporters in higher plants probably accounts for the elevated plasticity required to balance metal homeostasis in specific subcellular compartments during development and in response to environmental stimuli. Cu most likely enters the cytosol of root cells via a cell surface protein COPT-family transporter (Burkhead *et al*., 2009; Yruela, 2009), which has been identified in plants by sequence homology with the eukaryotic Cu transporters named Ctr or by functional complementation in yeast (Puig *et al*., 2007). Some of the COPT proteins may be active at the plasma membrane, whereas others may be active in internal membranes such as the vacuoles, facilitating release from intracellular stores (Burkhead *et al*., 2009). Perhaps COPT1, which is highly expressed in roots (Sancenón *et al*., 2004), and COPT2, which is highly expressed in shoots (Sancenón *et al.,* 2003) are the key Cu-regulated cell surface localized uptake systems, while COPT3 and COPT5 may function in mobilization of Cu from intracellular stores (Burkhead *et al*., 2009). However, metal competition experiments suggest that *Arabidopsis* COPT1 is a high-affinity transporter with specificity for Cu^+ ion (Sancenón *et al*., 2003) with a K_m in the lower micromolar range (Yruela, 2009). COPT transporters do not use ATP for Cu import, but their transport ability is stimulated by extracellular K^+ (Yruela, 2009). In addition to COPT transporters, ZIP2 and ZIP4 (members of the ATP-independent ZIP family of transporters) may function for high-affinity Cu uptake in plant cells (Wintz *et al*., 2003; Yruela, 2009). A link with Cu for ZIP2 and ZIP4 was found by expression profiling of plants grown on low Cu (Wintz *et al*., 2003). The same study reports that ZIP2 expression was relatively higher in roots, while ZIP4 was expressed more in the leaves (Wintz *et al*., 2003). In case of

high Cu, tolerant species can thrive and accumulate toxic metal in their shoots, such as some hyperaccumulators which use high metal levels to provide protection against herbivory or microbial attack (Burkhead *et al*., 2009). The ZIP2 and ZIP4 transporters were up-regulated at low Cu, but were even more influenced by Zn (Wintz *et al*., 2003). The same study found COPT2 to be up-regulated in leaves by both Cu and Zn deficiency.

Copper must be exported from the root symplast before entering the xylem. Transpiration would take Cu through the xylem to mature leaves where it could be loaded into the phloem in order to reach sink tissues such as newly developing leaves, flowers and seeds. Most likely, export from the root symplast involves the action of the Cu-transporting P-type ATPase HMA5 (Andrés-Colás *et al*., 2006), following the classical E1/E2 Albers-Post catalytical cycle (Yruela, 2009).

Typically, plants that are given excess Cu have elevated Cu in the roots and several studies report that excess Cu does not reach the shoot, at least in the first days of Cu exposure (Alaoui-Sossé *et al*., 2004; Navari-Izzo *et al*., 2006). Thus, export from root cells and long-distance transport to the shoot may be limited in the translocation of excess Cu. The P-type ATPases such as HMA5 most likely export Cu as Cu(I). However, it is not known in which oxidation state Cu travels in the xylem. Long-distance transport may involve chelators such as nicotianamine, a metal-chelating compound derived from methionine that is implicated in Cu transport in the xylem (Irtelli *et al*., 2009; Quartacci *et al*., 2009). However, few results have been obtained with respect to long-distance transport or transport processes taking place at root level. For instance, in a chelant-enhanced phytoextraction there is the formation of metal-chelant complexes in soils and it is unclear how plant roots take up these metal complexes. The progress on the knowledge of this mechanisms may lead to the development of new strategies for enhancing metal phytoextraction.

REFERENCES

Alaoui-Sossé, I.; Genet, P.; Vinit-Dunand, F.; Toussaint, M.L.; Epron, D.; Badot, P.M. Effect of copper on growth in cucumber plants (Cucumis sativus) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Sci*. **2004**, *166*, 1213–1218.

Andrés-Colás, N.; Sancenón, V.; Rodriguez-Navarro, S.; Mayo, S.; Thiele, D.J.; Ecker, J.R.; Puig, S.; Peñarrubia, L. The *Arabidopsis* heavy metal P-type ATPase HMA5 interacts with metallochaperones and functions in copper detoxification of roots. *Plant J.* **2006**, 45, 225–236.

Bandiera, M.; Mosca, G.; Vamerali, T. Phytotoxicity and metal leaching in EDDS-assisted phytoextraction from pyrite wastes with Ethiopian mustard and fodder radish. *Plant Bios*. **2010**, *144*, 490-498.

Baker, A.J.M.; Brooks, R.R. Terrestrial higher plants which hyperaccumulate metallic elements—a review of their distribution, ecology and phytochemistry. *Biorecovery* **1989**, *1*, 181–126.

Burkhead, J.L.; Reynolds, K.A.G.; Abdel-Ghany, S.E.; Cohu, C.M.; Pilon, M. Copper homeostasis. *New Phyt.* **2009**, *182*, 799-816.

Clemens, S. Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **2001**, *212*, 475-486.

Clemens, S.; Palmgren, M.G.; Krämer, U. A long way ahead: understanding and engineering plant metal accumulation. *Trends Plant Sci.* **2002**, *7*, 309-315.

Cuypers, A.; Smeets, K.; Ruytinx, J.; Opdenakker, K.; Keunen, E.; Remans, T.; Horemans, N.; Vanhoudt, N.; Van Sanden, S.; Van Belleghem, F.; Guisez, Y.; Colpaert, J.; Vangronsveld, J. The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. *J. Plant Physiol.* **2011**, *168*, 309-316.

Dominguez, M.T.; Madrid, F.; Maranon, T.; Murillo, J.M. Cadmium availability in soil and retention in oak roots: potential for phytostabilisation. *Chemosphere* **2009**, *76*, 480–486.

Evangelou, M.W.H.; Ebel, M.; Schaeffer, A. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity and fate of chelating agents. *Chemosphere* **2007**, *68*, 989-1003.

Grčman, H.; Velikonja-Bolta, Š.; Vodnic, D., Leštan, D. EDTA enhanced heavy metal phytoextraction: metal accumulation, leaching and toxicity. *Plant Soil* **2001**, *235*, 105–114.

Hall, J.L. Cellular mechanisms for heavy metals detoxification and tolerance. *J. Exp. Bot*. **2002**, *53*, 1-11.

Hall, J.L.; Williams, L.E. Transition metal transporters in plants. *J. Exp. Bot*. **2003**, *54*, 2601–2613.

Irtelli, B. *Different phytoextraction techniques of heavy metals*. Ph.D. thesis., University of Pisa, **2007**.

Irtelli, B.; Petrucci, W.A.; Navari-Izzo, F. Nicotianamine and histidine/proline are, respectively, the most important copper chelators in xylem sap of *Brassica carinata* under conditions of copper deficiency and excess. *J. Exp. Bot*. **2009**, *60*, 269-277.

Keller, C.; Ludwig, C.; Davoli, F.; Wochele, J. Thermal treatment of metal-enriched biomass produced from heavy metal phytoextraction. *Environ. Sci. Techno*l. **2005**, *39*, 3359–3367.

Kidd, P.; Barceló, J.; Bernal, M.P.; Navari-Izzo, F.; Poschenrieder, S.S.; Clemente, R.; Monterroso, C. Trace element behavior at the root-soil interface: Implications in phytoremediation. *Environ. Exp. Bot*. **2009**, *67*, 243-259.

Kos, B.; Leštan, D. Chelator induced phytoextraction and in situ soil washing of Cu. *Environ. Pollut*. **2004**, *132*, 333-339.

Leštan D.; Luo, C.; Li, X. The use of chelating agents in the remediation of metal-contaminated soils: A review. *Environ. Pollut*. **2008**, *153*, 3-13.

Luo, C.L.; Shen, Z.G.; Li, X.D. Plant uptake and leaching of metals during the hot EDDS-enhanced phytoextraction process. *Int. J. Phytorem*. **2006**, *9*, 181-196.

Madrid, F.; Liphadzi, M.S.; Kirkham, M.B. Heavy metal displacement in chelate-irrigated soil during phytoremediation. *J. Hydrol*. **2003**, *272*, 107–119.

Marchiol, L.; Sacco, P.; Assolari S.; Zerbi, G. Reclamation of polluted soil: phytoremediation potential of crop-related *Brassica* species. Water Air *Soil Pollut*. **2004**, *158*, 245-356.

Marschner, H. Mineral Nutrition of Higher Plants. Academic Press, London, **1995**.

McGrath, S.P.; Zhao, F.J. Phytoextraction of metals and metalloids from contaminated soils. *Curr. Opin. Biotechnol*. **2003**, *14*, 277-282.

McGrath, S.P.; Lombi, E.; Gray, C.W.; Caille, N.; Dunham, S.J.; Zhao, F.J. Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri*. *Environ. Pollut*. **2006**, *141*, 115-125.

Meers, E.; Vandecasteele, B.; Ruttens, A.; Vangronsveld, J.; Tack, F.M.G. Potential of five willow species (*Salix* spp.) for phytoextraction of heavy metals. *Environ. Exp. Bot*. **2007**, *60*, 57-68.

Meers, E.; Tack, F.M.G.; Verloo, M.G. Degradability of ethylenediamine disuccinic acid (EDDS) in metal contaminated soils: Implications for its use in soil remediation. *Chemosphere* **2008**, *70*, 358-363.

Meharg, A.A. The role of the plasmalemma in metal tolerance in angiosperms. *Physiol. Plantarum* **1993**, *88*, 191-198.

Memon, A.R.; Schröder, P. Implications of metal accumulation mechanisms of phytoremediation. *Environ. Sci. Pollut. Res*. **2009**, *16*, 162-175.

Miller, G.; Shulaev, V.; Mittler, R. Reactive oxygen signaling and abiotic stress. *Physiol. Plantarum* **2008**, *133*, 481-489.

Mosca, G.; Vamerali, T.; Ganis, A.; Coletto, L.; Bona, S. Miglioramento dell'efficienza agronomica della fitodecontaminazione di metalli pesanti. In: Zerbi G., Marchiol L. (eds.) *Fitoestrazione Di Metalli Pesanti – Contenimento Del Rischio Ambientale E Relazioni Suolo-Microrganismi-Pianta*. Forum Editrice Universitaria Udinese, Udine, Italy, **2004**, pp. 105-135.

Nascimento, C.W.A.; Xing, B. Phytoextraction: a review on enhanced metal availability and plant accumulation. *Sci. Agric*. **2006**, *63*, 299-311.

Navari-Izzo, F.; Quartacci, M.F. Phytoremediation of metals. Tolerance mechanisms against oxidative stress. *Minerva Biotec*. **2001**, *13*, 73-85.

Navari-Izzo, F.; Cestone, B.; Cavallini, A.; Natali, L.; Giordani, T.; Quartacci, M.F. Copper excess triggers phospholipase D activity in wheat roots. *Phytochemistry* **2006**, *67*, 1232–1242.

Navari-Izzo, F.; Rascio, N. Heavy metal pollution: damage and defense strategies in plants. In: Pessarakli, M. (Ed.), *Handbook of Plant and Crop Stress*. Taylor & Francis Publishing Company, Florida, **2010**, pp. 635-674.

Padmavathiamma, P.K.; Li, L.Y. Phytoremediation technology: hyper accumulation of metals in plants. *Water Air Soil Pollut* **2007**, *184*, 105–126.

Puig, S.; Mira, H.; Dorcey, E.; Sancenón, V.; Andrés-Colás, N.; Garcia-Molina, A.; Burkhead, J.L.; Gogolin, K.A.; Abdel-Ghany, S.E.; Thiele, D.J.; Ecker, J.R.; Pilon, M.; Peñarrubia, L. Higher plants possess two different types of ATX1-like copper chaperones. Biochem. *Biophys. Res. Commun.* **2007**, *354*, 385–390.

Quartacci, M.F.; Cosi, E.; Navari-Izzo, F., Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. *J. Exp. Bot.* **2001**, *52*, 77-84.

Quartacci, M.F.; Cosi, E.; Meneguzzo, S.; Sgherri, C.; Navari-Izzo, F. Uptake and translocation of copper in *Brassicaceae*. *J. Plant Nutr.* **2003**, *26*, 1065-1083.

Quartacci, M.F.; Argilla, A.; Baker, A.M.J.; Navari-Izzo, F. Phytoextraction of metals from a multiple contaminated soil by Indian mustard. *Chemosphere* **2006**, *63*, 918-925.

Quartacci, M.F.; Irtelli, B.; Baker, A.M.J.; Navari-Izzo, F. The use of NTA and EDDS for enhanced phytoextraction of metals from a multiply contaminated soil by *Brassica carinata*. *Chemosphere* **2007**, *68*, 1920-1928.

Quartacci, M.F.; Irtelli, B.; Gonnelli, C.; Gabbrielli, R.; Navari-Izzo, F. Naturally-assisted metal phytoextraction by *Brassica carinata*: Role of root exudates. *Environ. Pollut*. **2009**, *157*, 2697-2703.

Robinson, B.H.; Green, S.R.; Mills, T.M.; Clothier, B.E.; van der Velde, M.; Laplane, R.; Fung, L.; Deurer, M.; Hurst, S.; Thayalakumaran, T.; van den Dijssel, C. Phytoremediation: using plants as biopumps to improve degraded environments. *Aust. J. Soil Res*. **2003**, *41*, 599-611.

Robinson, B.H.; Schulin, R.; Nowack, B.; Roulier, S.; Menon, M.; Clothier, B.E.; Green, S.R.; Mills, T.M. Phytoremediation for the management of metal flux in contaminated sites. *Forest Snow Landscape Res*. **2006**, *80*, 221–234.

Robinson, B.H.; Bañuelos, G.; Conesa, H.M.; Evangelou, M.W.H.; Schulin, R. The phytomanagement of Trace Elements in Soil. *Crit. Rev. Plant Sci*. **2009**, *28*, 240-266.

Salt, D.E.; Smith, R.D.; Raskin, I. Phytoremediation. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **1998**, 49, 643-668.

Sancenón V.; Puig, S.; Mira, H.; Thiele, D.J.; Peñarrubia, L. Identification of a copper transporter family in *Arabidopsis thaliana*. *Plant Mol. Biol.* **2003**, *51*, 577–587.

Sancenón V.; Puig, S.; Mateu-Andrés, I.; Dorcey, E.; Thiele, D.J.; Peñarrubia, L. The *Arabidopsis* Copper Transporter COPT1 Functions in Root Elongation and Pollen Development. *J. Biol. Chem.* **2004**, *279*, 15348–15355.

Sgherri, C.; Milone, M.A.; Clijsters, H.; Navari-Izzo, F. Antioxidative enzymes in two wheat cultivars, differently sensitive to drought and subjected to subsymptomatic copper doses. *J. Plant Physiol*. **2001**, *158*, 1439-1447.

Sgherri, C.; Quartacci, M.F.; Navari-Izzo, F. Early production of activated oxygen species in root apoplast of wheat following copper excess. *J. Plant Physiol.* **2007**, *164*, 1152-1160.

Smeets, K.; Opdenakker, K.; Remans, T.; Van Sanden, S.; Van Belleghem, F.; Semane, B.; Horemans, N.; Guisez, Y.; Vangronsveld, J.; Cuypers, A.

Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. *J. Plant Physiol*. **2009**, *166*, 1982-1992.

Tandy, S.; Schulin, R.; Nowack, B. The influence of EDDS on the uptake of heavy metals in hydroponically grown sunflowers. *Chemosphere* **2006**, *62*, 1454-1463.

Vamerali, T.; Bandiera, M.; Mosca, G. In situ phytoremediation of arsenic- and metal-polluted pyrite waste with field crops: Effects of soil management. *Chemosphere* **2011**, *83*, 1241-1248.

Vangronsveld, J.; Clijsters, H. Toxic effects of metals. In Farago M.E. (ed.) *Plants and the chemical elements*. Weinheim: VCH Verlagsgesellschaft, **1994**, pp. 149-177.

Vangronsveld, J.; Cunningham, S.D. Introduction to the Concepts. In Vangronsveld J. and Cunningham S.D. (eds.) *Metal-Contaminated Soils: In Situ Inactivation and Phytorestoration*. Springer-Verlag and R.G. Landes Company, Georgetown, TX, **1998**, pp. 1-15.

Wei, S.; Zhou, Q. Trace elements in agro-ecosystems. In: Prasad M.N.V. (ed.) *Trace elements as contaminants and nutrients—consequences in ecosystems and human health*. Wiley, New Jersey, USA, **2008**, pp 55–80.

Wintz, H., Fox, T.; Wu, Y.Y.; Feng, V.; Chen, W.; Chang, H.S.; Zhu, T.; Vulpe, C. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. *J. Bio. Chem*. **2003**, *278*, 47644–47653.

Wu, L.H.; Luo, Y.M.; Xing, X.R.; Christie, P. EDTA-enhanced phytoremediation of heavy metal contaminated soil with Indian mustard and associated potential leaching risk. *Agric. Ecosyst. Environ*. **2004**, *102*, 307-318.

Yruela, I. Copper in plants: acquisition, transport and interactions. *Funct. Plant Biol.* **2009**, *36*, 409-430.

RESEARCH OUTLINE

Being most soils polluted by more metals rather than by one, there is increasing interest on the development of an environmentally-friendly technology able to clean up multiple metal-polluted soils. The main goal of the present research concerns the remediation through a chelant-enhanced phytoextraction programme of a waste highly contaminated by five metals (As, Co, Cu, Pb and Zn), located at Torviscosa and identified as a site of national interest for restoration. For this purpose, it has been investigated the use of tolerant plants able to survive and accumulate high amounts of metals in their harvestable tissues together with the application of an easily biodegradable chelant. The phytoextraction effectiveness of two accumulator species, *Brassica carinata* and *Raphanus sativus*, both belonging to *Brassicaceae* family, has been investigated using EDDS at different dosages and application times (*Chapter II*). To improve soil-plant transfer of metals the understanding of the mechanisms that underlie metal complexes uptake and translocation such as CuEDDS (*Chapter III*) together with the localization of metals in the tissues (*Chapter IV*) and the defence responses at transcriptional and metabolic levels of the plants against oxidative injury (*Chapter V*) were investigated.

APPLICATION TIME AND DOSAGE AS TOOLS FOR ENHANCING EDDS-ASSISTED PHYTOEXTRACTION OF METALS BY *BRASSICACEAE* SPECIES

Benedetta Cestone,[†] Mike F. Quartacci,[†] Teofilo Vamerali,[‡] Marianna Bandiera,[§] Paola Lucchini,[§] and Flavia Navari-Izzo[†]

[†] *Dipartimento di Biologia delle Piante Agrarie, Università di Pisa, Via del Borghetto 80, 56121 Pisa, Italy,* [‡] *Dipartimento di Scienze Ambientali, Università di Parma, Viale Usberti 11/A, 43100 Parma, Italy, and* [§] *Dipartimento di Agronomia Ambientale e Produzioni Vegetali, Università di Padova, Viale dell'Università 16, 35020 Legnaro, Padova, Italy*

Article in preparation for Environ. Sci. Technol.

Abstract

In order to improve the chelant-enhanced phytoextraction technique, we investigated the use of the biodegradable chelating agent (S,S)-N,N'-ethylenediaminedisuccinic acid (EDDS) and *Brassicaceae* species (*Brassica carinata* and *Raphanus sativus*) for the cleaning up of a multiple metal-contaminated soil (As, Co, Cu, Pb, Zn). The two accumulator crops were grown in pots filled with a soil-sand mixture (1:1, w/w) for 75 days and amended with EDDS distributed at different growth stages as a single (2.5 or 5 mmol kg^{-1}) or repeated doses (1 mmol kg^{-1} every 5 or 10 days). Shoot biomass decreased for both species at all treatments except for *B. carinata* when treated with the lowest single dose one week before the harvest. Among metals, Cu resulted to be the highest bioavailable metal. At harvest, both metal and EDDS leaching were evaluated along the soil profile and in the percolation water. For both crops repeated treatments made worse the leachate quality by increasing both EDDS and metal contents. The application of 2.5 mmol kg^{-1} EDDS and the use of

B. carinata resulted an optimal compromise to improve metal uptake (+ 31%) without causing growth depression and risk of leaching.

Introduction

The use of biodegradable chelants in improving the uptake of metals by plants and in limiting the leaching of metals from soil has been proposed as an effective tool to remediate metal-contaminated soils. However, environmental and economic concerns require that the addition of chelants should be kept to a minimum. The EDTA structural isomer (S,S)-N,N'-ethylenediamine disuccinic acid (EDDS) has received attention as potential replacement for EDTA as it is a strong chelant and unlike EDTA it is easily biodegradable (*1*), the persistence depending on soil characteristics, microbial activity, type of metal as well as chelant amount (*2*, *3*). The half-life of EDDS applied to soils at concentrations ranging from 3 to 6 mmol kg^{-1} was estimated to be 2.5-7.5 days depending on the amount of chelating agent added (*4*). On the other hand, it was reported that only 18-42% of EDDS (20 mmol kg^{-1}) was biodegraded in soils after 7 weeks (*5*).

Even though biodegradable chelants increase the desorption of metals from the soil and make them available for root uptake, only a small fraction of the mobilized metals is effectively taken up by the plant and subsequently translocated to the shoots (*6*). In sunflower, the addition to soil of a moderate dose of EDDS (1.6 mmol kg^{-1}) resulted in a shoot recovery of only 2.2, 0.8 and 7.3% of mobilized Zn, Cu and Ni, respectively (*7*). Metal excess in soil may result in plant toxicity and consequent metabolic disorders which lead to reduced growth and metal uptake by roots (*8*), thus limiting phytoextraction effectiveness. In addition, the high degree of mobile metals left in the soil after remediation increases the possibility of leaching into the lower soil horizons and groundwater. In a soil column experiment, the addition of 5 mmol kg^{-1} EDDS caused leaching of about one-fifth of the initial Cu in soil (*9*).

To enhance metal accumulation capacities by high biomass species and to prevent the possible movement of metal complexes into groundwater, fundamental features to take into consideration are the selection of suitable chelants, their dosages and time of application (*10*). The different chelant distribution methods may have a significant impact on the efficiency of metal phytoextraction. The splitting of the application in several small dosages (instead of one application), besides reducing metal leaching, may simultaneously decrease the effectiveness of the treatment (*2*). On the other hand, the optimal moment for chelant amendment is the result of a compromise between i) postponing the treatment near harvest to minimize potential growth depression induced by metal toxicity and ii) fixing the time of application at an early plant phenological stage to guarantee sufficient exposure time for uptake and to prevent post-harvest mobility of metals (*7*).

The aim of this work was to investigate the phytoextraction effectiveness of the two accumulator crops *Brassica carinata* and *Raphanus sativus* grown in multiple metal-contaminated pyrite wastes and amended with EDDS distributed in different growth stages as a single or repeated doses. In addition, both metal and EDDS leaching were evaluated along the soil profile and in the percolation water.

Experimental section

Soil Characteristics. The metal-contaminated wastes were collected from a site located at Torviscosa (Udine, North-East Italy) used as a dump for pyrite cinders derived from the ore roasting processes for sulphur extraction. At present, the site is included within the perimeter of the polluted area named 'Grado and Marano lagoon and neighbouring water-courses' identified as a site of national interest by the Italian Cabinet Decree 468/2001 'National Programme for Environmental Restoration of Polluted Sites'. The pyrite cinders extended for a depth of 0.7 m over a deep horizon of impermeable clay and had been covered

with a less polluted gravelly soil 0.15-0.2 m in depth. Air-dried cinder samples were sieved (2 mm), about 1 g of dried weight was microwave acid-digested (*11*), filtered (PTFE pore size 0.45 μm) and total metal concentrations were determined by a Spectro CirOS Vision ICP-OES (Spectro Analytical Instruments Gmb H KG, Kleve D, Germany). Cinders were also analysed for DTPA-extractable metals by adding 5 mM DTPA (diethylenetriaminepentaacetic acid), 10 mM $CaCl_2$ and 0.1 M triethanolamine (pH 7.3) to give a 1:2 (w/v) soil:solution ratio (*12*). After shaking for 2 h (60 cycles min^{-1}), tubes were centrifuged at 17400 *g* for 10 min and analyzed by ICP-OES. The total and DTPA-extractable concentrations of metals exceeding the Italian Guideline Values (IGV) for soil contamination for agricultural uses (Italian Legislative Decree 152/2006) are reported in Table 1.

TABLE 1. Total and DTPA-extractable Metal Concentrations in Pyrite Cinders

Metal	Total (mg kg^{-1})	IGV[a] (mg kg^{-1})	DTPA-extractable (mg kg^{-1})
As	886	20	N.D.[b]
Co	100	20	N.D.
Cu	1735	120	142
Pb	493	100	70
Zn	2404	150	45

[a] Italian Guideline Values for soils for agricultural uses (Legislative Decree 152/2006).

[b] Not determinable.

Besides the five metals in excess also Cd, Cr, Mn and Ni were detected, even though all of them at concentrations below the legal thresholds (for Mn the National legislation does not fix any limit). A certified reference material (ERM-CC141, JRC-IRMM, Belgium) was used to ensure accuracy and precision in the analyses. Cinder characterization was carried out according to the Italian

Methods of Chemical Analysis of Soil (*12*). Devoid of organic matter content, with high bulk density (1.65 g cm^{-3}), poor in nutritional status, pH sub-alkaline (7.3), and relatively low electrical conductivity (0.3 S m^{-1}), the cinders were colonised by a sparse vegetation cover (*13*).

Experimental Setup. The experimental trials were conducted at the experimental farm 'Lucio Toniolo' of the University of Padova. Pyrite cinders were mixed with sand (1:1, w/w) to facilitate water drainage and to prevent the poor performance of plants grown in pure wastes. After mixing, the mixture was allowed to equilibrate for four weeks, undergoing three cycles of saturation with water and air-drying before being re-mixed and finally planted. Fodder radish (*Raphanus sativus* L. var. *oleiformis* cv Siletta nova) and Ethiopian mustard (*Brassica carinata* A. Braun cv 079444) were grown in a greenhouse for 75 days in cylindrical, insulated, opaque PVC pots (57 mm inner diameter, 520 mm height). Pots were filled to a depth of 490 mm with the cinder-sand mixture (2 kg dry weight mixture per pot) and their bases were connected by black tubing to 1.5 L opaque PVC bottles in order to collect the leachate. Each species was sown separately in pots (five seeds per pot) and following emergence pots were thinned to one plant per pot. Plants were regularly watered with 100 mL of a 1:2 diluted Hoagland solution (*14*). Compared to untreated controls, four EDDS treatments (five replicates each) were tested: i) 1 mmol kg^{-1} repeated five times at 10 days interval starting from 28 after sowing (1x5-10d); ii) 1 mmol kg^{-1} repeated five times at 5 days interval starting from 48 after sowing (1x5-5d); iii) a single 2.5 mmol kg^{-1} application one week before harvest (2.5x1); iv) a single 5 mmol kg^{-1} application one week before harvest (5x1). The different EDDS doses were added to the soil by diluting chelant stock solution in 100 mL of distilled water, an amount that did not cause percolation. Control plants were irrigated with an equivalent amount of water. Throughout the experimental period, to each pot a total of 3.8 L of water was distributed as the sum of Hoagland and EDDS solutions (water for the control). At harvest the above-ground parts of

plants were collected, washed carefully with distilled water to remove any soil splash. One set of shoots from both species was oven-dried at 110 °C for 24 h, whereas a second group was immersed in liquid N_2 and stored at –80°C till analyzed. Samples from the cinder-sand mixture were collected at 7, 28 and 42 cm of depth for DTPA-extractable metal and EDDS determinations. In addition, percolation water from each container was collected and the volume measured.

Sequential Extraction of Metals. The European Community Bureau of Reference three step sequential extraction procedure was followed (*15*). Briefly, the acid-soluble phase (step 1) was extracted with a 0.11 M acetic acid solution, the reducible phase (step 2) with 0.5 M hydroxylammonium chloride, whereas the oxidisable phase (step 3) was obtained with 8.8 M H_2O_2 and 1 M ammonium acetate extractions. After the sequential extraction steps, the residual metal content (fraction 4) was determined by microwave digestion with HNO_3 (65%) and HCl (37%) (1:3, v/v); this phase is the difference between the total metal content and the sum of the contents in the three previous phases. The metals found primarily in this fraction are those that are associated with minerals, forming part of their crystalline structure and, which, as a result, are unlikely to be released from soils (*14*). Metals in the solutions obtained following sequential extractions (fractions 1-3) and sample digestion (fraction 4) were determined by ICP-OES. Also in this case, a certified reference material (ERM-CC141, JRC-IRMM, Belgium) was used to ensure accuracy.

Metal Analysis. DTPA-extractable metals at different depth (14, 28 and 42 cm) of the cinder-sand mixture were determined as previously reported. For shoot metal determinations dried ground plant material was added with 5 mL HNO_3 (70%) and 1.5 mL H_2O_2 (30%) and digested in a microwave oven following EPA method 3052 (*16*). After being filtered (0.45 μm PTFE) samples were analyzed by ICP-OES. Certified reference materials (ERMCD281 and BRC-402, JRC-IRMM, Belgium) were used to ensure accuracy.

EDDS Analysis. For EDDS analysis of shoots the frozen material was lyophilized. The dry material was then extracted in a mortar with 50% ethanol (1:5 w/v), transferred to a test tube, and sonicated for 2 h with a Bransonic 3510 ultrasonic apparatus (Branson, Danbury, CT, USA) at room temperature to avoid degradation or cyclization of EDDS (*17*). After centrifugation at 3000 *g* for 3 min, the supernatant was filtered through a 0.45 μm PTFE filter. EDDS derivatization and analysis were carried out as described by Metsärinne et al. (*18*). Analyses were carried out by HPLC with a Waters HPLC system consisting of two 515 pumps and a 2487 programmable UV detector. EDDS was separated using a Waters Spherisorb ODS2 C18 reverse-phase column (25 x 0.46 cm, 5 μm) and detected at 254 nm wavelength. A 15% methanol and 85% tetrabutylammoniumbromide (0.02 M) eluent at a flow rate of 1 mL min^{-1} was used as the mobile phase. Identification of EDDS was obtained by comparison of its retention time with that of standards. Quantification of EDDS was obtained by comparing calibration curves. Chromatogram analysis was performed by Millennium 32 software (Waters).

EDDS determination in the cinder-sand mixture was carried out adding 10 mL deionized water to 1 g air-dried mixture. Tubes were shaken for 30 min and centrifuged at 14000 *g* for 10 min. The supernatants were then collected and the residues re-suspended in 10 mL water. The above step was repeated twice for a total of three consecutive extractions. EDDS in the combined supernatants was measured by the addition of 5 mM $CuSO_4$ (*19*). After the addition of the reagent, samples were incubated for 12 h at room temperature in the dark to allow the displacement of other metals from EDDS. Samples were filtered (0.45 μm PTFE) and acidified to pH 5.0 with HNO_3. For chelant determination absorption of the complex was measured at 670 nm using a Varian Cary 1E UV-Vis spectrophotometer. EDDS quantification was obtained by comparison with a standard (CuEDDS complex) curve in the 0.1-2.0 mM range.

Determination of complexed EDDS in the leachate was carried out directly on filtered and acidified aliquots reading the absorption at 670 nm. Total EDDS (free and complexed) was determined spectrophotometrically following the addition of 5 mM $CuSO_4$ as above reported.

Statistical Analysis. Statistical analysis was carried out with Costat 6.4 (CoHort software). The error bars represent the standard error of the mean of three independent experiments (n=3) each analyzed twice. Differences among means were evaluated by one-way ANOVA and LSD test at the $P \leq 0.05$ level.

Results

Sequential Extraction of Metals. The sequential extraction of metals from the cinder-sand mixture (Figure 1) show that As and Co were equally distributed in the three forms – exchangeable and bound to carbonates (1st step), bound to iron and manganese oxides (2nd step), and bound to organic matter and sulphides (3rd step). Extractable Cu and Zn were present mainly in the weakly-bound acid-soluble form, whereas the highest Pb concentration was detected in the reducible form. For each element the sum of the three extractable forms was less than 20% of the total concentration.

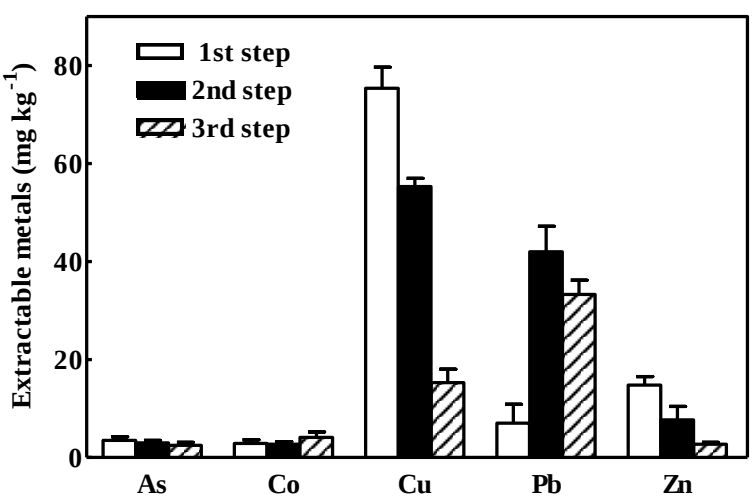


FIGURE 1. Metal concentrations in the different soil fractions following sequential extraction. 1st step, acid-soluble phase; 2nd step, reducible phase; 3rd step, oxidisable phase. Results are means ± SE (*n* = 3).

EDDS in the Percolation Water. In both species the total amount of EDDS detected in the percolation water collected throughout the different amendment periods showed the same trend (Figure 2A,B).

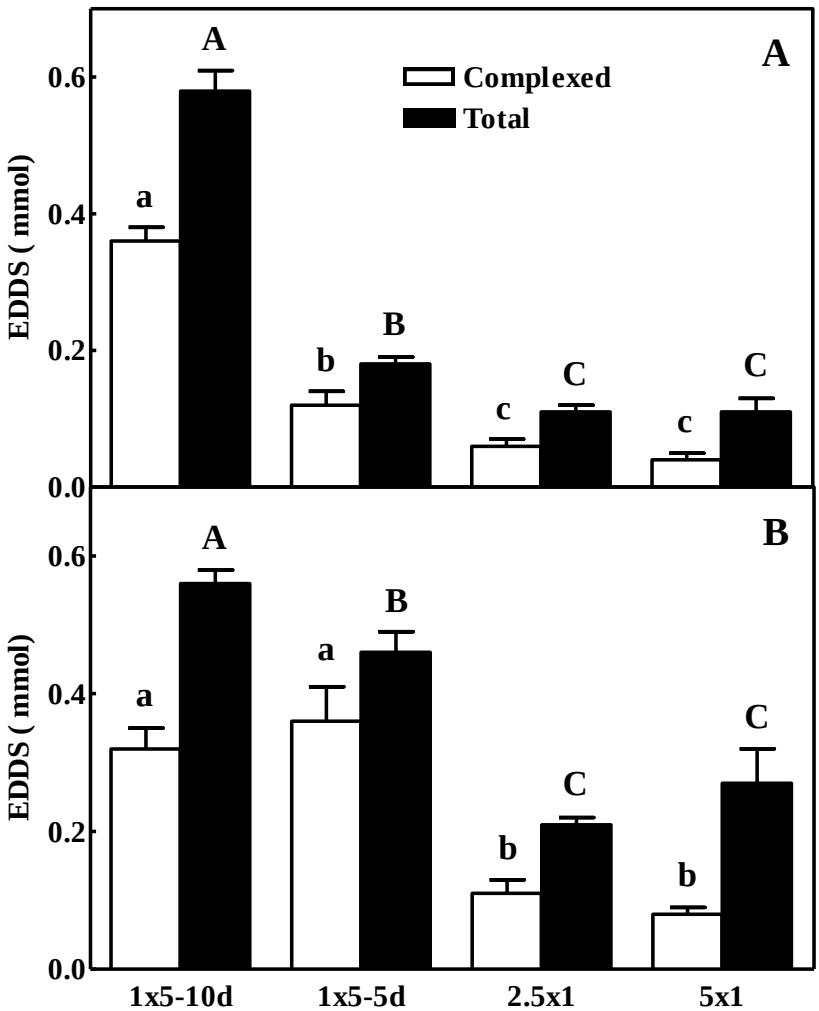


FIGURE 2. Total and complexed EDDS amounts in the percolation water collected from pots planted with *B. carinata* (**A**) and *R. sativus* (**B**) following amendments. Results are means ± SE (*n* = 3). For each chelant fraction means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). 1x5-10d, 1 mmol EDDS kg^{-1} added every 10 days starting 28 days after sowing; 1x5-5d, 1 mmol EDDS kg^{-1} added every 5 days starting from 48 days after sowing; 2.5x1, 2.5 mmol EDDS kg^{-1} added one week before harvest; 5x1, 5 mmol EDDS kg^{-1} added one week before harvest.

The highest chelant amount was observed for the early treatment (1x5-10d), whereas the lowest was found in the two single dose additions. As total EDDS also metal-complexed EDDS amounts showed a similar pattern in the percolation waters of the two crops. The repeated chelant applications caused higher EDDS levels in comparison with the single ones (Figure 2A,B). On average, in both species the percentages of complexed EDDS ranged from 60% (1x5-10d) to 33% (5x1) of total EDDS amounts. The remarkable reductions of total and complexed EDDS in the leachate collected from pots planted with *B. carinata* after the 1x5-5d amendment (about 70% compared to the 1x5-10d treatment) is worth noting.

EDDS and Metals in the Cinder-Sand Mixture. *EDDS Concentration.* In general, the single applications caused higher chelant concentrations in the more superficial parts of the mixture planted with both species. On the contrary, the repeated amendments resulted in an accumulation of EDDS as the depth increased (Figure 3A,B).

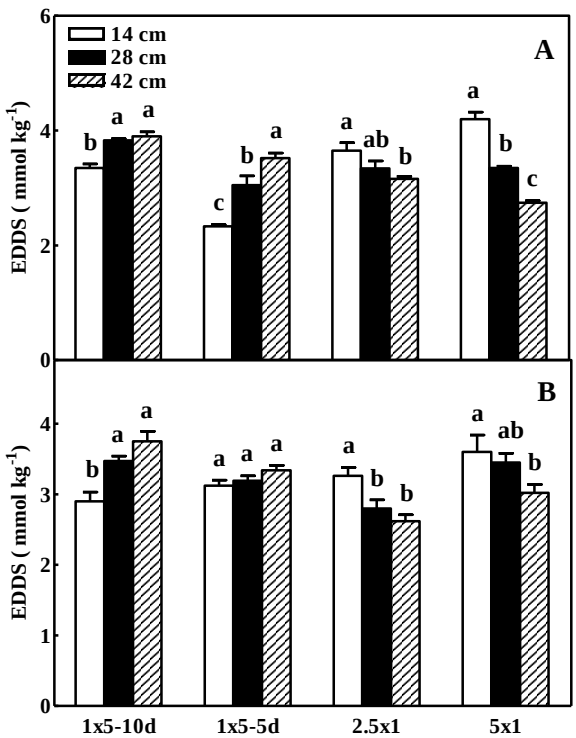


FIGURE 3. EDDS concentrations in the cinder-sand mixture at three different depths following amendments of pots planted with *B. carinata* (**A**) and *R. sativus* (**B**). Results are means ± SE (*n* = 3). For each treatment means followed by the same letter are not significantly different by ANOVA (LSD test, P ≤ 0.05). For abbreviations see Figure 2.

Available Metal Concentration. In Figure 4 are reported the mean concentrations of the available metals found in the mixture after plant harvest. Compared to the control chelant amendments resulted in a significantly higher total available metal concentrations only in the *B. carinata* 5x1 treatment. For both crops and all treatments Cu was the metal showing the highest concentration, followed by Pb and to a lesser extent by Zn, the latter element not evidencing differences between species. Available As, Co and Mn were present at concentrations 100 to 1000-fold lower (Figure 4A,B).

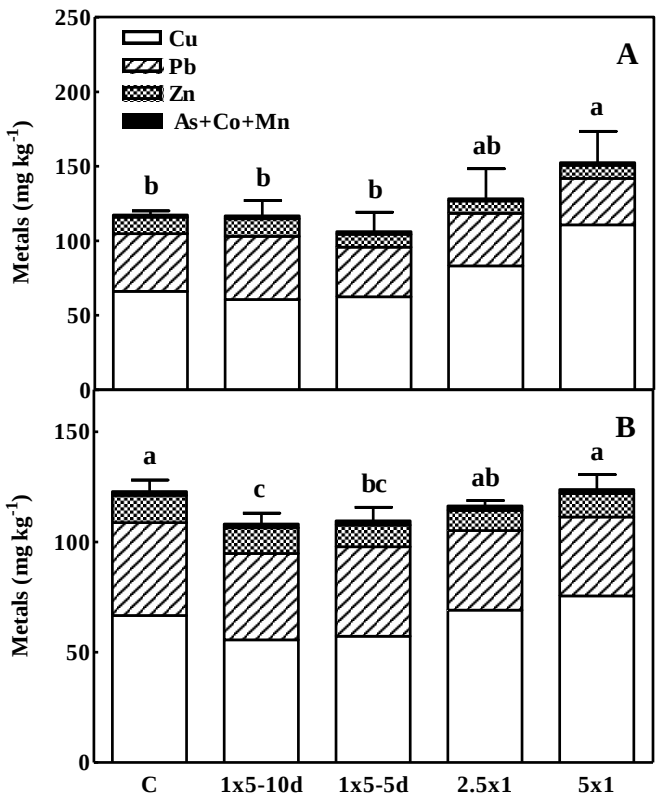


FIGURE 4. Mean concentrations of available metals in the cinder-sand mixture following amendments of pots planted with *B. carinata* (**A**) and *R. sativus* (**B**). Results are means ± SE (*n* = 3). For each treatment means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). For abbreviations see Figure 2.

In the mixture planted with *B. carinata* and amended with 2.5 and 5 mmol EDDS kg^{-1} copper represented 65 and 73% of total available metals, respectively (Figure 4A). In the multiple treatments (1x5-10d and 1x5-5d) Cu accumulated mostly at lower depths (28 and 42 cm), whereas in the two single amendments a remarkable accumulation of Cu occurred at 28 cm of depth (Supporting Information: Figure 7A,B). The chelant treatments did not result in any appreciable variation of Pb and Zn mean concentrations in pots planted with both crops (Figure 4A,B). Available Pb concentration was enhanced in the mixture lowest horizons only following the 5x1 treatment (about 40% in comparison with the most superficial layer), the others not causing any significant change in the metal concentration (Supporting Information: Figure 7A,B). Except for the early treatment (1x5-10d), EDDS applications resulted in a similar accumulation of Zn at 28 and 42 cm of depth (about 3- and 2-fold compared to the 14 cm-depth for *B. carinata* and *R. sativus*, respectively).

EDDS and metals in shoots. *Dry Weight.* As reported in Table 2, the single lowest application (2.5x1) resulted in a slight reduction (*R. sativus*) or no loss (*B. carinata*) of shoot dry biomass compared to the untreated plants. The other amendments caused a progressive reduction of dry matter in shoots, which reached the value of about 80% in the early chelant application (1x5-10d).

TABLE 2. Shoot Dry Biomass at Harvest[a]

Species	C[b]	1x5-10d	1x5-5d	2.5x1	5x1
			(g plant^{-1})		
B. carinata	1.6 ± 0.2 a[c]	0.3 ± 0.1 c	0.8 ± 0.1 b	1.6 ± 0.2 a	1.1 ± 0.1 b
R. sativus	0.9 ± 0.2 a	0.2 ± 0.1 c	0.3 ± 0.1 bc	0.7 ± 0.1 ab	0.5 ± 0.1 bc

[a] Results are means ± SE. [b] For abbreviations see Figure 2. [c] For each species means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$).

EDDS Uptake. For the same chelant treatment, EDDS amount in the shoots was higher in *R. sativus* compared to *B. carinata*, the latter species showing very small differences among treatments, although a significant increase of EDDS resulted in the single lowest amendment (2.5x1) (Figure 5). In *R. sativus* the two single dose amendments resulted in a higher chelant accumulation (about 33%) compared to the multiple ones.

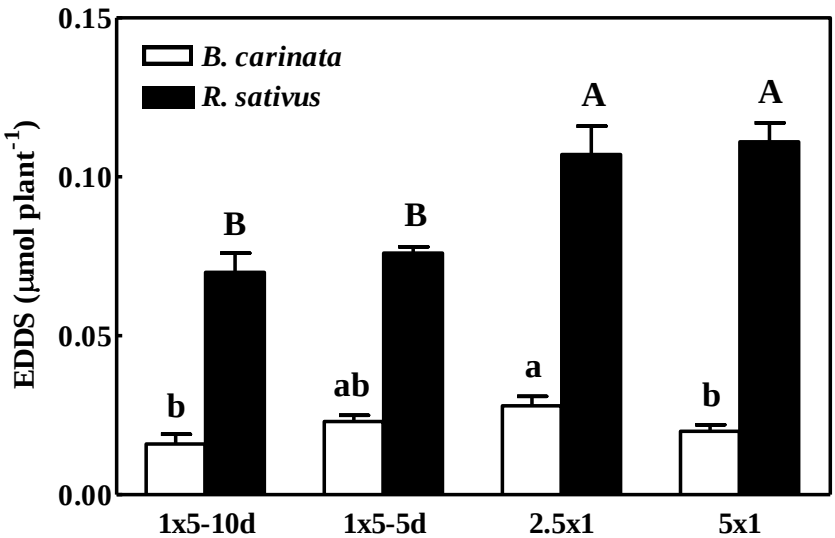


FIGURE 5. EDDS concentrations in shoots of *B. carinata* (**A**) and *R. sativus* (**B**) plants following amendments. Results are means ± SE (n = 3). For each plant organ means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). For abbreviations see Figure 2.

Metal Uptake. Together with Cu and Zn, the shoots of the two species accumulated remarkable levels of Mn (Figure 6). With the exception of Pb and to a lesser extent As, metals were taken up more effectively by *B. carinata* (Supporting Information: Figure 8). In this crop the amendment effects (namely a higher uptake compared to the control) were detectable for Co (1x5-5d), Pb (5x1), and Cu (1x5-5d, 2.5x1 and 5x1), whereas in *R. sativus* a positive response to chelant application was detected for Cu (1x5-5d and 2.5x1) and Pb (5x1) (Supporting Information: Figure 8). Among the four treatments, the 2.5x1

amendment was in general more effective in enhancing shoot metal accumulation (+31% compared to the control in *B. carinata*) (Figure 6). For all metals and both species the 1x5-10d treatment was the less effective amendment.

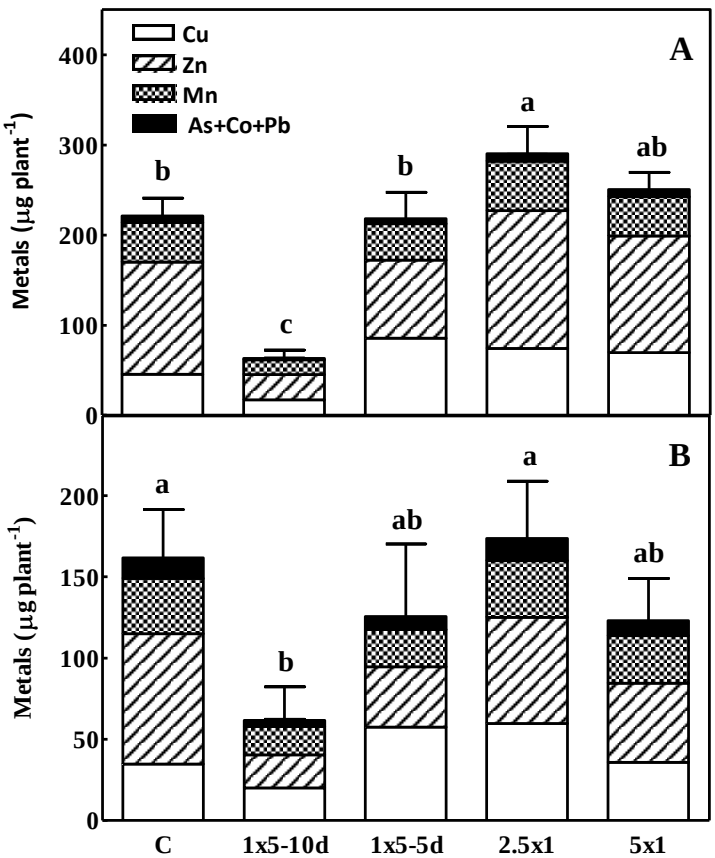


FIGURE 6. Metal concentrations in shoots of *B. carinata* (**A**) and *R. sativus* (**B**) plants following amendments. Results are means ± SE (n = 3). For each species means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). For abbreviations see Figure 2.

Discussion

EDDS Application Time and Dosage. Although EDDS is one of the most biodegradable among metal chelants, the choice of the right application time and dosage plays a pivotal importance because under not optimal conditions

ligand persistence or ligand effect (i.e. the concentration of metal(s) mobilised into the soil solution following chelant addition) can increase dramatically (*7*). In our site the presence of a thick impermeable clay layer at 70 cm of depth prevented significant leaching of contaminants, although the safety of EDDS-enhanced phytoextraction should be verified (*13*, *20*). Pyrite cinders are an inhospitable substrate for plant growth owing to their anomalous chemical-physical properties, the high Fe and S contents and the concurrent presence of more metals (*1*, *13*, *14*, *20*). In order to make up for the asphyxial soil conditions and allow plant growth, the pyrite waste was mixed with sand. In the mixture Cu resulted to be the highest bioavailable metal (Table 1). Indeed, extractable Cu, as well as Zn, was mainly bound to carbonates and thus weakly bound to soil particles and easily exchangeable, whereas Pb, which was manly bound to iron and manganese oxides, resulted less bioavailable (Figure 1). The amounts of DTPA-extractable metals detected in the mixture immediately after the harvest of the two *Brassicaceae* species showed that the different EDDS amendments had a different effect on mobilisation of metals (Figure 4) and on the growth of plants (Table 2). In agreement with comparative experiments (*7*, *14*, *21*, *22*, *23*), desorption of metals in the soil mixture was related to complexation with chelants. However, only a limited fraction of mobilised metals was extracted from the soil solution and effectively absorbed and translocated in the plants, whereas the remaining portion might have been re-stabilized by biodegradation of the ligand (*7*). Similarly to the results obtained in previous experiments (*14*), for all EDDS treatments Cu was the metal desorbed in the highest amount in the soil mixture after the growth of both crops, probably due to the relatively high stability constant (log K 18.4) of this metal with EDDS, followed by Pb and to a lesser extent by Zn, whose chelation constants were lower (log K 12.7 and 13.4, respectively) (*14*, *21*). After the harvest of *R. sativus*, the reduction (Figure 4B) and localization in depth of available metal concentrations (Supporting Information: Figure 7) following repeated treatments

were probably linked to a higher metal leaching (*20*). At the same time, the decrease in mobilisation patterns of metals may also be caused by sorption of the chelate to soil particles or competition of other electropositive elements for chelation (*7*). Evaluating EDDS leaching along the soil profile (Figure 3), the EDDS behavior in the mixed soil might be directly related to the EDDS amounts in the percolation water collected from pots planted with the two crops following amendments (Figure 2). In both plants, either the total and complexed EDDS were more leached in case of repeated applications, confirming the increase in EDDS amounts along the soil profile (Figures 2 and 3). On the contrary, both the single doses resulted in a lower leaching and an accumulation of EDDS in the superficial layers (Figures 2 and 3), thus minimizing the risks of leaching.

The single, moderate dose of EDDS (2.5x1) positively affected uptake and translocation of metals which induced higher metal removal. This amendment applied one week before harvest, in the pre-flowering stage, increased the available metal pool without causing extreme risk of leaching. The same results were already observed by Meers et al. (*7*), indicating that for optimal extraction soil amendments should be applied in pre-harvest rather than during the plant cycle.

Phytoextraction Effectiveness. In the two *Brassicaceae* species grown in the presence of several toxic metals, the shoot dry weight (Table 2) turned out to be inversely related to the volumes of the percolation water collected throughout their life cycle (*20*). As previously observed (*14*), in both plants growth depression might be caused by metal/chelant toxicity. Indeed, impaired growth may be considered a direct consequence of the higher metal accumulation in plant shoots which negatively influenced metal removal. *R. sativus* was previously grown in pyrite wastes showing good phytoextraction abilities (*24*), but several authors have highlighted the progressive reduction of shoot biomass as well as its rapid senescence under EDDS amendment (*25*). The overall results show that the extended applications (1x5-10d, 1x5-5d) reduced the

phytoextraction effectiveness, increasing the potential risk of leaching. EDDS measurements in shoots of both species clearly indicate that substantial uptake of EDDS occurred, even if EDDS uptake was higher in *R. sativus* and, in particular, if this species was treated with the single doses (2.5x1, 5x1) (Figure 5). A possible explanation of this higher accumulation of EDDS in the shoots of radish (+33%) compared to mustard may be related to a general higher root damage as a consequence of early senescence (*25*). Consequently, EDDS passed through the root cortex, reached the xylem along a nonselective passive apoplastic pathway at breaks in the root endodermis and the Casparian strip, and then rapidly was transported to the plant shoots (*14*, *17*, *22*). In *B. carinata*, the highest concentration of EDDS was detected following the 2.5x1 application (Figure 5), dosage at which no shoot reduction occurred (Table 2), suggesting a better tolerance of this species to high metal concentrations. The uptake of essential metals, such as Cu and Zn, depends on the absence or presence of chelating agents. The effects of EDDS on metal uptake could be explained by a shift of the main transport route from the symplastic (selective uptake) to the apoplastic pathway (nonselective uptake) (*17*, *21*, 26, *27*). With a high dissolved metal concentration, the nonselective uptake in the presence of EDDS would exceed selective uptake along the symplastic pathway (*14*, *22*).

A hurdle for phytoextraction might be plant growth before and after chelant addition. Notwithstanding chelanting agents are thought to protect cells from the oxidative damaging effects of metals inactivating and minimizing the negative impact of free metal ions (*14*, *17*, *28*), high chelant concentrations are known to decrease plant growth (*21*, *25*, *29*). In this experiment, the single treatments were not long-lasting and were added shortly before harvest. Indeed, a high biomass at the moment of chelant amendment is important to provide a high transpirational flow for chelant uptake (*21*). In according to Grčman et al. (*30*), the splitting of EDDS distribution in repeated low doses decreased the phytoextraction effectiveness (Figure 6), probably owing to a temporary high

concentration of EDDS and bioavailable metals in the shallow layer at first and then in the lower layers of pots. This difference resulted in a greater toxicity for the sensitive young plants as highlighted by the higher shoot biomass reduction (Table 2). At the same time, differently from Grčman et al. (*30*), EDDS amendments in separate doses did not reduce leaching but increased the volumes of percolation water and leachate (*20*). Previous studies evidenced that the presence of EDDS increases the translocation of metals (*14*, *17*, *21*, *31*, *32*), although it slows down the uptake of metals such as Cu (*17*). The affinity constant of the chelants and the composition of the soil determine the speciation of the chelant in soil solution, which in turn is related to the metal uptake. In multiple metal-contaminated soils available metal concentrations can be very different from those in single polluted soils (*21*). The EDDS presence increased the biodisponibility of metals, above all of Cu, being the chelation constant very high and the consequent CuEDDS complex very strong. Because of their lower affinity for the chelant, ZnEDDS and PbEDDS are weaker than CuEDDS and therefore competition starts to play a role. As a consequence of this competition together with a different metal mobility, in both crops the metal concentrations in shoots were different (Figure 6). In xylem free ions such as Cu and Zn were probably complexed again with free EDDS, thus increasing the rate of translocation from roots to shoots using transpiration as the main driving force (*10*, *17*, *21*, *31*, *32*, *33*). EDDS accelerated metal transport, probably because of formation of electro-neutral complexes between metal free ions and EDDS, which probably prevented metal ions to react with negatively charged cell wall components (as carboxylic and hydroxylic groups), therefore facilitating transport via transpiration stream.

Despite some authors highlighted the fact that research focused on chelant-enhanced phytoextraction of metals from polluted soils has reached a dead-end (*2*, *34*), the data obtained in this experiment show the possibility to go on in this direction. Differently from *R. sativus*, *B. carinata* treated with a single moderate

dose (2.5x1) did not suffer any biomass reduction, demonstrating its ability to survive the toxic effects of more metals with the simultaneous accumulation of them.

Acknowledgements

EDDS (Octaquest E30) was kindly obtained from Innospec Limited (Chesire, UK) as the Na_3EDDS salt. Seeds of *Brassica carinata* cv. 180 were kindly provided by Prof. M. Mazzoncini (Dipartimento di Agronomia e Gestione dell'Agrosistema, University of Pisa). This study was funded by the University of Pisa (Fondi di Ateneo 2008).

Literature Cited

(1) Quartacci, M.F.; Irtelli, B.; Gonnelli, C.; Gabbrielli, R.; Navari-Izzo, F. Naturally-assisted metal phytoextraction by *Brassica carinata*: Role of root exudates. *Environ. Pollut*. **2009**, *157*, 2697-2703.

(2) Evangelou, M.W.H.; Ebel, M.; Schaeffer, A. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity and fate of chelating agents. *Chemosphere* **2007**, *68*, 989-1003.

(3) Meers, E.; Tack, F.M.G.; Verloo, M.G. Degradability of ethylenediamine disuccinic acid (EDDS) in metal contaminated soils: Implications for its use in soil remediation. *Chemosphere* **2008**, *70*, 358-363.

(4) Meers, E.; Tack, F.M.G.; Van Slycken, S.; Ruttens, A.; Vangronsveld, J.; Verloo, M.G. Chemically assisted phytoextraction: a review of potential soil amendments for increasing plant uptake of heavy metals. *Internat. J. Phytorem*. **2008**, *10*, 390-414.

(5) Hauser, L.; Tandy, S.; Schulin, R.; Nowack, B. Column extraction of heavy metals from soils using the biodegradable chelating agent EDDS. *Environ. Sci. Technol*. **2005**, *39*, 6819-6824.

(6) Zhao, Z.; Xi, M.; Jiang, G.; Liu, X.; Bai, Z.; Huang, Y. Effects of IDSA, EDDS and EDTA on heavy metals accumulation in hydroponically grown maize (*Zea mays* L.). *J. Hazard. Mater*. **2010**, *181*, 455-459.

(7) Meers, E.; Ruttens, A-; Hopgood, M.J.; Samson, D.; Tack, F.M.G. Comparison of EDTA and EDDS as potential soil amendments for enhanced phytoextraction of heavy metals. *Chemosphere* **2005**, *58*, 1011-1022.

(8) Robinson, B.H.; Bañuelos, G.; Conesa, H.M.; Evangelou, M.W.H.; Schulin, R. The phytomanagement of trace elements in soil. *Crit. Rev. Plant Sci*. **2009**, *28*, 240-266.

(9) Kos, B.; Leštan, D. Chelator induced phytoextraction and in situ soil washing of Cu. *Environ. Pollut*. **2004**, *132*, 333-339.

(10) Leštan D.; Luo, C.; Li, X. The use of chelating agents in the remediation of metal-contaminated soils: A review. Environ. Pollut. 2008, 153, 3-13.

(11) USEPA. EPA method 3051, microwave assisted acid digestion of sediments, sludges, soils, and oils. In *Test Methods for Evaluating Solid Waste*, 3rd ed.; Washington, DC, **1995**.

(12) Mi.P.A.F. (Ministero delle Politiche Agricole e Forestali). *Metodi di Analisi Chimica del Suolo*. Franco Angeli (ed.), Milan, Italy, **2000**.

(13) Vamerali, T.; Bandiera, M.; Mosca, G. In situ phytoremediation of arsenic- and metal-polluted pyrite waste with field crops: Effects of soil management. *Chemosphere* **2011**, *83*, 1241-1248.

(14) Quartacci, M.F.; Irtelli, B.; Baker, A.M.J.; Navari-Izzo, F. The use of NTA and EDDS for enhanced phytoextraction of metals from a multiply contaminated soil by *Brassica carinata*. *Chemosphere* **2007**, *68*, 1920-1928.

(15) Rauret, G.; Lopez-Sanchez, J.F.; Sahuquillo, A.; Barahona, E.; Lachica, M.; Ure, A.; Muntau, H.; Quevauviller, P. **2000**. EUR Report 19503 EN, CEC, Brussels.

(16) USEPA. EPA method 3052, microwave assisted acid digestion of siliceous and organically based matrices. In *Test Methods for Evaluating Solid Waste*, 3rd ed.; Washington, DC, 1995.

(17) Cestone, B.; Quartacci, M.F.; Navari-Izzo, F. Uptake and translocation of CuEDDS complexes by *Brassica carinata*. *Environ. Sci. Technol*. **2010**, *44*, 6403-6408.

(18) Metsärinne, S.; Tuhkanen, T.; Aksela, R. Photodegradation of ethylenediaminetetraacetic acid (EDTA) and ethylenediamine disuccinic acid (EDDS) within natural UV radiation range. *Chemosphere* **2001**, *45*, 949-955.

(19) Vandevivere, P.C.; Saveyn, H.; Verstraete, W.; Feijtel, T.C.J.; Schowanek, D.R. Biodegradation of metal-[S,S]-EDDS complexes. *Environ. Sci. Technol.* **2001**, *35*, 1765-1770.

(20) Bandiera, M.; Mosca, G.; Vamerali, T. Phytotoxicity and metal leaching in EDDS-assisted phytoextraction from pyrite wastes with Ethiopian mustard and fodder radish. *Plant Bios.* **2010**, *144*, 490-498.

(21) Tandy, S.; Schulin, R.; Nowack, B. Uptake of metals during chelant-assisted phytoextraction with EDDS related to the solubilized metal concentration. *Environ. Sci. Technol.* **2006**, *40*, 2753-2758.

(22) Nowack, B.; Schulin, R.; Robinson, B.H. Critical assessment of chelant-enhanced metal phytoextraction. *Environ. Sci. Technol*. **2006**, *40*, 5225-5232.

(23) Luo, C.; Shen Z.; Baker, A.J.M.; Li, X. A novel strategy using biodegradable EDDS for the chemically enhanced phytoextraction of soils contaminated with heavy metals. *Plant Soil* **2006**, 285, 67-80.

(24) Bandiera, M.; Mosca, G.; Vamerali, T. Humic acids affect root characteristics of fodder radish (*Raphanus sativus* L. Var. *oleiformis* Pers.*)* in metal-polluted wastes. *Desalination* **2009**, *247*, 79–92.

(25) Kos, B.; Grčman, H.; Leštan, D. Phytoextraction of lead, zinc and cadmium from soil by selected plant. *Plant Soil Environ*. **2003**, *49*, 548-553.

(26) Collins, R.N.; Merrington, G.; McLaughlin, M.J.; Knudsen, C. Uptake of intact zinc-ethylenediaminetetraacetic acid from soil is dependent on plant species and complex concentration. *Environ. Toxicol. Chem*. **2002**, *21*, 1940-1945.

(27) Tanton, T.W.; Crowdy, S.H. The distribution of lead chelate in the transpiration stream of higher plants. *Pestic. Sci*. **1971**, *2*, 211-213.

(28) Ruley, A.T.; Sharma, N.C.; Sahi, S.V.; Singh, S. R.; Sajwan, K.S. Effects of lead and chelators on growth, photosynthetic activity and Pb uptake in *Sesbania drummondii* grown in soil. *Environ. Pollut*. **2006**, *144*, 11-18.

(29) Luo, C.; Shen, Z.; Li, X. Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. *Chemosphere* **2005**, *59*, 1-11.

(30) Grčman, H.; Velikonja-Bolta, Š.; Vodnic, D., Leštan, D. EDTA enhanced heavy metal phytoextraction: metal accumulation, leaching and toxicity. *Plant Soil* **2001**, *235*, 105–114.

(31) Tandy, S.; Schulin, R.; Nowack, B. The influence of EDDS on the uptake of heavy metals in hydroponically grown sunflowers. *Chemosphere* **2006**, *62*, 1454-1463.

(32) Bell, P.F.; McLaughlin, M.J.; Cozens, G.; Stevens, D.P.; Owens, G.; South, H. Plant uptake of ^{14}C-EDTA, ^{14}C-Citrate , and ^{14}C-Histidine from chelator-buffered and conventional hydroponic solutions. *Plant Soil* **2003**, *253*, 311-319.

(33) Nowack, B.; Schwyzer, I.; Schulin, R. Uptake of Zn and Fe by wheat (*Triticum aestivum* var. Greina) and transfer to the grains in the presence of chelating agents (ethylenediaminedisuccinic acid and ethylenediaminetetraacetic acid). *J. Agr. Food Chem*. **2008**, *56*, 4643-4649.

(34) Komarek, M.; Vaněk, A.; Mrnka, L.; Sudová, R.; Száková, J.; Tejnecký, V.; Chrastný, V. Potential and drawbacks of EDDS-enhanced phytoextraction of copper from contaminated soils. *Environ. Pollut*. **2010**, *158*, 2428-2438.

Supporting Information

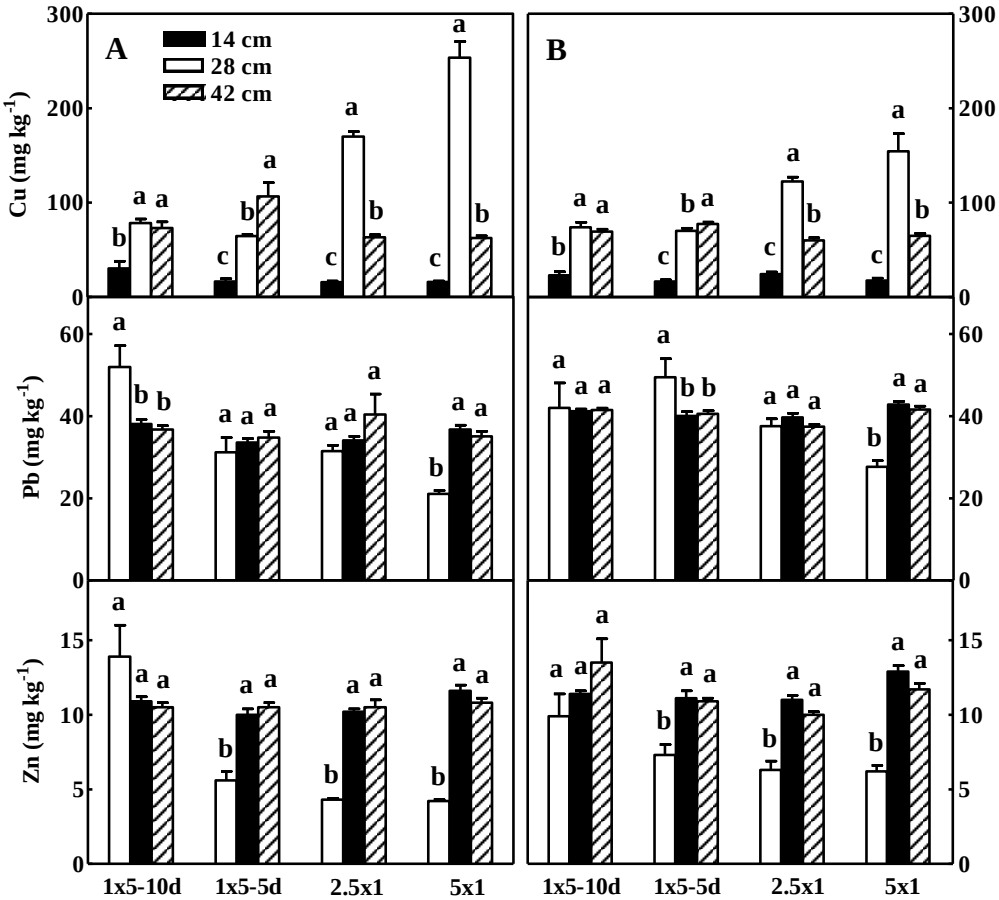


FIGURE 7. Metal concentrations in the cinder-sand mixture at three different depths following amendments of pots planted with *B. carinata* (**A**) and *R. sativus* (**B**). Results are means ± SE (*n* = 3). For each treatment means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). For abbreviations see Figure 2.

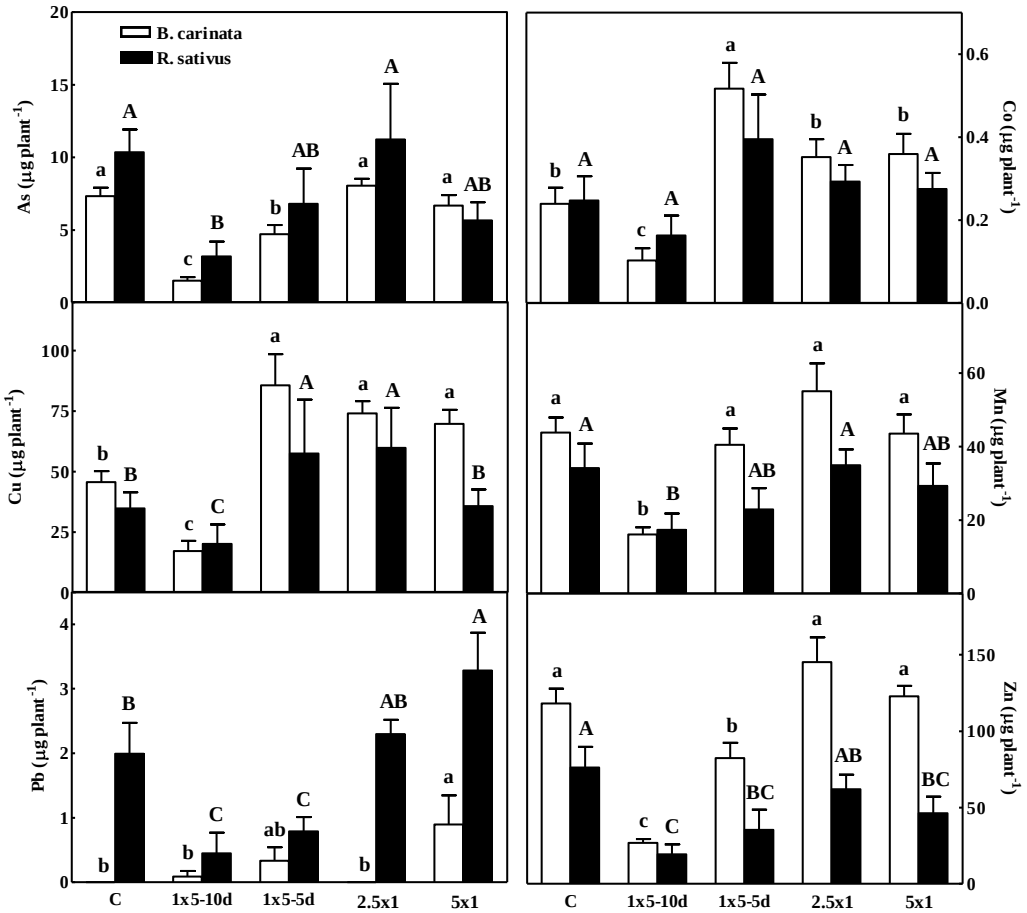


FIGURE 8. Metal concentrations in shoots of *B. carinata* and *R. sativus* plants following amendments. Results are means ± SE (*n* = 3). For each species means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). For abbreviations see Figure 2.

UPTAKE AND TRANSLOCATION OF CuEDDS COMPLEXES BY *BRASSICA CARINATA*

Benedetta Cestone, Mike F. Quartacci, and Flavia Navari-Izzo

Dipartimento di Biologia delle Piante Agrarie, Università di Pisa, Via del Borghetto 80, 56121 Pisa, Italy

Environ. Sci. Technol. **2010**, *44*, 6403-6408

Abstract

The knowledge of the mechanisms that underlie metal complex uptake may lead to the development of new strategies for enhancing metal phytoextraction. As metals such as copper are actively taken up by roots, by inhibiting the proton driving force it is possible to obtain preliminary indications on the metal complex uptake mechanism. For this, Cu, EDDS and Cu-EDDS uptake kinetics of *Brassica carinata* excised roots incubated in 30 and 150 μM solutions of either the metal, the chelant, and the complex were determined in the presence or not of the ATPase inhibitor vanadate. Following both Cu and CuEDDS treatments, metal uptake was negatively influenced by vanadate, whereas EDDS uptake did not, suggesting that Cu and the chelant did not enter the roots in their complexed form but by two different routes. The incubation in the same solutions of *B. carinata* intact plants showed that, differently from Cu, EDDS was largely translocated to shoots but its low concentration resulted in a Cu to EDDS molar ratio ranging from 2 to 4 depending on metal complex concentration in the solution confirming that the uptake pathways of the two compounds were different.

Brief: Results suggest that copper and EDDS do not enter the roots in their complexed form but by two different routes.

Introduction

Chelant-enhanced phytoextraction aims to clean up metal-polluted soils by stimulating plants to accumulate the contaminating metal(s) in the harvestable parts owing to the addition of chelating agents to the soil. This technique exerts its effects by solubilizing target metals from soil and making them more available for plant uptake and translocation to shoots (*1*, *2*). The EDTA structural isomer (S,S)-N,N'-ethylenediamine disuccinic acid (EDDS) has received attention as potential replacement for EDTA as it is a strong chelant and unlike EDTA it is easily biodegradable (*3*).

Differently from the active mechanisms involved in the uptake of essential metals such as Cu and Zn, it is widely accepted that synthetic chelants and their metal complexes pass through the root cortex and reach the xylem along a fully non-selective apoplastic pathway. It is unlikely that the anionic metal-ligand complexes pass through the cell membrane due to its large size and to the fact that no specific transporters are known. In spite of this, complexes can cross the Casparian strip barrier at the root tip where it is not fully differentiated or damaged by lateral roots formation, by high chelant concentrations and seedling transplantation. This nonselective passive apoplastic uptake allows the complex to reach the xylem and to be translocated to the shoots using transpiration as the main driving force (*4*–*8*). However, a not fully understood selective route of uptake was also suggested (*9*). According to this pathway, complexes actively move through some endodermal passage cells adjacent to the Casparian strip to the other side of the strip and then (again) extracellularly to the xylem.

An active mechanism is also at the basis of the split-uptake mechanism by which only free metal ions are taken up by roots (*8*). As the free metal ion activity decreases, metals are released from the complex to compensate for the shift in the equilibrium, maintaining a supply for their uptake in the rhizosphere. Indeed, Fe-EDTA is known to dissociate before plant uptake (*8*, *10*).

Hydroponic solutions are useful research tools because of the easiness in which toxic elements and chelants are manipulated to investigate metal complex uptake into the roots and translocation to the above-ground parts (*4*, *6*, *7*, *11*). In addition, the use of equimolar or high concentrations of the chelant in the solution eliminates free metal availability. However, hydroponic trials alone cannot be transferred directly to phytoextraction in the field where many soil factors affect the process.

To improve Cu phytoextraction effectiveness a better understanding of the key mechanisms of chelant-stimulated metal acquisition is needed. To predict soil-plant transfer of metals the understanding of the uptake mechanisms of metal complexes is fundamental for enhancing metal phytoextraction potential and for applying the more appropriate technology for chelant distribution (doses and application times respectful of the environment). The aim of this work was to investigate the short-term influx of free Cu ions, EDDS or CuEDDS complexes into excised roots of *Brassica carinata* at equimolar concentrations of both the metal and the chelant. Inhibitory experiments concerning selective metal uptake mechanism and solute leakage measurements were also carried out. In addition, on whole plants the translocation of Cu and CuEDDS complexes from roots to the above-ground parts was studied.

Experimental section

Plant Growth. Seeds of *Brassica carinata* cv 180 were surface sterilized for 10 min with NaClO (about 2% of active chlorine) and sown directly in polystyrene trays with holes containing wet expanded clay. The trays were placed in plastic bins filled with 6 L of a continuous aerated nutrient solution (*2*). Plants were grown at 16-h photoperiod, 400 µmol m^{-2} s^{-1} photon flux density, 23 ± 1 °C temperature, and 70-75% relative humidity.

Experimental Setup. In order to select the Cu concentrations to be used in the experimental solutions 3-week-old plants were collected from the nutrient

solution and excised roots were incubated in experimental solutions at increasing Cu concentrations (0.25-300 μM Cu as $CuSO_4$) for 30 min in the above-reported conditions. In the experimental solutions all micronutrients other than Cu and $NH_4H_2PO_4$ were omitted to avoid competition among metals and precipitation of copper phosphate. At the end of the treatments adsorbed metals were desorbed from roots by soaking them in 5 mM $Pb(NO_3)_2$ for 30 min under continuous stirring and rinsed with distilled water. Roots were then oven-dried for metal determination.

In Experiment I plants were collected from the nutrient solution and the excised roots were incubated separately in experimental solutions containing Cu (as $CuSO_4$), EDDS (as Na_3EDDS) or CuEDDS at two concentrations of both the metal and the chelant (30 and 150 μM) for increasing time (0-12 h) at the growth conditions above-reported. Control excised roots were incubated in the nutrient solution (0.12 μM Cu). In order to inhibit ATPase activity 500 μM Na_3VO_4 and 50 mM KCl were added to each experimental solutions before excised roots were incubated. At the end of the treatments metals were desorbed and rinsed with distilled water. Roots were then frozen in liquid N_2 and stored at –80°C until EDDS analysis or oven-dried for Cu determination.

In Experiment II whole plants collected from the nutrient solution were incubated in controlled conditions for 48 h using both the experimental solutions (Cu, EDDS or CuEDDS in the presence or not of 500 μM vanadate) at the concentrations reported for Experiment I. Control plants were incubated in the nutrient solution containing 0.12 μM Cu. After desorption of metals from roots by 5 mM $Pb(NO_3)_2$ for 30 min under continuous stirring shoots were cut 1 cm above roots, and shoots and roots were collected after washing with distilled water. Shoots and roots were then frozen in liquid N_2 and stored at –80°C until EDDS analysis or oven-dried for Cu determination.

Copper Analysis. Shoots and roots were oven-dried at 110°C for 24 h. Dried ground material was then microwave digested with a mixture (3:1, v/v) of HNO_3

(65%) and H_2O_2 (30%) in a capped Teflon pressure digestion vessel at 200°C for 20 min and analysed for Cu using a Perkin-Elmer Optimal DV 2100 ICP OES. Standards (National Institute of Standards and Technology, MD, USA) and reagent blanks were run with all samples to ensure accuracy and precision in the analyses (*12*). The standard reference material used was SRM 1570a (spinach leaves).

EDDS Analysis. For EDDS analysis frozen shoots and roots were first lyophilized. The dry material was then extracted in a mortar with 50% ethanol (1:5, w/v), transferred to a test tube, and sonicated for 2 h with a Bransonic 3510 ultrasonic apparatus (Branson, Danbury, CT, USA) at room temperature to avoid degradation or cyclization of EDDS (*13*). After centrifugation at 3000 *g* for 3 min, the supernatant was filtered through 0.45 μm nylon syringe filters (Millipore). EDDS derivatization and analysis were carried out as described by Metsärinne et al. (*14*). Analyses were carried out by HPLC with a Waters HPLC system consisting of two 515 pumps and a 2487 programmable UV detector. EDDS was separated using a Waters Spherisorb ODS2 C18 reverse-phase column (25 x 0.46 cm, 5 μm) and detected at 254 nm wavelength. A 15% methanol and 85% tetrabutylammoniumbromide (0.02 M) eluent at a flow rate of 1 ml min^{-1} was used as the mobile phase. Identification of EDDS was obtained by comparison of its retention time with that of standards. Quantification of EDDS was obtained by comparing calibration curves. Chromatogram analysis was performed by Millennium 32 software (Waters).

Relative Leakage Ratio Measurement. Relative leakage ratio (RLR) was evaluated by determining solute release from roots (*15*). Solute release was measured by determining diffusate conductivity using a Janway 4010 Conductivity Meter. RLR was expressed as the ratio of conductivity of solute leakage after 24 h to total conductivity following liquid N_2 treatment.

Determination of ATPase Activity. The H^+-ATPase hydrolytic activity of root microsomal fractions was carried out following the rate of NADH oxidation at 340 nm (*16*).

Statistical Analysis. All statistical analysis was carried out with Costat 6.4 (CoHort software). The error bars represent the standard deviation of the mean of three independent experiments (n=3) each analyzed twice. The effects of experimental factors were evaluated by one-way ANOVA and comparisons between means were carried out using the LSD test at the significance level of $P \leq 0.05$.

Results

Concentration-dependent uptake of copper. Excised roots of *B. carinata* showed two concentration-dependent uptake mechanisms for free Cu^{+2} ions (Figure 1) which kinetics fitted well to a typical Michaelis-Menten curve (R^2 = 0.98). A first uptake saturation curve showing a K_M value of 1.4 μM was observed at Cu concentrations up to 60 μM, whereas a second more flat curve was present at higher concentrations (K_M = 217.7 μM).

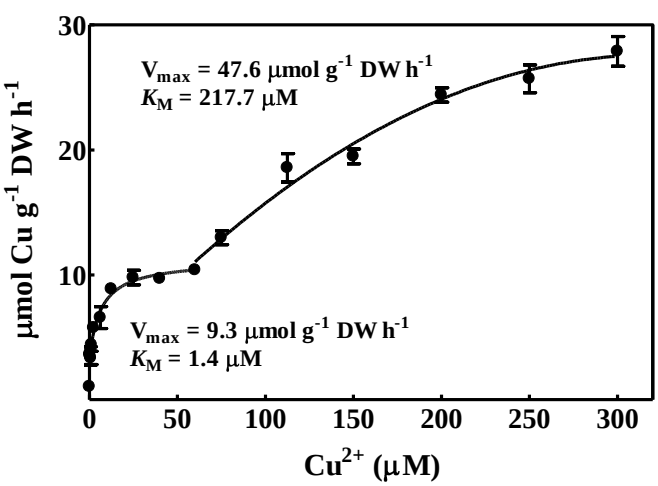


FIGURE 1. Copper uptake by *Brassica carinata* excised roots incubated for 30 min in solutions containing increasing concentrations of Cu. Data points are means ± SD (*n* = 3).

Experiment I. *Root Solute Leakage.* The relative leakage ratio of excised roots incubated for 12 h in solutions containing 30 or 150 μM Cu, CuEDDS or EDDS is reported in Figure 2. Compared to control roots, free Cu ions caused an early leakage of solutes at both concentrations (already after 1 h of incubation) showing a continuous increase during time (Figure 2A), the 150 μM incubation resulting in a higher leakage. In contrast, CuEDDS and EDDS treatments resulted less toxic inducing a significant membrane leakage only at 12 h (Figure 2B,C).

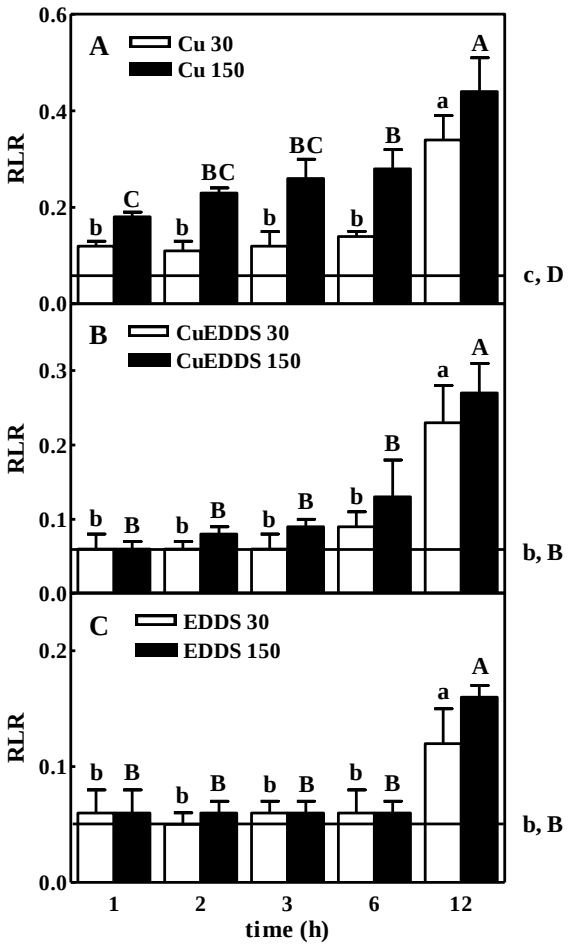


FIGURE 2. Relative leakage ratio (RLR) of *B. carinata* excised roots incubated for increasing time in solutions containing 30 or 150 μM Cu (**A**), CuEDDS (**B**) or EDDS (**C**). Results are means ± SD (*n* = 3). For each treatment means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). The horizontal lines represent the mean value of control roots (0.12 μM Cu) throughout the treatment and related significance.

Root Copper Influx. In order to select the vanadate concentration to be used in the experimental solutions the ATPase inhibitor was added at increasing doses, and RLR of roots was determined after 12 h of incubation. Up to 500 μM vanadate no significant differences in comparison with the control were

observed (data not shown), concentration beyond which an enhanced solute leakage occurred. At 500 μM vanadate (the concentration selected for the inhibitory experiments) an about 90% inhibition of ATPase activity was found.

Uptake of free Cu ions by excised *B. carinata* roots was monitored over 12 h (Figure 3A). The influx displayed a saturation curve at both concentrations in the presence or not of vanadate. At both concentrations metal uptakes resulted in a rapid initial phase (up to 1 h) followed by a steady-state phase in which the rate of influx progressively lowered (150 μM) or remained almost constant (30 μM).

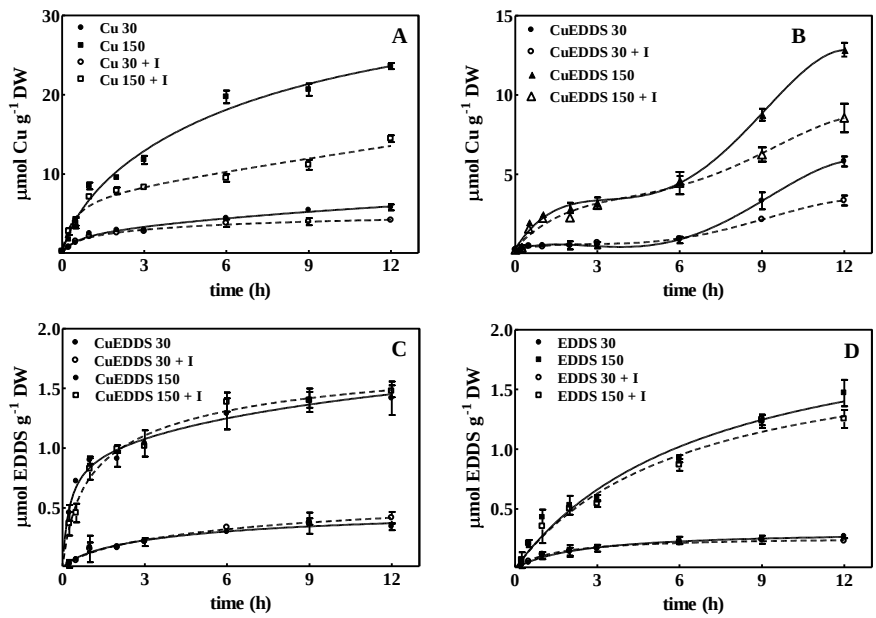


FIGURE 3. Copper and EDDS uptake by *B. carinata* excised roots incubated for increasing time in 30 or 150 μM Cu (**A**), CuEDDS (**B**, **C**) or EDDS (**D**) solutions in the presence or not of vanadate (I, inhibitor). Data points are means ± SD (*n* = 3).

The inhibition of Cu uptake induced by vanadate was observed at different times (6 h at the lowest and 1 h at the highest metal concentration). At the end of the incubation the addition of vanadate reduced Cu uptake by 28 and 40% in the 30 and 150 μM treatments, respectively. The time course of copper influx into CuEDDS-treated roots (Figure 3B) showed a trend somewhat different from

that observed for Cu ions. In this treatment the initial influx phase was followed by an almost constant uptake up to 6 h, after which a second influx period occurred. Also in this case the uptake of the metal into excised roots was reduced by vanadate (Figure 3B). The inhibition rates at 12 h were 42 and 33% in the 30 and 150 μM CuEDDS treatment, respectively. Compared to free Cu ion incubation, the lowest complex concentration in the absence of vanadate resulted in a similar final metal level, whereas at the highest concentration there was a 1.8-fold reduction (from 23.6 to 12.8 μmol Cu g^{-1} DW).

Root EDDS Influx. In both CuEDDS and EDDS treatments the chelant influx into roots showed a similar behavior at both concentrations (Figures 3C,D) reaching at the end of the incubation period almost the same values. The presence of vanadate did not influence EDDS uptake. The CuEDDS treatment resulted in chelant molar concentrations about 10 and 17-fold lower (at 30 and 150 μM, respectively) compared to those detected for Cu in the same treatment (Figures 3B,C). Following CuEDDS incubation a linear relationship between metal and chelant uptake was observed up to 6 h (R^2 = 0.87 and R^2 = 0.91 at 30 and 150 μM, respectively), time after which chelant influx stopped whereas Cu one continued.

Experiment II. *Solute Leakage.* The root RLR values of intact plants incubated for 48 h at 30 and 150 μM Cu, CuEDDS or EDDS are shown in Figure 4. Compared to control plants, free Cu ions induced a significant solute leakage at both levels. On the contrary, CuEDDS and EDDS alone treatments caused an enhancement of the RLR value only at the highest concentration. In any case, the increase in the leakage was relatively slight not exceeding the value of 0.3.

Plant Copper Uptake. In comparison with control plants, Cu incubation caused an increase in root metal concentration (6- and 76-fold at 30 and 150 μM treatments, respectively), whereas in shoots the enhancement was more than halved (Figure 5A). The presence of the inhibitor vanadate drastically reduced copper concentrations in roots (–64 and –83%, respectively) and shoots.

Treatments did not increase metal translocation to shoots which remained constant at a value of about 3% of total copper.

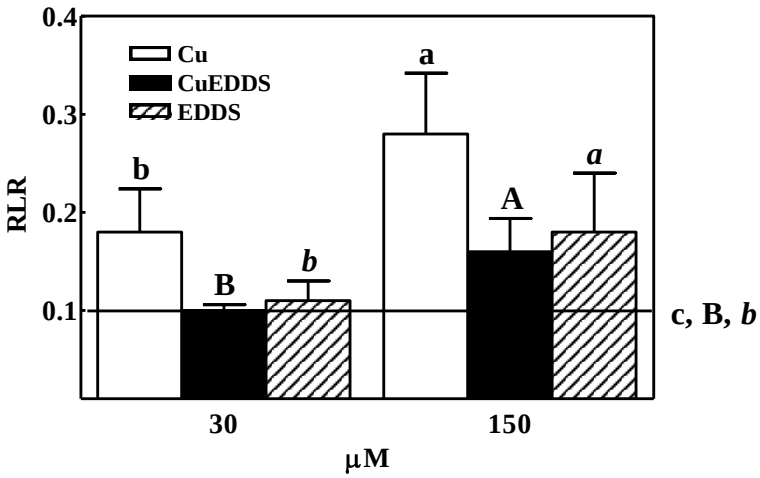


FIGURE 4. Root relative leakage ratio (RLR) of *B. carinata* intact plants incubated for 48 h in solutions containing 30 or 150 μM Cu, CuEDDS or EDDS. Results are means ± SD (n = 3). For each treatment means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$). The horizontal line represents the mean value of control roots (0.12 μM Cu) and related significance.

Also in the CuEDDS treatment an increase in root and shoot copper concentrations was observed when compared to control plants (Figure 5B). However, at 30 and 150 μM CuEDDS the amount of metal in roots was 67 and 90% lower than that detected following Cu incubation, whereas shoot copper concentrations did not show differences. As a consequence the amount of copper translocated to the shoots increased to about 10%. As for Cu incubation, vanadate inhibited metal uptake by roots at almost the same percentages.

Plant EDDS Uptake. In the CuEDDS treatment chelant uptake by roots did not show differences at both concentrations (Figure 5C). On the contrary, in shoots an about 7-fold increase at the highest metal complex concentration was observed. Compared to copper EDDS was present in much lesser amount both in

roots and in shoots. In addition, it was prevalently detected in shoots (74 and 95% of total concentration, respectively).

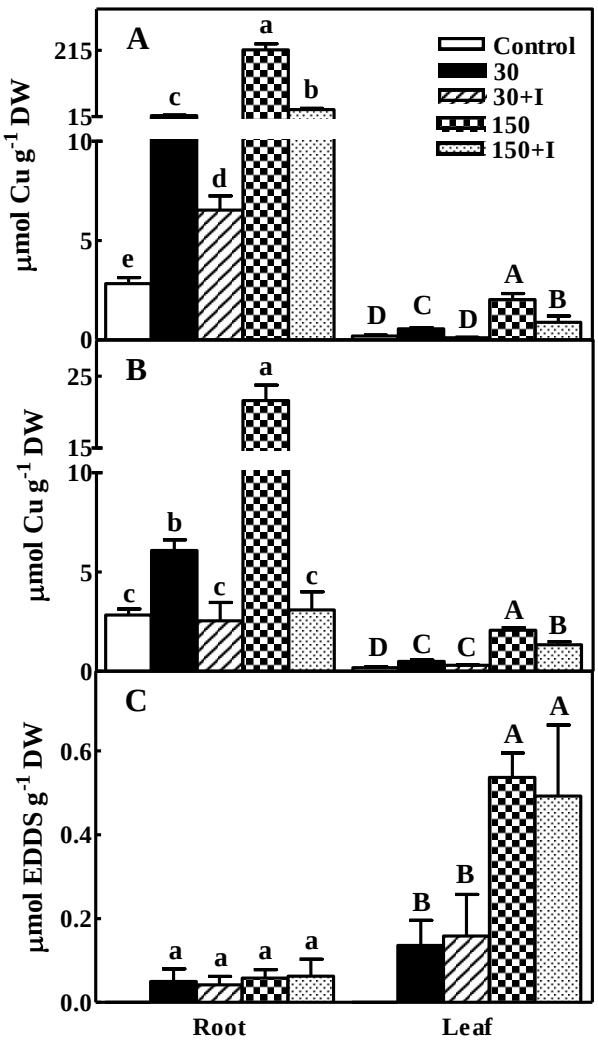


FIGURE 5. Copper and EDDS concentrations in roots and shoots of *B. carinata* intact plants incubated for 48 h in 30 or 150 µM Cu (**A**) or CuEDDS (**B**, **C**) solutions in the presence or not of vanadate (I, inhibitor). Results are means ± SD (*n* = 3). For each plant organ means followed by the same letter are not significantly different by ANOVA (LSD test, P ≤ 0.05).

Discussion

Copper and EDDS Influx into Excised Roots. The determination of metal influx kinetics is a fundamental step toward the modeling of soil-plant transfer in polluted environments. In nutrient acquisition two steps have to be distinguished: i) passive binding of ions within the free space of the cell wall and ii) active uptake of ions into the cells across membranes. The latter step might be

mediated by multiphasic mechanisms (*17*) and for its correct understanding removal of ions from the apoplasm by suitable desorption solutions is necessary. In addition, time course studies on trace metal uptake showed that it should be measured over periods sufficiently short to reflect inward unidirectional flux (*17*, *18*) and to avoid modifications of uptake kinetics that are expected to occur in the range of metal toxicity due to structural damages (*19*). For these reasons an incubation time of 30 min was chosen to study concentration-dependent influx of Cu. The use of excised roots is justified by the fact that in this case metal influx into roots is not affected by its translocation to the shoots.

The change in the influx pattern when the external Cu concentration was higher than 60 μM (Figure 1) might be explained in terms of a biphasic mechanism, consisting of a high-affinity uptake system present at lower metal concentrations and a low-affinity system active at higher concentrations. The existence of a biphasic or multiphasic kinetic pattern for Cu uptake by roots of several species was already observed and related to active mechanisms (*17*, *20*). Copper absorption is most likely mediated by the high-affinity Cu^{+} transporter COPT1 and the Cu^{2+} transporter ZIP2 as recently described for *Arabidopsis* (*21*, *22*). The hyperbolic shape of the two kinetic curves (Figure 1), both showing a saturable component, suggests that Cu uptake - in the tested concentration range - was metabolically driven by root plasma membrane carrier proteins. The K_M value (1.4 μM) derived from the first curve of the biphasic kinetics fell within the lower micromolar range described for the Ctr1 family of transporters, which COPT1 belongs to (*22*). The high K_M observed in the second phase likely originated from an inactivation of the binding sites responsible for the active transport (*17*). The calculated kinetic parameters K_M and V_{max} were not much different from those observed for intact barley roots incubated for 2 h in solutions containing Cu in the range 1.6 - 315 μM (*23*).

The 30 and 150 μM copper concentrations, representative of the two influx mechanisms (Figure 1), were selected being them the concentrations at which

CuEDDS caused, after 12 h of incubation, progressive significant increases in solute leakage compared to control excised roots. It was previously observed (*24*) that Cu induced in the elongation zone of roots both rhizoderm and outer cortex ruptures after 12 h of exposure to low levels of Cu (about 1-2 μM).

It was reported that metal complexes are less phytotoxic than free metal ions as they reduce the interactions of metals with plant metabolic targets (*7*, *12*). Free Cu, which is a redox metal responsible of oxidative damage to cellular metabolism, resulted in a much higher and earlier solute leakage compared to CuEDDS (Figure 2A,B), the latter showing increased RLR values only after 12 h compared to the control. In contrast to uncomplexed EDTA, which reduced plant growth and transpiration by removal of essential divalent ions from the plasma membrane (*11, 12*), in this study free protonated EDDS resulted in a low toxicity to excised roots (Figure 2C), likely due to the lowest concentrations and shortest time of exposure. Similarly, hydroponically grown sunflower did not show any reduction of shoot and root biomass compared to the control when treated with 500 μM EDDS (*7*).

The proton-pumping ATPase (H^+-ATPase) of the plant plasma membrane generates the proton electrochemical gradient that is necessary to activate ion and metabolite secondary transport. Vanadate, a potent inhibitor of P-type ATPases, is thought to mimic P_i and block the enzyme in its E2 form preventing the solute release. The time course influxes of both Cu and EDDS following H^+-ATPase inhibition by vanadate suggest that whereas the metal was taken up by an active mechanism, the chelant entered the roots by a non-selective passive mechanism (Figure 3). Indeed, following both Cu and CuEDDS treatments, metal influx was negatively influenced by vanadate suggesting the involvement of an active mechanism. The different times at which the inhibition was detected (1 and 6 h for Cu and CuEDDS treatments, respectively) might be explained by the fact that, besides the time needed to inactivate ATPases, the metal complex had likely to be split and free Cu ions released before active uptake occurred.

This uptake route was previously observed for another redox metal such as iron following FeEDTA treatment (*9*, *11*), but not for Pb and Zn complexes which cannot be split through the reduction or oxidation of the metals. Compared to CuEDDS treatment, the highest RLR values following free Cu incubation (Figure 2A,B) did not significantly influence Cu uptake mechanism. A saturation pattern of copper uptake, comparable with the uptake pattern of rice roots, was already observed in cell suspension cultures of bean incubated for 28 h in solutions containing Cu concentrations up to 2 mM. The saturation time was reached at significantly different time intervals depending on the metal concentration (*25*). Starting from 16 h a linear enhanced uptake of the metal was detected in both treatments (data not shown) as consequence of a disorganized root system induced by Cu - and to a lesser extent by EDDS - toxicity, which allowed a large nonselective influx.

In contrast to Cu uptake, EDDS was not influenced by vanadate throughout the two treatments (CuEDDS and EDDS) suggesting in both cases a passive mechanism (Figure 3C,D). At both levels, CuEDDS incubation resulted in chelant concentrations about one order of magnitude lower than those detected for copper (Figure 3A,B). Even if in the presence of equimolar concentrations of both the metal and the chelant in the solution, a remarkable higher Cu influx was observed suggesting that in the first 12 h of CuEDDS incubation, EDDS and Cu did not enter the roots in their complexed form but mainly by two different routes, and confirms that chelant influx was due to an apoplastic pathway (*5*, *9*) as suggested by the similar amounts of EDDS in the two treatments. The saturation pattern of the curves observed after about 9 h of incubation were likely linked to the attainment of the chelant binding capacity of root apoplasm. Similarly to Cu, the large linear influx of the chelant during CuEDDS incubation starting from 16 h (data not shown) was likely due to membrane alterations and tissue disorganization induced by the toxicity of both the metal and the chelant. When Cu molar concentrations were plotted against EDDS ones throughout the

CuEDDS treatment, a linear correlation up to 6 h was obtained at both 30 and 150 μM levels. The Cu to EDDS molar ratios, ranging from 2.3 to 2.5, further suggest that the metal and the chelant were taken up by different mechanisms.

Copper and EDDS Uptake by Intact Plants. Intact plants treated for 48 h with Cu, CuEDDS or EDDS showed root solute leakage values lower than those determined for excised roots incubated at the same concentrations for 12 h (Figures 2 and 4). All the treatments resulted in a very slight toxicity to roots even at the highest concentrations. It may be suggested that, differently from excised roots, during the incubation period intact roots were able to activate defense/repair antioxidative mechanisms which allowed functioning of membranes and cell metabolism (*3*, *15*).

At both concentrations, CuEDDS incubation determined a lower metal accumulation in roots compared to Cu likely due to both reduced membrane disorganization - as evidenced by the low root leakage - and/or the absence of free Cu ions in the solution (Figure 5A,B), which might have limited uncontrolled Cu inflow. According to other hydroponic studies (*7*, *26*, *27*), Cu uptake in the presence of EDDS was reduced compared to free Cu. As the hypothesis of a switch to an apoplastic less effective route (*28*) is not supported by the vanadate-induced inhibition of Cu uptake an active mechanism in the CuEDDS treatment may be suggested. Large apoplastic uptake of metal complexes is a function of the complex concentration in the solution (*7*) as well as metal and plant species. Above a threshold chelant concentration, a linear relationship between the solution concentration and the amount taken up is expected due to damage to membranes of root cells which normally function to control the uptake and translocation of solutes (*11*, *29*).

Following both Cu and CuEDDS incubation, shoot metal concentrations showed similar values (Figure 5A,B), even though Cu translocation in the CuEDDS treatment was, at 30 and 150 μM level, 3- and 10-fold higher, respectively, compared to the metal alone treatment. The low copper accumulation in shoots

of Cu and CuEDDS-treated plants might be related to the low RLR values and the consequent slight damage to root cells which did not allow uncontrolled inflow of solution into the stele by the apoplastic pathway (*30*). In addition, the reduced metal translocation suggests that at the two concentrations used non-selective uptake of Cu in the presence of the chelant did not occur or did not exceed active uptake along the symplastic pathway (*28*). The low translocation value of Cu-treated plants (about 3%) is in accordance with that observed for hydroponically grown sunflower (*7*), whereas the highest translocation found in sunflower following CuEDDS treatment (about 40% in comparison with 10% of the present study) may be due to a higher chelant concentration (500 μM) and a longer duration (6 days).

The presence of EDDS in different plant organs was already observed (*5–7*). Differently from Cu, the chelant was largely translocated to shoots (Figure 5C), but its very low concentration resulted in a Cu to EDDS molar ratio ranging from 2 to 4 depending on metal complex concentration (Figure 5,C). This may be considered a further indication that the uptake pathways of the two compounds were different and that only a reduced fraction of total absorbed Cu was complexed by free EDDS after passing into the xylem and then transported to the shoots (*31*). Alternatively, it cannot be turned out the possibility that part of the metal might have been taken up in the complexed form. Shoot chelant uptake increased in proportion to the dissolved EDDS, whereas root uptake did not (Figure 5C). To explain the root behavior it may be suggested that the chelant was adsorbed to the roots which become saturated at relatively low EDDS concentrations (*6*).

Acknowledgements

EDDS (Octaquest E30) was kindly obtained from Innospec Limited (Chesire, UK) as the Na_3EDDS salt. Seeds of *Brassica carinata* cv. 180 were kindly provided

by Prof. M. Mazzoncini (Dipartimento di Agronomia e Gestione dell'Agrosistema, University of Pisa). This study was funded by the University of Pisa (Fondi di Ateneo 2008).

Literature Cited

(1) Meers, E.; Tack, F.M.G.; Verloo, M.G. Degradability of ethylenediamine disuccinic acid (EDDS) in metal contaminated soils: Implications for its use in soil remediation. *Chemosphere* **2008**, *70*, 358-363.

(2) Quartacci, M.F.; Irtelli, B.; Baker, A.M.J.; Navari-Izzo, F. The use of NTA and EDDS for enhanced phytoextraction of metals from a multiply contaminated soil by *Brassica carinata*. *Chemosphere* **2007**, *68*, 1920-1928.

(3) Quartacci, M.F.; Irtelli, B.; Gonnelli, C.; Gabbrielli, R.; Navari-Izzo, F. Naturally-assisted metal phytoextraction by *Brassica carinata*: Role of root exudates. *Environ. Pollut.* **2009**, *157*, 2697-2703.

(4) Nowack, B.; Schwyzer, I.; Schulin, R. Uptake of Zn and Fe by wheat (*Triticum aestivum* var. Greina) and transfer to the grains in the presence of chelating agents (ethylenediaminedisuccinic acid and ethylenediaminetetraacetic acid). *J. Agr. Food Chem.* **2008**, *56*, 4643-4649.

(5) Tandy, S.; Schulin, R.; Nowack, B. Uptake of metals during chelant-assisted phytoextraction with EDDS related to the solubilized metal concentration. *Environ. Sci. Technol.* **2006**, *40*, 2753-2758.

(6) Tandy, S.; Schulin, R.; Nowack, B. The influence of EDDS on the uptake of heavy metals in hydroponically grown sunflowers. *Chemosphere* **2006**, *62*, 1454-1463.

(7) Bell, P.F.; McLaughlin, M.J.; Cozens, G.; Stevens, D.P.; Owens, G.; South, H. Plant uptake of ^{14}C-EDTA, ^{14}C-Citrate , and ^{14}C-Histidine from chelator-buffered and conventional hydroponic solutions. *Plant Soil* **2003**, *253*, 311-319.

(8) Leštan D.; Luo, C.; Li, X. The use of chelating agents in the remediation of metal-contaminated soils: A review. *Environ. Pollut.* **2008**, *153*, 3-13.

(9) Tanton, T.W.; Crowdy, S.H. The distribution of lead chelate in the transpiration stream of higher plants. *Pestic. Sci.* **1971**, *2*, 211-213.

(10) Vassil, A.D.; Kapulnik, Y.; Raskin, I.; Salt, D.E. The role of EDTA in lead transport and accumulation by Indian mustard. *Plant Physiol.* **1998**, *117*, 447-453.

(11) Hernández-Allica, J.; Garbisu, C.; Barrutia, O.; Becerril, J.M. EDTA-induced heavy metal accumulation and phytotoxicity in cardoon plants. *Environ. Exp. Bot.* **2007**, *60*, 26-32.

(12) Markert, B. *Instrumental Element and Multi-Element Analysis of Plant Samples – Methods and Applications*; John Wiley & Sons: Chichester, New York, Brisbane, Toronto, Singapore, **1996**.

(13) Kolleganov, M.; Kolleganova, I.G.; Mitrofanova, N.D.; Martynenko, L.I.; Nazarov, P.P.; Spitsyn, V.I. Influence of cyclization on *N,N'*-ethylenediaminedisuccinic acid on its complex forming properties. *Bull. Acad. Sci. USSR Div. Chem. Sci.* **1983**, *32*, 1167-1175.

(14) Metsärinne, S.; Tuhkanen, T.; Aksela, R. Photodegradation of ethylenediaminetetraacetic acid (EDTA) and ethylenediamine disuccinic acid (EDDS) within natural UV radiation range. *Chemosphere* **2001**, *45*, 949-955.

(15) Sgherri, C.; Quartacci, M.F.; Navari-Izzo, F. Early production of activated oxygen species in root apoplast of wheat following copper excess. *J. Plant Physiol*. **2007**, *164*, 1152-1160.

(16) Briskin, D.P.; Reynolds-Niesman I. Determination of H^+/ATP stoichiometry for the plasma membrane H^+-ATPase from red beet (*Beta vulgaris* L.) storage tissue. *Plant Physiol.* **1991**, *95*, 242-250.

(17) Lidon, F.C.; Henriques F.S. Effects of increasing concentrations of Cu on metal uptake kinetics and biomass yields. *Soil Sci.* **1992**, *154*, 4-49.

(18) Reid, R.J.; Smith, F.A. Measurement of calcium fluxes in plants using ^{45}Ca. *Planta* **199**2, *186*, 558–566.

(19) Bravin, M.N; Le Merrer, B.; Denaix, L.; Schneider, A.; Hinsinger, P. Copper uptake kinetics in hydroponically-grown durum wheat (*Triticum turgidum durum* L.) as compared with soil's ability to supply copper. *Plant Soil* **2010**, *331*, 91-104.

(20) Marquenie-van der Werff, M.; Ernst, W.H.O. Kinetics of copper and zinc uptake by leaves and roots of an aquatic plant, *Elodea nuttallii*. *Z. Pflanzenphysiol. Bd.* **1979**, *92*, 1-10.

(21) Burkhead, J.L.; Reynolds, K.A.G.; Abdel-Ghany S.E.; Cohu, C.M.; Pilon, M. Copper homeostasis. *New Phytol.* **2009**, *182*, 799-816.

(22) Yruela, I. Copper in plants: acquisition, transport and interactions. *Funct. Plant Biol.* **2009**, *36*, 409-430.

(23) Veltrup, W. Concentration dependent uptake of copper by barley roots. *Physiol. Plant.* **1976**, *36*, 217-220.

(24) Kopittke, P.M.; Blamey, F.P.C.; Menzies, N.W. Toxicities of soluble Al, Cu and La include ruptures to rhizodermal and root cortical cells of cowpea. *Plant Soil* **2008**, *303*, 217-227.

(25) Veltrup, W.; Austenfeld, F.-A. The uptake of copper by cell suspension cultures of *Phaseolus vulgaris* L. cv. SAXA. *Plant Cell Rep*. **1981**, *1*, 31-33.

(26) Wenger, K.; Gupta, S.K.; Furrer, G.; Schulin, R. The role of nitrilotriacetate in copper uptake by tobacco. *J. Environ. Qual.* **2003**, *32*, 1669-1676.

(27) Taylor, G.J.; Foy, C.D. Differential uptake and toxicity of ionic and chelated copper in *Triticum aestivum*. *Can. J. Bot*. **1985**, *63*, 1271-1275.

(28) Nowack, B.; Schulin, R.; Robinson, B.H. Critical assessment of chelant-enhanced metal phytoextraction. *Environ. Sci Technol*. **2006**, *40*, 5225-5232.

(29) Luo, C.; Shen Z.; Baker, A.J.M.; Li, X. A novel strategy using biodegradable EDDS for the chemically enhanced phytoextraction of soils contaminated with heavy metals. *Plant Soil* **2006**, *285*, 67-80.

(30) Collins, R.N.; Merrington, G.; McLaughlin, M.J.; Knudsen, C. Uptake of intact zinc-ethylenediaminetetraacetic acid from soil is dependent on plant species and complex concentration. *Environ. Toxicol. Chem*. **2002**, *21*, 1940-1945.

(31) Luo, C.; Shen, Z.; Li, X. Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. *Chemosphere* **2005**, *59*, 1-11.

SPATIAL DISTRIBUTION OF COPPER BY MICRO-PIXE IN *BRASSICA CARINATA* PLANTS EXPOSED TO $CuSO_4$ OR CuEDDS

Benedetta Cestone[a], Katarina Vogel-Mikuš[c], Mike Frank Quartacci[a], Nicoletta Rascio[b], Paula Pongrac[c], Primož Pelicon[d], Primož Vavpetič[d], Nataša Grlj[d], Luka Jeromel[d], Peter Kump[d], Marijan Nečemer[d], Marijan Regvar[c] & Flavia Navari-Izzo[a]

[a]Dipartimento di Biologia delle Piante Agrarie, Università di Pisa, Via del Borghetto 80, 56124 Pisa, Italy, [b]Dipartimento di Biologia, Università di Padova, Via Ugo Bassi 58/B, 35121 Padova, Italy, [c]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, S1-1000 Ljubljana, Slovenia, and [d]Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia.

Submitted to Environ. Exp. Bot.

Abstract

A better understanding of the mechanisms governing Cu uptake, distribution and tolerance in *B. carinata* plants in the presence of chelants is needed before significant progresses in Cu phytoextraction could be made. Therefore, the aims of this study were to characterize (S,S)-N,N'-ethylenediamine disuccinic acid (EDDS)-assisted Cu uptake and to compare Cu spatial distribution patterns in roots and leaves of plants treated with $CuSO_4$ or CuEDDS.

Quantitative copper distribution maps as well as concentration profiles across root and leaf cross-sections of desorbed plants following incubation with 30 or 150 μM $CuSO_4$ or CuEDDS acquired by micro-PIXE showed different behaviours. In roots, both 30 μM treatments resulted in higher Cu levels in epidermal/cortical regions. At 150 μM $CuSO_4$ the metal mainly accumulated in root vascular bundles, whereas in the similar CuEDDS treatment Cu was detected in endodermis and adjacent inner cortical cell layer. In leaves, both treatments

and concentrations induced Cu accumulation in veins. The 150 μM CuEDDS treatment enhanced metal translocation to shoots in comparison with $CuSO_4$, differently from the 30 μM treatments. Inhibition of H^+-ATPases in EDDS-assisted treatments resulted in reduced Cu accumulation in roots and altered Cu distribution pattern in leaves, where Cu was no more localized in veins but was present in large concentrations in the rib parenchyma. The physiological significance of EDDS-assisted Cu uptake is discussed.

1. Introduction

Among metals frequently found in polluted soils, copper (Cu), notwithstanding its role as essential nutrient, is becoming a rising problem due to its widespread use. Metal toxicity is linked to metal persistence and dangerousness, as they cannot be chemically or biologically degraded and are very difficult to remove from soils. Therefore, there is growing interest in the development of efficient Cu phytoextraction technologies that use plants able to extract Cu from contaminated soils.

Metal accumulation capacity of fast growing and high-biomass plants requires a thorough understanding of the biological processes involved in metal acquisition from soils, metal translocation from roots to shoots, as well as tolerance to and accumulation of high concentrations of metals, important features which could be used to improve phytoextraction efficiency (Quartacci et al., 2007, 2009).

Chelant-enhanced phytoextraction exerts its effects by solubilizing target metals from soil and making them more available for plant uptake and translocation to the shoots (Cestone et al., 2010; Meers et al., 2008; Quartacci et al., 2007). The EDTA structural isomer (S,S)-*N,N*'-ethylenediamine disuccinic acid (EDDS) has received attention as a potential replacement for EDTA as it is a strong chelant and unlike EDTA it is easily biodegradable (Quartacci et al., 2009). *Brassica carinata* is a widespread annual species that is able to accumulate and

tolerate significant amounts of copper without biomass reduction following the addition of a biodegradable organic chelator such as EDDS. Differently from the active mechanisms involved in the uptake of essential metals such as Cu and Zn, it is widely accepted that synthetic chelants and their metal complexes pass through the root cortex and reach the xylem along a fully non selective apoplastic pathway. It is unlikely that the anionic metal-ligand complexes pass through the cell membrane due to their large size and to the fact that no specific transporters are known. Indeed, previous studies have demonstrated that Cu and EDDS do not enter into the root of *B. carinata* in their complexed form but they can be translocated to the shoots only after splitting of the complex (Cestone et al., 2010). This indicates that Cu and EDDS are taken up into the root by two separate pathways. In xylem free Cu ions are suggested to complex again with free EDDS, thus increasing the rate of translocation from roots to shoots (Cestone et al., 2010). Entry in the xylem may be favoured by the electro-neutrality of the CuEDDS complex, which prevents binding of free Cu ions to the negatively charged xylem cell wall components. However, additional studies on Cu localization and distribution in *B. carinata* root and leaf tissues are needed.

Element imaging techniques that allow mapping of element distribution in plant tissues with high spatial resolution and sensitivity are best suited to depict the individual composition of tissues and their variability arising from cellular and sub-cellular organization. Micro-proton-induced X-ray emission spectroscopy with a focused proton beam, frequently referred to as micro-PIXE, has been proven as an efficient technique to determine spatial elemental distribution within biological tissues (Przybylowicz et al., 1997, 2004; Vogel-Mikuš et al., 2007, 2008a, 2008b, 2009a, 2009b, 2010). Since it features both high lateral resolution (of the order of 1 μm) and high elemental sensitivity (in the range of 1 mass ppm), it is appropriate for simultaneous localization and quantification of elements within distinct morphological structures in plant tissues. In combination with scanning transmission ion microscopy (STIM), which

gives data on the lateral thickness distribution of the sample, micro-PIXE provides unique information on the quantitative spatial distribution of major and trace elements within plant organs (Vogel-Mikuš et al., 2007, 2008a, 2008b, 2009a, 2009b, 2010).

In order to make further progresses in the understanding of the mechanism of EDDS-assisted Cu uptake (Cestone et al., 2010) we used micro-PIXE (i) to reveal the spatial distribution of Cu within root and leaf cross-sections of *B. carinata* plants treated with $CuSO_4$ or CuEDDS, (ii) to link the observed Cu accumulation patterns to the mechanisms underlying Cu uptake and translocation from roots to shoots and detoxification mechanisms enabling the growth of *B. carinata* plants in the presence of high Cu concentrations, and (iii) to characterize the mode of Cu uptake and transport using a H^+-ATPase inhibitor.

2. Materials and Methods

2.1. Plant material

Brassica carinata (cv 180) seedlings were hydroponically grown according to Cestone et al. (2010). After one month, the plants were collected from the nutrient solution and transferred into a pre-experimental solution in which all micronutrients other than Cu and $NH_4H_2PO_4$ were omitted (Cestone et al., 2010). Plants were then incubated separately for 72 h in experimental solutions containing 30 or 150 µM $CuSO_4$ or CuEDDS, the latter treatment in the presence or not of 500 µM of the H^+-ATPase inhibitor sodium orthovanadate (Na_3VO_4). Control plants were incubated in a nutrient solution containing 0.12 µM $CuSO_4$. After treatments, plants were quickly washed with ultrapure MilliQ water. In one set of plants apoplastically bound copper was desorbed from roots by soaking them in 5 mM $Pb(NO_3)_2$ for 30 min under continuous stirring. After that, the plants were rinsed with ultrapure MilliQ water. One set of desorbed and not

desorbed plants were then separated (roots and shoots) and immediately oven-dried at 105°C till constant weight for X-ray fluorescence (XRF) analysis. Another set of desorbed and not desorbed plants was kept in a growth chamber (75% relative humidity; 200 μmol m^{-2} s^{-1} photon flux density; 16/8 h day/night; 23°C) and prepared within few days after sampling for micro-PIXE measurements.

2.2. Sample preparation and elemental analyses

2.2.1. XRF analysis

Dried plant tissues were pulverized and homogenized in a mortar. Depending on material's disposal, about 100 or 500 mg of powdered plant material (roots or shoots) was pressed in pellets using pellet die and hydraulic press. Before XRF analysis root and shoot pellets were weighed. As primary fluorescence excitation, the annular radioisotope excitation source Cd-109 (25 mCi) from Isotope Products Laboratories (USA) was utilized. The emitted fluorescence radiation was measured by an energy dispersive X-ray spectrometer, composed of a Si(Li) detector (Canberra, Meriden, USA) with a 25 μm-thick Be window, a spectroscopy amplifier (Canberra M2024), ADC (Canberra M8075) and PC based MCA (Canberra S-100). The energy resolution of the spectrometer at count rates below 1000 c s^{-1} was 175 eV at 5.9 keV. XRF analysis was performed in air and the samples were irradiated for 1000 s (Nečemer et al., 2008). The analysis of complex X-ray spectra was performed by AXIL spectra analysis program (van Espen and Janssens, 1993), included in the QXAS (Vekemans et al., 1994) software package. Metal quantification of the measured spectra was performed using the fundamental parameter QAES (Quantitative Analysis of Environmental Samples) developed by Kump et al. (2007).

2.2.2. Micro-PIXE analysis

Selected roots and leaves were detached from intact plants, quickly washed with ultrapure MilliQ water and sectioned with a sharp razor blade in small

pieces of approximately 2 x 5 mm^2. The roots pieces were placed in aluminium beds filled with Jung tissue freezing medium (Leica), while the leaf pieces were immediately inserted into 2 mm stainless steel needles with polished tips. The samples were then rapidly frozen in propane cooled by liquid nitrogen (Schneider et al., 2002; Vogel-Mikuš et al. 2008a, 2008b, 2009a, 2009b). After freezing, the samples were sectioned with a Leica CM3050 cryotome (Leica, Bensheim, Germany). The temperature of cryo-microtome head and chamber varied between -25°C to -20°C, depending on the tissue water content, and the section thickness was 30-60 µm. Afterwards, the sectioned specimens were placed into specially pre-cooled aluminium holders and carefully transferred to an Alpha 2-4 Christ freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) via a cryo-transfer-assembly cooled by liquid nitrogen and freeze-dried first for 3 hours at -180°C and 0.04 mbar, and then for 1 day at -50°C and 0.04 mbar. During the freeze-drying process, the low temperature and pressure applied were aimed to minimize shrinkage of specimens. The freeze-dried specimens were then individually mounted into aluminium holders between two thin layers of Pioloform foil (SPI supplies, West Chester, PA, USA). The foil was prepared by dissolving 1 g Pioloform in 75 ml chloroform as described in Vogel-Mikuš et al. (2007).

Micro-PIXE analysis was run using the nuclear microprobe at the Jožef Stefan Institute (Ljubljana) according to Pelicon et al. (2005) and Simčič et al. (2002) on 3 different samples per treatment. In the results session only maps of a representative specimen are presented.

A proton beam with energy of 3 MeV and a diameter varying from 1 to 1.5 µm at ion currents ranging from 40 to 500 pA was used. Simultaneously, an on-off axis STIM (Pallon et al., 2003) was carried out to determine beam exit energy from the sample, which is related to the sample local area density. In combination with known matrix composition, proton exit energy measured by STIM was used for determination of specimen thickness. The stopping power of

3 MeV protons in cellulose is 114 keV cm^2 mg^{-1}. Proton energy loss in the sample was within the order of 120 keV, which corresponds to a cellulose area density of 1.05 mg cm^{-2}. Assuming the cellulose bulk density of 1.6 g cm^{-3} this result yields 6.5 µm of equivalent bulk cellulose thickness. Heterogeneous morphology of the sample results in uneven area density after freeze-drying and is reflected in broadening and asymmetry of the peak in the STIM spectra. Uncertainty in the centroid energy determination was better than 10 keV, which yields 8 % error in average sample thickness.

The detection of X-ray energies from 0.9 keV up to 25 keV was provided by a pair of X-ray detectors. These include a high-purity germanium X-ray detector with an active area of 95 mm^2, a 25 µm-thick beryllium window and a 100 µm-thick polyimide absorber positioned at an angle of 135° with respect to the beam direction. Simultaneously, a Si(Li) detector with an area of 10 mm^2 and an 8 µm-thick Be window was installed at the angle of 125° with respect to the beam direction, for the detection of low-energy X-rays, in the energy range from 0.9 to 4 keV. To avoid sample charging during measurements and time consuming carbon coating of specimen, the samples were sprayed with low-energy electrons originating from a hot tungsten filament (Vogel-Mikuš et al., 2008a).

Precise proton dose determination is required for quantitative micro-PIXE analysis. For this reason, an in-beam chopping device was positioned in the beam line after the last collimation of the beam before it hits the sample (Vogel-Mikuš et al., 2009a, 2009b). The rotating chopper consisted of gold-plated graphite that periodically intersected the beam with a frequency of approx. 10 Hz, which made the method insensitive to beam intensity fluctuations. The spectrum of backscattered protons from the chopper was recorded in parallel with PIXE spectra in the list mode. The high-energy part of the spectrum consisted of protons scattered from the Au layer and appeared as a separate peak. Its area was proportional to the proton flux. During off-line data processing, the proton dose corresponding to an arbitrary scanning area

selection could be extracted from the list-mode results simultaneously with the PIXE spectra. Micro-PIXE data analysis was performed using GEOPIXE II (Ryan, 2000) software package generating quantitative element distribution maps and copper concentrations profiles across *B. carinata* root and leaf cross-sections.

2.3. Statistical analysis

Statistical analysis was carried out with Costat 6.4 (CoHort software). The error bars represent the standard error of the mean of three independent experiments (n=3) each analyzed twice. The significance of the differences among means was evaluated by one-way ANOVA. Comparisons among means were carried out using the LSD test at the significance level of $P \leq 0.05$.

2.4. Colocalization analysis

Quantitative colocalization analysis was performed by ImageJ programme using plug-in 'Intensity correlation analysis' generating Pearson's correlation coefficients (r), Mander's Overlap coefficient (R) and Intensity correlation quotient (ICQ) (http://rsbweb.nih.gov/ij/plugins/colocalization.html). Pearson's correlation coefficients range from 1 to -1, where the value 1 represents perfect correlation/colocalization, -1 represents perfect exclusion and zero represents random localization. Mander's Overlap coefficient ranges between 1 and zero, the values meaning high and low colocalization, respectively. The ICQ values are distributed between -0.5 and +0.5 by subtracting 0.5 from this ratio. Random localization is represented by ICQ values ~0, segregated localization by $0>ICQ\geq-0.5$, and dependent localization by $0<ICQ\leq+0.5$.

3. Results and Discussion

3.1. Copper uptake and translocation

In order to be transported to the shoots, metals have to be first imported into the cytosol. This mainly involves energy-dependent ion transport processes across plasma membrane. At root tip, where endodermal barrier is not yet fully developed or damaged by lateral root formation, nonselective passive apoplastic transport may also occur. In order to increase bioavailability of essential metals plants exude a variety of small organic molecules ranging from organic acids, histidine, nicotianamine to phytosiderophores into the soil (Haydon and Cobett, 2007; Irtelli et al., 2009; Quartacci et al., 2009). These organo-metallic complexes are then actively transported into the symplast via suitable transport systems (Haydon and Cobett, 2007). However, when metals are chelated to synthetic ligands such as EDTA or EDDS, it is widely accepted that these anionic metal-ligand complexes cannot pass through the cell membrane due to their large size and to the fact that specific transporters are not known. Therefore, as shown by Cestone et al. (2010), CuEDDS complexes need to dissociate before entering into the root separately.

In desorbed *B. carinata* roots the highest Cu concentrations were observed in plants treated with $CuSO_4$, followed by CuEDDS and CuEDDS-I (CuEDDS treatment with inhibitor) at both 30 or 150 μM treatments (Fig. 1a). Differently from the exposure to 30 μM $CuSO_4$, the 150 μM one resulted in an increased metal uptake due to membrane damage caused by the higher concentration of free Cu ions and consequent increased passive Cu symplast uptake (Cestone et al., 2010). In contrast, lower Cu root uptake in the CuEDDS treatments suggests that Cu was not transported into the symplast as a complex (Cestone et al., 2010). In addition, in the CuEDDS treatments Cu toxicity was delayed or mitigated and the complex resulted much less harmful to plasma membranes, also lowering passive Cu influx (Cestone et al., 2010).

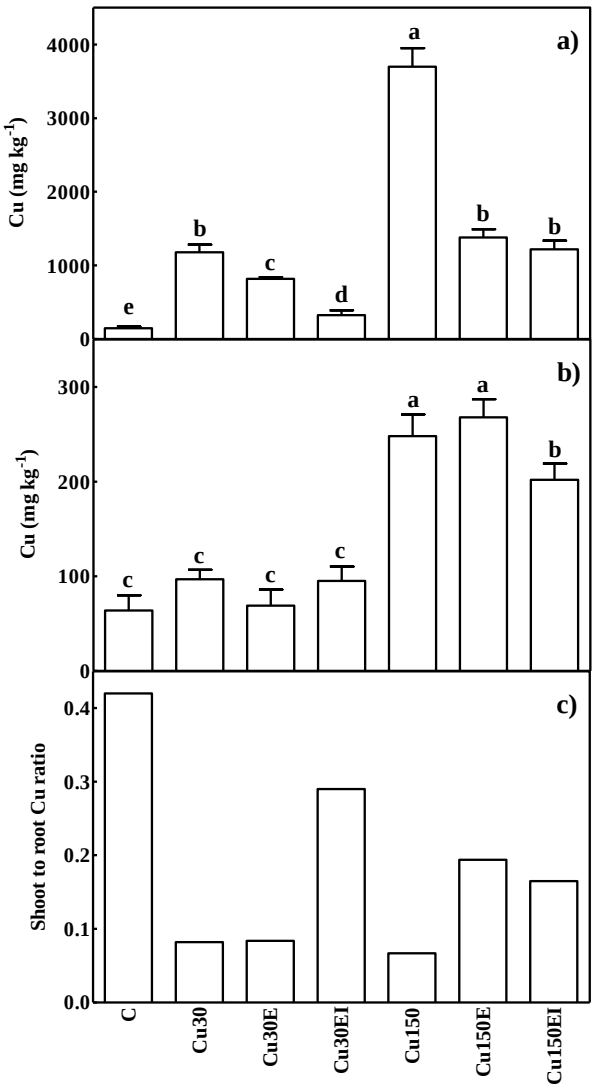


FIGURE 1. Copper concentrations in **a**) roots and **b**) shoots of *B. carinata* plants (mean ± standard error), and **c**) shoot to root Cu ratio. C, control; Cu30, 30 μM $CuSO_4$; Cu150, 150 μM $CuSO_4$; Cu30E, 30 μM CuEDDS; Cu150E, 150 μM CuEDDS; Cu30EI, 30 μM CuEDDS plus vanadate; Cu150EI, 150 μM CuEDDS plus vanadate. Results are means ± SE (n = 3). For each plant organ means followed by the same letter are not significantly different by one-way ANOVA (LSD test, $P \leq 0.05$).

Vanadate was expected to disturb the activity of proton-pumping ATPases (H^+-ATPases) of the plant plasma membranes that generates the proton electrochemical gradient needed to sustain ion and metabolite secondary active transport (Cestone et al., 2010). In the 30 μM CuEDDS-I treatment energy-dependent processes involved in metal transport were clearly inhibited, resulting in a significant lower root Cu concentration compared to roots treated with 30 μM $CuSO_4$ or CuEDDS. In roots treated with 150 μM CuEDDS-I, however, the decrease of Cu concentration was not pronounced compared to the similar

CuEDDS treatment. This effect may be mainly attributed to the time needed for deactivation of H^+-ATPases. Due to the higher Cu concentration in the nutrient solution, a portion of Cu could be taken into the root symplast before the inhibition of H^+-ATPases was complete (Cestone et al., 2010).

In leaves of plants treated with 30 μM $CuSO_4$, CuEDDS or CuEDDS-I, Cu concentrations did not differ significantly from those of control plants, pointing out that Cu retention processes in the roots were still efficient (Fig. 1b) and the functionality of the endodermal barrier was intact. In plants treated with 150 μM $CuSO_4$ or CuEDDS, significantly higher leaf Cu concentrations were observed in comparison with the control, with a slight reduction in Cu concentration in the CuEDDS-I treatment (Fig. 1b). Although Cu concentrations in leaves of $CuSO_4$ or CuEDDS treated plants were comparable, shoot to root ratios showed that in the 150 μM CuEDDS treatment Cu transport from roots to shoots was favoured when compared to $CuSO_4$ (Fig. 1c).

The 150 μM CuEDDS-I treatment slightly inhibited the accumulation of Cu in leaves (Fig. 1b) probably due to inhibition of energy-dependent root symplast Cu import, while root to shoot transport via xylem was comparable to the CuEDDS treatment (Fig. 1c). Once in the symplast, Cu transport with bulk flow through the xylem seemed to be less impaired by vanadate as it depends more on pressure differences than on energy-dependent processes (Fig. 1a-c). Vanadium was traced in roots of CuEDDS-I treated plants, where concentrations reached 985±174 μg g^{-1} in 30 μM CuEDDS-I and 2500±235 μg g^{-1} in 150 μM CuEDDS-I treatments, respectively, and also in the shoots, where concentrations reached 916 ± 185 μg g^{-1} in 30 μM CuEDDS-I and 679 ± 184 μg g^{-1} in 150 μM CuEDDS-I treatments, respectively. Micro-PIXE analysis (Fig. 8) complemented with Intensity correlation analysis showed high colocalization level of vanadium with phosphorus in *B. carinata* leaves (Pearson's r=0.806, Manders R=0.863 and intensity correlation quotient ICQ=0.387) confirming its affinity for binding to phosphate groups.

3.2. Copper localization in roots and shoots

Tissue morphology structures of *B. carinata* roots and leaves were determined using light microscopy (Figs. 2-7). Within roots and leaves the different tissues have distinct affinities for Cu as revealed by the localization maps (Figs. 3, 5 and 7). In elemental maps xylem and phloem were distinguished on the basis of light–microscopy examination of specimens and mineral distribution maps (Fig. 8), since in phloem phosphorus is much more abundant then in xylem (Vogel-Mikuš et al., 2008b).

In order to better understand processes involved in active uptake of Cu ions into the root cells across membranes, removal of ions from the apoplasm by lead nitrate solution was necessary. This desorption may have resulted in the remobilization of light cations such as K^+ and Ca^{2+} in root apoplast or the altered distribution of other weakly bound elements, but it has not influenced redistribution of Cu ions between apoplast and symplast, as revealed by Cu distribution maps and Cu concentration profiles across root and leaf tissues of not desorbed plants treated with 150 μM $CuSO_4$ or CuEDDS (Figs. 6 and 7).

Micro-PIXE analysis of Cu localization in desorbed *B. carinata* root cross-sections showed that in control and in all 30 μM treated plants ($CuSO_4$, CuEDDS or CuEDDS-I) the metal was more or less evenly distributed, with a slight enhancement of Cu concentrations in the epidermal/cortical region (Figs. 2 and 3). On the contrary, in the 150 μM $CuSO_4$ treatment Cu was mainly concentrated in the vascular tissues (Figs. 2 and 3), indicating its increased uptake into the symplast. This may be mainly attributed to the previously mentioned high concentration of free Cu ions in the nutrient solution and high Cu toxicity. In contrast, the same effect was not observed in the 30 μM $CuSO_4$ treatment (Figs. 2 and 3), further supporting that this treatment was much less harmful to the root membranes (Cestone et al., 2010), so passive uptake of Cu into the symplast was also much lower.

FIGURE 2 (below). Quantitative distribution maps of copper in root cross-sections of desorbed *B. carinata* plants. Sample position is depicted by the green borderline. Maps were generated using GeoPIXE II and the dynamic analysis method. Concentrations are reported in wt %. LM, light micrography. For other abbreviations see Fig. 1.

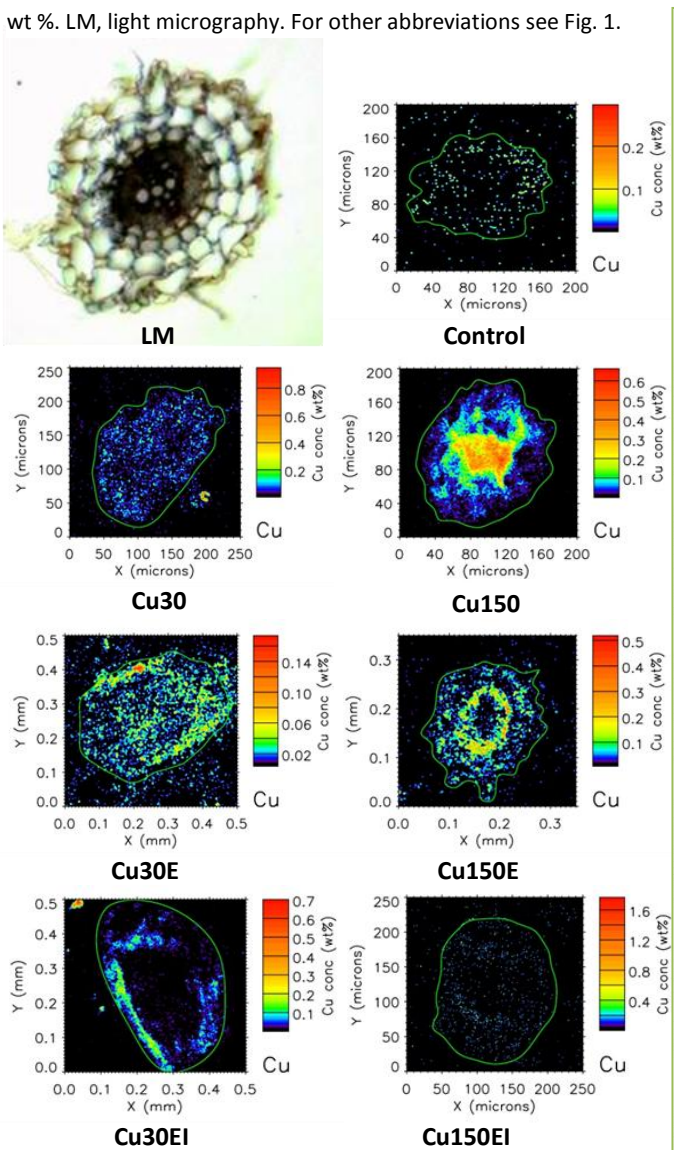
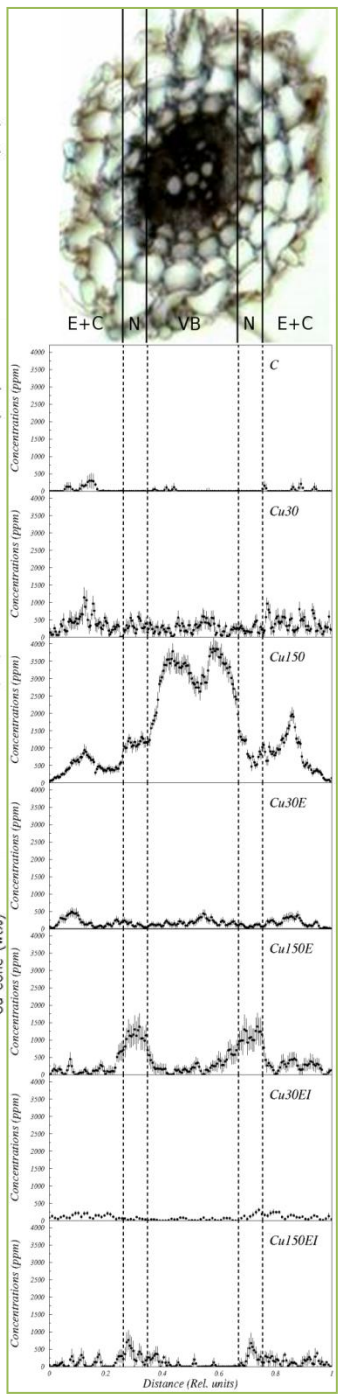


FIGURE 3 (right). Copper concentration profiles across root cross-sections of desorbed *B. carinata* plants. Root tissues on light micrography and corresponding marked regions on Cu concentration profiles. **E+C**, epidermis and cortex; **N**, endodermis with adjacent layer of inner cortical cells; **VB**, vascular bundles. For other abbreviations see Fig. 1.



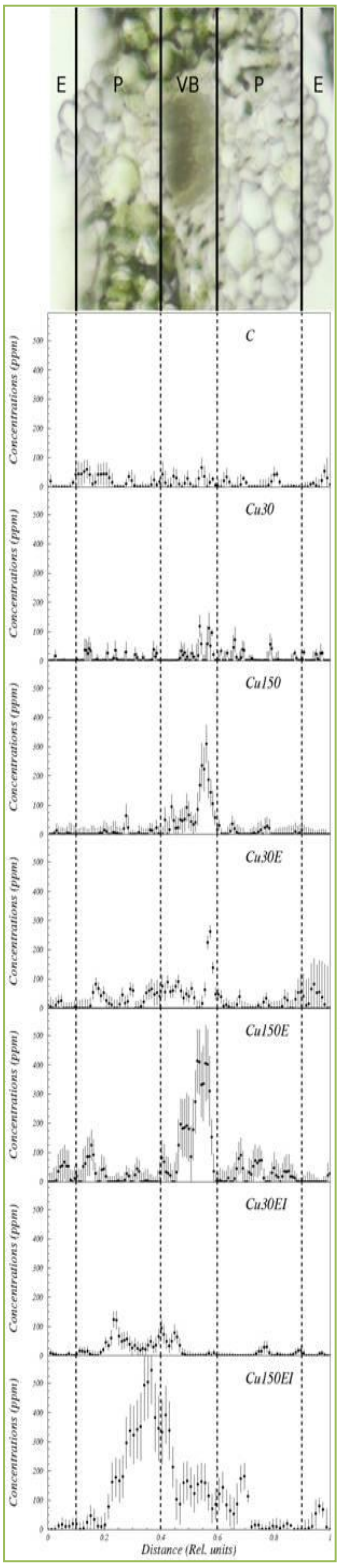


FIGURE 4 (below). Quantitative distribution maps of copper in leaf cross-sections of desorbed *B. carinata* plants. Sample position is depicted by the green borderline. Maps were generated using GeoPIXE II and the dynamic analysis method. Concentrations are reported in wt % or ppm. For abbreviations see Fig. 1.

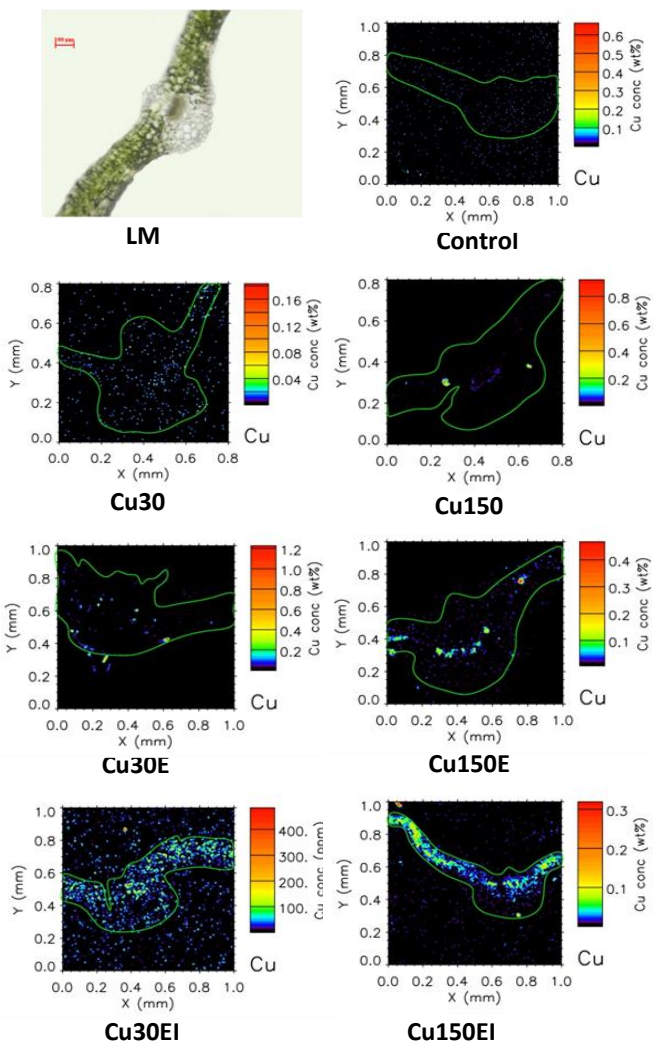


FIGURE 5 (left). Copper concentration profiles across leaf cross-sections of desorbed *B. carinata* plants. Leaf tissues on light micrography and corresponding marked regions on Cu concentration profiles. **E**, epidermis; **P**, rib parenchyma; **VB**, vascular bundles. For other abbreviations see Fig. 1.

High cortical Cu concentrations shown by the Cu elemental map of not desorbed 150 μM $CuSO_4$ treated roots (Figs. 6 and 7a), compared to the same treatment in desorbed roots (Figs. 2 and 3), may be ascribed to the apoplastic Cu of cortical cells, which resulted in much higher Cu concentration differences between vascular and cortical cells of desorbed roots. In roots of 150 μM CuEDDS and CuEDDS-I treated plants, increased Cu concentrations were not observed in vascular bundles but in endodermis and inner cortical cell layer adjacent to the endodermis (Figs. 2 and 3), indicating that efficient apoplastic barrier was still preserved. Similarly, only trace Cu concentrations were observed in vascular bundles of the 150 μM CuEDDS not desorbed roots (Figs. 6 and 7a), where the highest Cu concentrations were observed in epidermal and cortical layers and not in inner cortical and endodermal layers.

This could be, as in the 150 μM $CuSO_4$ not desorbed roots, mainly attributable to extensive Cu apoplast binding.

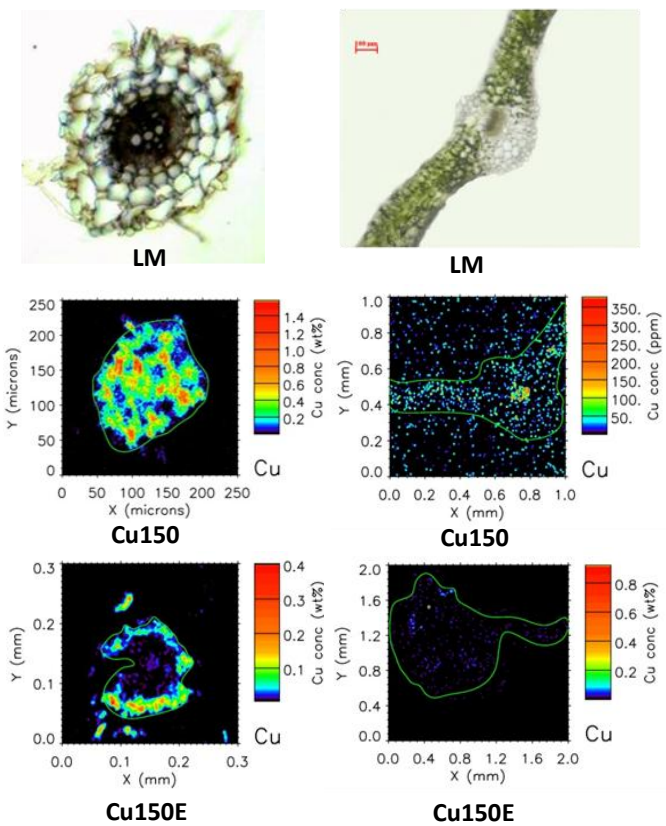


FIGURE 6. Quantitative distribution maps of copper in root and leaf cross-sections of not desorbed *B. carinata* plants. Sample position is depicted by the green borderline. Maps were generated using GeoPIXE II and the dynamic analysis method. Concentrations are reported in wt % or ppm. For abbreviations see Fig. 1.

Despite extensive differences in Cu localization were observed between CuEDDS and $CuSO_4$ treatments, Cu concentrations in leaves remained comparable (Figs. 1, 4, 5, 6 and 7b), evidencing different root to shoot Cu translocation mechanisms in EDDS-assisted and not-assisted Cu uptake. In the 150 μM $CuSO_4$ treatment, increased leaf Cu accumulation in desorbed plants was mainly a consequence of the high concentration of toxic free Cu ions and passive symplast accumulation of Cu due to membrane damage, while in the 150 μM CuEDDS treatment root apoplastic barrier remained well preserved (Figs. 2,

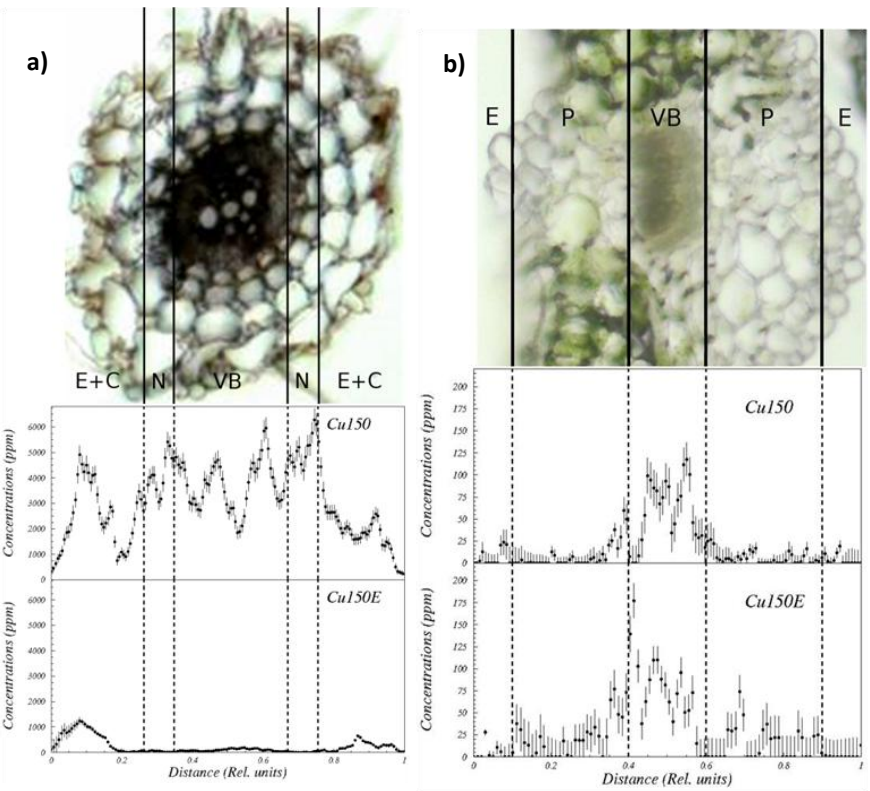


FIGURE 7. Copper concentration profiles across **a**) root and **b**) leaf cross-sections of not desorbed *B. carinata* plants. Root tissues on light micrography and corresponding marked regions on Cu concentration profiles. **E+C**, epidermis and cortex; **N**, endodermis with adjacent layer of inner cortical cells; **VB**, vascular bundles. Leaf tissues on light micrography and corresponding marked regions on Cu concentration profiles. **E**, epidermis; **P**, rib parenchyma; **VB**, vascular bundles. For other abbreviations see Fig. 1.

3, 6 and 7a). This observation stresses the importance of organo-metallic complexes for metal detoxification in plant tissues. Re-complexation of Cu and EDDS back to CuEDDS could already occur in the symplast and/or in the xylem at root level. Formation of electro-neutral complexes between free Cu ions and EDDS probably prevented Cu ions to react with negatively charged cell wall components (as carboxylic and hydroxylic groups), therefore facilitating transport of Cu via transpiration stream into the shoots.

In leaves Cu was mainly found in vascular tissues (Figs. 4, 5, 6 and 7b), especially in the 150 μM $CuSO_4$ or CuEDDS treatments, while almost no Cu was detected in rib parenchyma with the exception of CuEDDS-I treated plants (Figs. 4 and 5). Similar Cu distribution pattern in leaves of $CuSO_4$ and CuEDDS treated plants indicates that in both cases Cu was only poorly unloaded from the vascular cylinder and transported further into symplast of photosynthetically active mesophyll cells. In the process of xylem unloading, metals are first unloaded into the apoplast and then transported through the leaf tissues either by apoplastic pathway via transpiration stream, or symplastic pathway which includes a first transport of ions or small metal organic complexes (e.g. metal-citrate or metal-nicotianamine) across plasma membranes of mesophyll cells via energy-dependent processes involving metal transporters (Conte and Walker et al. 2011; Irtelli et al., 2009). In some metal-tolerant species metals (e.g. Cd, Vollenweider et al., 2006) were shown to accumulate primarily in the collenchyma cell wall of the leaf veins, which adapted to metal sinks by thickening their cell walls. When vein sinks became saturated, Cd accumulation appeared at sites in the leaf blade next to veins. Similarly, in *B. carinata* leaves preferential accumulation of Cu in vascular tissues may inhibit metal translocation to photosynthetically active mesophyll cells, therefore enabling plant tolerance to copper (Seregin and Ivanov, 2001; Vollenweider et al., 2006). At increasing concentration of free Cu (as $CuSO_4$), when all the binding sites in

cell walls of vascular tissues would be occupied Cu would probably start to intensively accumulate in the mesophyll.

If we assume that metals complexed to synthetic ligands as EDDS cannot be transported across plant membranes, CuEDDS complexes probably need to dissociate before Cu can enter into the leaf symplast. Dissociation of CuEDDS complexes in mesophyll cell apoplast may depend on many factors as stabilization energy of the ligand and pH as well as the rate of Cu uptake into the symplast, which is also connected to the number of metal transporters present in the cell membranes. In addition, retention of complexes or ions in vascular and perivascular tissues may also depend on the rate of sequestration in vascular parenchyma and collenchyma cells, consequently affecting concentration gradients. Interestingly, the CuEDDS-I treatments resulted in completely altered Cu distribution in leaves. Cu was not detected in veins any more but was localized mainly in leaf mesophyll and rib parenchyma (Figs. 4 and 5), although leaf bulk Cu concentrations were comparable (at 30 μM) or only slightly lower (at 150 μM) in comparison with $CuSO_4$ and CuEDDS treatments (Fig. 1). Besides Cu distribution pattern, distribution patterns of other elements as P, S, Ca and Zn were also altered following treatment with vanadate, with more intensive localization in the mesophyll compared to leaf veins (Fig. 8). Instead of active transport in cell symplast, these elements were presumably flown to the mesophyll by an apoplastic pathway via transpiration stream, where they were deposited.

In the EDDS-I treatments, Cu was unloaded from the xylem to the adaxial part of the rib parenchyma (Fig. 5, P region) and distributed within parenchyma cells, whereas this was not observed in $CuSO_4$ and CuEDDS-treatments. Besides saturation kinetics of metal binding sites, which allow translocation of metals to mesophyll only after all the binding sites in vascular tissues are saturated (Vollenweider et al., 2006), energy-dependent mechanisms may be also involved in sequestration of metals in leaf vascular tissues, enabling protection of

photosynthetically active tissues from metal toxicity and consequentially increasing tolerance to heavy metals.

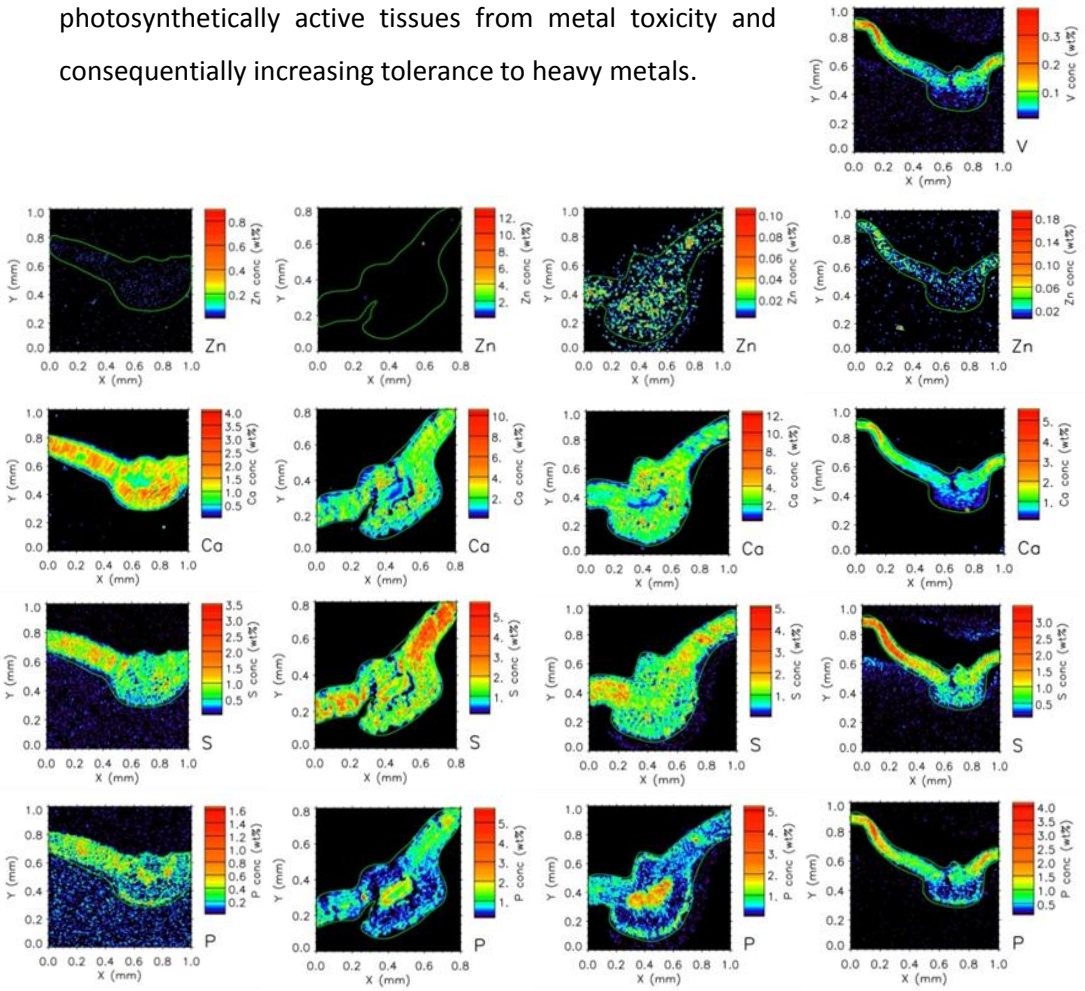


FIGURE 8. Quantitative elemental maps of phosphorus, sulphur, calcium, zinc and vanadium in leaf cross-sections of desorbed *B. carinata* plants. Sample position is depicted by the green borderline. Maps were generated using GeoPIXE II and the dynamic analysis method. Concentrations are reported in wt %. For abbreviations see Fig. 1.

4. Conclusions

According to micro-PIXE Cu localization maps, EDDS-assisted Cu uptake and transport resulted in preserved root endodermal barrier indicating that CuEDDS complexes are much less toxic to the plant than free Cu ions, while at the same

time Cu concentrations in leaves and consequentially Cu phytoextraction capacity remain comparable.

EDDS accelerates Cu transport from roots to shoots, probably because of a weaker affinity of the metal for binding to the cell wall components in plant tissues.

Besides saturation kinetics governing the dynamics of Cu xylem unloading in leaves, energy-dependent processes may be involved in Cu sequestration within leaf veins, thus preventing its mobilization into the photosynthetically active mesophyll cells and conferring tolerance of *B. carinata* to Cu.

Acknowledgements

EDDS (Octaquest E30) was kindly obtained from Innospec Limited (Chesire, UK) as the Na_3EDDS salt. Seeds of *Brassica carinata* cv. 180 were kindly provided by Prof. M. Mazzoncini (Dipartimento di Agronomia e Gestione dell'Agrosistema, University of Pisa). This work has been supported by the University of Pisa (Fondi di Ateneo 2009 and 2010) and European Community as an Integrating Activity 'Support of Public and Industrial Research Using Ion Beam Technology (SPIRIT)' under EC contract no. 227012. Support of Slovenian Research Agency for the research project J7-0352, research programmes P1-0112 and P1-0212, and research infrastructure of Microanalytical Centre is acknowledged.

References

Cestone, B., Quartacci, M.F., Navari-Izzo, F., 2010. Uptake and translocation of CuEDDS complexes by *Brassica carinata*. Environ. Sci. Technol. 44, 6403-6408.

Conte, S.S., Walker, E.L., 2011. Transporters contribution to Iron Trafficking in Plants. Mol. Plant 4, 464-476.

Haydon, M.J., Cobbett, C.S., 2007. Transporters of ligands for essential metal ions in plants. New Phytol. 174, 499-506.

Irtelli, B., Petrucci, W.A., Navari-Izzo, F., 2009. Nicotianamine and histidine/proline are, respectively, the most important copper chelators in xylem sap of *Brassica carinata* under conditions of copper deficiency and excess. J. Exp. Bot. 60, 269-277.

Kump, P., Nečemer, M., Vogel-Mikuš, K., Rupnik, Z., Ponikvar, D., Pelicon, P., Regvar, M., Pongrac, P., Simčič, J., Budnar, M., 2007. Improvement of the XRF quantification and enhancement of the combined applications by EDXRF and Micro PIXE. First research co-ordination meeting under coordinated research project on *Unification of nuclear spectrometries: integrated techniques as a new tool for materials research*. IAEA, Vienna, pp. 91–95.

Meers, E., Tack, F.M.G., Verloo, M.G., 2008. Degradability of ethylenediamine disuccinic acid (EDDS) in metal contaminated soils: Implications for its use in soil remediation. Chemosphere 70, 358–363.

Nečemer, M., Kump, P., Ščančar, J., Jačimović, R., Simčič, J., Pelicon, P., Budnar, M., Jeran, Z., Pongrac, P., Regvar, M., Vogel-Mikuš, K., 2009. Application of X-ray fluorescence analytical techniques in phytoremediation and plant biology studies. Spectrochim. Acta B 63, 1240-1247.

Pallon, J., Auzelyte, V., Elfman, M., Garmer, M., Kristiansson, P., Malmqvist, K., Nilsson, C., Shariff, A., Wegden, M., 2003. An off-axis STIM procedure for precise mass determination and imaging. Nucl. Instrum. Meth. B 219, 988-993.

Pelicon, P., Simčič, J., Jakšič, M., Medunic, Z., Naab, F., McDaniel, F.D., 2005. Spherical chamber-effective solution for multipurpose nuclear microprobe. Nucl. Instrum. Meth. B 231**,** 53–59.

Przybyłowicz, W.J., Mesjasz-Przybyłowicz, J., Prozesky, V.M., Pineda, C.A., 1997. Botanical applications in nuclear microscopy. Nucl. Instrum. Meth. B 130, 335–345.

Przybyłowicz, W.J., Mesjasz-Przybyłowicz, J., Migula, P., Turnau, K., Nakonieczny, M., Augustyniak, M., Glowacka, E., 2004. Elemental microanalysis in ecophysiology using ion microbeam. Nucl. Instrum. Meth. B 219–220, 57–66.

Ryan, C.G., 2000. Quantitative trace element imaging using PIXE and the nuclear microprobe. Int. J. Imag. Syst. Tech. 11, 219-230.

Schneider, T., Scheloske, S., Povh, B., 2002. A method for cryosectioning of plant roots for proton microprobe analysis. Int. J. PIXE 12, 101–107.

Seregin, I.V., Ivanov, V.B., 2001. Physiological aspects of cadmium and lead toxic effects on higher plants. Russ. J. Plant Physiol. 48, 523–544.

Simčič, J., Pelicon, P., Budnar, M., Šmit, Ž., 2002. The performance of the Ljubljana ion microprobe. Nucl. Instrum. Meth. B 190**,** 283–286.

Quartacci, M.F., Irtelli, B., Baker, A.M.J., Navari-Izzo, F., 2007. The use of NTA and EDDS for enhanced phytoextraction of metals from multiply contaminated soil by *Brassica carinata*. Chemosphere 68, 1920-1928.

Quartacci, M.F.; Irtelli, B.; Gonnelli, C.; Gabbrielli, R.; Navari- Izzo, F., 2009. Naturally-assisted metal phytoextraction by *Brassica carinata*: Role of root exudates. Environ. Pollut. 157, 2697–2703.

van Espen, P.J.M., Janssens, K.H.A., 1993. Spectrum evaluation. In: van Grieken RE, Markowicz AA (eds) Handbook of X-ray Spectroscopy; Methods and Techniques. Marcel Dekker New York, pp. 181–293.

Vekemans, B., Janssens, K., van Espen, P., Adams, F., 1994. Quantitative X-ray analysis system (QXAS). Distributed by the International Atomic Energy Agency, Vienna.

Vogel-Mikuš, K., Pongrac, P., Kump, P., Nečemer, M., Simčič, J., Pelicon, J., Budnar, M., Povh, B., Regvar, M., 2007. Localisation and quantification of elements within seeds of Cd/Zn hyperaccumulator *Thlaspi praecox* by micro-PIXE. Environ. Pollut. 147**,** 50–59.

Vogel-Mikuš, K., Simčič, J., Pelicon, P., Budnar, M., Kump, P., Nečemer, M., Mesjasz-Przybylowicz, J., Przybylowicz, W.J, Regvar, M., 2008a. Comparison of essential and non-essential element distribution in leaves of the Cd/Zn hyperaccumulator *Thlaspi praecox* as revealed by micro-PIXE. Plant Cell Environ. 31, 1484-1496.

Vogel-Mikuš, K., Regvar, M., Mesjasz-Przybylowicz, J., Przybylowicz, W.J., Simčič, J., Pelicon, P., Budnar, M., 2008b. Spatial distribution of cadmium in leaves of metal hyperaccumulating *Thlaspi praecox* using micro-PIXE. New Phytol. 179, 712-721.

Vogel-Mikuš, K., Pelicon, P., Vavpetič, P., Kreft, I., Regvar, M., 2009a. Elemental analysis of edible grains by micro-PIXE: Common buckwheat case study. Nucl. Instrum. Meth. B 267, 2884-2889.

Vogel-Mikuš, K., Pongrac, P., Pelicon, P., Vavpetič, P., Povh, B., Bothe, H., Regvar, M., 2009b. Micro-PIXE analysis for localisation and quantification of elements in roots of mycorrhizal metal-tolerant plants, In: A. Varma, A.C. Kharkwal (Eds.), Symbiotic Fungi: Principles and Practice (Soil Biology, 18), Springer, Heidelberg, pp. 227-242.

Vogel-Mikuš, K., Kump, P., Nečemer, M., Pelicon, P., Arčon, I., Pongrac, P., Povh, B., Regvar, M., 2010. Quantitative analyses of trace elements in environmental samples: options and (im)possibilities. In: I. Sherameti, A. Varma (Eds.), Soil Heavy Metals (Soil biology, 19), pp. 113-138.

Vollenweider, P., Cosio, C., Günthardt-Goerg, M.S., Keller, C., 2006. Localization and effects of cadmium in leaves of a tolerant *Salix viminalis* L. Part II. Microlocalization and cellular effect of cadmium. Environ. Exp. Bot. 58, 25–40.

TOXIC AMOUNTS OF $CuSO_4$ OR CuEDDS CAUSE DIFFERENT METABOLIC AND TRANSCRIPTIONAL RESPONSES IN *BRASSICA CARINATA*

Benedetta Cestone[1], Ann Cuypers[2], Jaco Vangronsveld[2], Cristina Sgherri[1], Flavia Navari-Izzo[1]

[1]*Dipartimento di Biologia delle Piante Agrarie, Università di Pisa, Via del Borghetto 80, I-56124 Pisa, Italy,* [2]*Centre for Environmental Sciences, Hasselt University, Agoralaan Building D, 3590 Diepenbeek, Belgium*

Submitted to Environ. Exp. Bot.

Abstract

To improve our knowledge concerning the responses of *Brassica carinata* to different forms of copper (Cu), two-week-old seedlings were exposed for 24 h to 30 μM $CuSO_4$ or CuEDDS. $CuSO_4$ showed to be more toxic than CuEDDS, as illustrated by higher levels of thiobarbituric acid reactive substances (TBARS) and an enhanced relative leakage ratio (RLR), although the superoxide dismutase (SOD) activity increased following both treatments. This higher toxicity was underlined by increased transcription of lipoxygenase (*LOX*) and respiratory burst oxidase homolog (*RBOH*) in $CuSO_4$-exposed seedlings. Furthermore, decreases in catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities were observed in roots of $CuSO_4$-exposed seedlings, while CuEDDS-exposure induced a general increase in enzyme activities. In the primary leaves, both exposures caused a decrease of antioxidative enzyme activities, although this was not always reflected at the transcriptional level. Roots exposed to $CuSO_4$ showed increases of reduced glutathione (GSH) and tocopherols and a reduction of lipoic acid (LA), while ascorbate (AsA) remained constant. On the contrary, CuEDDS-exposure induced decreases in AsA and tocopherol levels, whereas amounts of GSH and LA amounts did not change. In

the primary leaves, both exposures caused a decrease in tocopherols and after $CuSO_4$-exposure a reduction of LA was observed. All together, these results demonstrate that EDDS plays a crucial role in the tolerance of *B. carinata* to oxidative stress induced by Cu and might be important with regard to Cu phytoextraction.

1. Introduction

Industrialization and agriculture have caused the release of high amounts of copper (Cu) leading to soil and water contamination and even reductions in crop yield. Copper removal from soils requires effective approaches. Remediation with conventional methods is expensive and environmentally invasive (Quartacci et al., 2003). Phytoremediation, which covers several different plant-based strategies, among which chelator-enhanced phytoextraction (Quartacci et al., 2007; Cestone et al., 2010), has gained increasing attention in recent years as an environmentally-friendly, cost effective and carbon neutral alternative for remediation of metal-polluted soils (Vangronsveld et al*.,* 2009).

Plants require Cu as a redox-active micronutrient essential to maintain normal growth and development (Yruela, 2009; Cuypers et al., 2011). However, Cu triggers a wide variety of plant responses, ranging from altered gene expression to changes at cellular metabolism. In fact, there are considerable evidences that at high concentrations Cu can become extremely toxic (Sgherri et al., 2001, 2002; Smeets et al., 2009), and can induce directly oxidative injury (Smeets et al., 2009; Navari-Izzo and Rascio, 2010; Cuypers et al., 2011) or indirectly metabolic perturbations (Collin et al., 2008). Copper redox cycling can give rise to a high production of harmful reactive oxygen species (ROS) through the Fenton and Haber-Weiss reactions leading to oxidative stress (Sgherri et al., 2007; Smeets et al., 2009; Cuypers et al., 2011). These free radicals are able to initiate peroxidation of polyunsatured fatty acids, damaging cell membranes, nucleic acids, proteins and other biomolecules (Quartacci et al., 2001;

Navari-Izzo et al., 2006; Yruela, 2009). The production of ROS has been categorized for a long time as an exclusively negative event, although the ROS are genetically programmed and are also produced in plant cells under non-stressful conditions (Foyer and Noctor, 2005). They have also a role in signalling processes (Foyer and Noctor, 2005; Navari-Izzo and Rascio, 2010; Cuypers et al., 2011). Mittler et al. (2004) described in *Arabidopsis* a wide gene network, which controls the balance between ROS toxicity and ROS signaling.

Copper-induced ROS can be generated by the action of several enzymes bound or associated with the cell plasma membrane, among which lipoxygenases (LOX) (Quartacci et al., 2001) and NAD(P)H oxidases (Porta and Rocha-Sosa, 2002; Smeets et al., 2009; Cuypers et al., 2011). The latter are also called respiratory burst oxidase homologs (RBOH) because they are similar to those present in mammalian neutrophils (Sagi and Fluhr, 2006).

To prevent ROS-induced damage, plant cells are equipped with a complex battery of enzymatic and non-enzymatic antioxidant systems that can protect them from oxidative injury and are essential in maintaining cellular redox equilibrium. Enzymatic scavengers are protective enzymes, which operate in several compartments of the cell and include superoxide dismutases (SOD), catalases (CAT), glutathione reductases (GR) and several classes of peroxidases (PX), such as ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and syringaldazine peroxidase (SPX). SOD are located in different cellular compartments with specific metal co-factor (Alscher et al., 2002) and act as the first line of defense against ROS, dismutating superoxide radicals ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2). CAT, PX and the enzymes involved in the ascorbate (AsA)-glutathione (GSH) cycle play a role in the removal of H_2O_2. The effect of Cu on the activities of these enzymes and their involvement in various defense mechanisms of plant tissues against Cu-induced damage remains controversial. Many authors have indicated that the activities of SOD, CAT and PX are increased under Cu stress (Cuypers et al., 2002; Srivastava et al., 2006 and references

therein), while in other studies, it has been reported that Cu excess does not increase or even inhibits the activities of SOD, CAT and PX (Palma et al., 1987; Chaoui and El Ferjani, 2005). APX uses AsA as reducing substrate in the AsA-GSH cycle. Oxidized ascorbate produced in this way is reduced by GSH, that is regenerated from oxidized glutathione (GSSG) by GR using NAD(P)H (Foyer and Noctor, 2005; Lomonte et al., 2010). Hence, GR serves to maintain the antioxidants in their reduced functional state.

In addition to AsA and GSH, non-enzymatic radical-scavengers are composed by other low molecular weight compounds, such as tocopherols and lipoic acid. Lipoic acid has a protective function both in the oxidized (LA) and reduced form (DHLA). Lipoic acid, due to its solubility in both water and lipid phases, connects the activity of antioxidants in the cell membrane (tocopherols) to the antioxidants in the cytoplasm (AsA and GSH), strengthening the antioxidants network (Navari-Izzo et al., 2002; Sgherri et al., 2002). DHLA is able to donate an electron to oxidized forms of GSH and AsA, thus regenerating these compounds in their reduced powerful antioxidant forms (Navari-Izzo and Rascio, 2010). Tocopherols (α-, β-, γ-, δ-tocopherol) are produced mainly in chloroplasts. As lipophilic antioxidants they prevent the propagation of peroxidation in thylakoid membranes through two different oxidation mechanisms involving the AsA-GSH antioxidant system (Navari-Izzo et al., 1997; Navari-Izzo and Rascio, 2010; Krieger-Liszkay and Trebst, 2006).

For the remediation of degraded or contaminated soils, stress-tolerant plants are required. Plants possessing a higher antioxidative capacity can cope better with metal toxicity and may take up for a longer time significant amounts of pollutants. Thus, to predict soil-plant transfer of metals the understanding of the defense mechanisms against metal-induced oxidative injury is important for improving metal phytoextraction potential in the presence of a chelator. To date, different cultivars of *Brassicaceae* species exposed to relatively high Cu concentrations have been studied in hydroponics by several researchers

(Quartacci et al., 2003; Wang et al., 2004; Russo et al., 2008; Li et al., 2009), but little has been published so far on the gene expression and changes of some metabolites of *Brassicaceae* species used as metal accumulators in phytoremediation programs (Quartacci et al., 2007, 2009; Jahangir et al., 2008). In order to investigate the defense mechanisms involved in Cu response, the content of antioxidant compounds, the antioxidative enzyme activities and transcriptional levels of ROS-inducing enzymes and antioxidative enzymes have been investigated in roots and primary leaves of two-week-old *Brassica carinata* seedlings exposed for 24 h to 30 µM $CuSO_4$ or Cu-(S,S)-*N,N'*-ethylenediamine disuccinic acid (CuEDDS). The plants and the chelator were selected from previous studies (Quartacci et al., 2007, 2009; Cestone et al., 2010). Growth- and exposure-period were chosen to monitor early specific transcriptional levels of ROS-producing and antioxidative enzymes which occur in young plants exposed to excess Cu.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise and were analytical grade or reverse-phase high performance liquid chromatography (RP-HPLC) grade for the solvents. EDDS (Octaquest E30) was kindly obtained from Innospec Limited (Chesire, UK) as the Na_3EDDS salt. All solutions were made with high purity water (Millipore, Bedford, MA, USA). All solvents and water were accurately degassed before use in RP-HPLC analysis.

2.2. Plant material and growth conditions

Seeds of *Brassica carinata* cv 180 were surface sterilized for 15 min with diluted NaClO (about 1% of active chlorine) and placed on moist filter paper in

dark for five days at 4°C in order to synchronize germination. Afterwards, the seeds were germinated in Petri dishes on a filter paper soaked with tap water directly in the growth chamber with alternated light under a 12 h photoperiod at 70-75% relative humidity (photosynthetic photon flux density of 400 µmol m^{-2} s^{-1} at leaf level, delivered by cool white fluorescent lamps, L140W/20SA, Osram, Augsburg, Germany) and at 22°C/18°C day/night. After three days, the seedlings were planted on perforated polystyrene substrate filled with rock wool (1 plant/pot). The substrate was placed in black pots containing aerated Raskin solution (Quartacci et al., 2007). The solutions were continuously aerated with an air pump and renewed every three days. After two weeks of growth the seedlings were exposed for 24 h on solutions containing 30 µM $CuSO_4$ or CuEDDS. In the experimental solutions all micronutrients other than Cu and $NH_4H_2PO_4$ were omitted to avoid competition among metals and precipitation of Cu phosphate (Cestone et al., 2010). As a control, one set of seedlings was incubated only in the nutrient solution (0.12 µM $CuSO_4$). At the end of the treatments, a set of the seedlings was used fresh for the relative leakage ratio (RLR) measurements, and another set of seedlings was desorbed as reported by Cestone et al. (2010), separated (roots and primary leaves) and immediately oven-dried at 70°C till constant weight for metal analysis: fresh and dry weights were determined. Prior to biological measurements other seedlings were subdivided in roots and primary leaves, and snap-frozen in liquid nitrogen before storage at -70°C.

2.3. Relative leakage ratio

RLR was measured by determining solute release from roots as reported by Cestone et al. (2010).

2.4. Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) of the plant tissues (roots and primary leaves) were determined spectrophotometrically using a UV-Visible Spectrophotometer (Model UV-1602, Shimadzu) according to Smeets et al. (2009).

2.5. Element analysis

Dried ground material was digested with a mixture (3:1, v/v) of HNO_3 (69-70%) and H_2O_2 (30-32%) using a pressure and temperature control microwave digestion system (Milestone, mega 240 EM-45) at power from 250 up to 600 Watt for 15 min. The element concentrations (Ca, Cu, Fe, K, Mg, Mn, P, S, Zn) were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (PerkinElmer Optima, 3000 DV). SRM 1570a (spinach leaves) was used as a reference.

2.6. Enzyme analysis

Samples were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.8) containing 1mM EDTA, 1 mM dithiothreitol, and 4% insoluble polyvinylpyrrolidone (1 ml buffer 100 mg^{-1} fresh weight). The homogenate was squeezed through a nylon mesh and centrifuged for 10 min at 13,500*g* and 4°C. The enzyme activities were measured spectrophotometrically (Model UV-1602, Shimadzu) in the supernatants at 25°C. SOD (EC 1.15.1.1) activity was based on the inhibition of cytochrome c at 550 nm (Smeets et al., 2009). APX (EC 1.11.1.11), GPX (EC 1.11.1.9), SPX (EC 1.11.1.7), GR (EC 1.6.4.2) and CAT (EC 1.11.1.6) activities were measured at 298, 436, 530, 340 and 240 nm, respectively, according to Smeets et al. (2009). Proteins were determined as reported by Bradford (1976) using serum bovine albumin as a standard.

2.7. Gene expression

Frozen plant tissues (1-75 mg) stored in 2 ml microcentrifuge tubes were disrupted under frozen conditions using two stainless steel beads of 2 mm diameter in each sample and the Retsch Mixer Mill MM2000 (Haan, Germany). RNA was extracted from the crushed tissues using the RNAqueous Plant Mini Kit (Ambion, Applied Biosystems). RNA concentration and purity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Isogen Life, Science), measuring the RNA concentration at 260 nm and checking that the 260/280-ratio was higher than 1.80. One µg of total RNA was used in a 10 µl Quantitect Reverse Transcription reaction (Qiagen) using random hexamer primers to produce cDNA for further analysis. This step included the use of TURBO DNA-free kit (Ambion, Applied Biosystems), necessary to remove the contaminating genomic DNA. A 10-fold dilution of the cDNA was made using 1/10 diluted Tris-EDTA buffer (1 mM Tris–HCl, 0.1 mM EDTA at pH 8.0) and stored at -20°C. A complete gene map of *Brassica carinata* does not exist. The high level of genetic resemblance between *Brassica* and *Arabidopsis* (Jahangir et al., 2008) allowed us to use primers of *Arabidopsis thaliana* in *Brassica carinata*, after testing on beforehand their efficiency in *Brassica*. Reference genes and their primer sequences were based on Remans et al. (2008). Quantitative PCR (RT-PCR) was performed in optical 96-well plates with the Applied Biosystems ABI Prism 7900HT Fast sequence detection system (SYBR Green chemistry), using universal cycling conditions (10 min at 95°C (AmpliTaq Gold Activation), 40 cycles of 15 s at 95°C and 1 min at 60°C) according to manufacturer's conditions. The reaction specificity was detected by the generation of a melting or dissociation curve. As a negative control, RNase free water was used instead of the cDNA template. The following genes were determined (Atg numbers and NCBI reference numbers are in brackets): *LOX3* (*At1g17420*), *LOX4* (*At1g72520*), *RBOHE* (*At1g19230*), *RBOHF* (*At1g64060*), copper/zinc *SOD2* (*CSD2*: *At2g28190*), iron *SOD1* (*FSD1*: *At4g25100*), manganese *SOD1* (*MSD1*: *At3g10920*), *CAT2*

(*At4g35090*), *CAT3* (*At1g20620*), *APX1* (*At1g07890*), *GR1* (*At3g24170*) and *GR2* (*At3g54660*). In order to determine expression levels accurately, relative gene expression in root and primary leaves of each sample was calculated as $2^{-\Delta Ct}$ and expressed relative to a normalization factor based on the expression levels of the best-performing housekeeping genes determined using geNorm v3.4 (Vandesompele et al., 2002). In the roots three reference genes (UBC9, *At5g25760*; Sand Family, *At2g28390* and EF-1α, *At5g60390*) were considered stable by geNorm, instead in the primary leaves besides these three there was also another one for a total of four reference genes (Actin2, *At3g18780*).

2.8. Antioxidants

2.8.1. Glutathione and ascorbic acid (vitamin C)

Plant tissues were homogenized in a cold mortar with inert sand at 4°C in ice-cold 5 % (v/v) trichloroacetic acid. After centrifugation at 12,000 *g* for 15 min, the supernatants were collected and the metabolites were determined according to Lomonte et al. (2010). Total glutathione and glutathione disulphide (GSSG) contents were detected at 412 nm at 25°C. The amount of reduced glutathione (GSH) was calculated from the difference between the total glutathione and GSSG. Reduced ascorbate (AsA) and total ascorbate contents were determined at 534 nm after 90 min of incubation at 30°C. The amount of dehydroascorbate (DHA) was calculated from the difference between the total and reduced ascorbate.

2.8.2. Lipoic acid

Reduced (DHLA) and oxidized (LA) forms were extracted from *B. carinata* roots and primary leaves by acidic hydrolysis followed with chloroform extraction as reported by Sgherri et al. (2010). The resultant organic fraction was evaporated to dryness under vacuum and stored before the determination at -20°C under nitrogen. Both contents in the extracts were determined by RP-HPLC

(Shimadzu LC-20AD) with an electrochemical detector (model 791, Metrohm, Herisan, Switzerland) equipped with a glassy-carbon working electrode and LC Solution Software (Shimadzu) for peak integration. Chromatography separations were performed at +1.1 V with a Nova-Pak C18 column (3.9 x 150 mm, 4 µm, Waters, Milford, MA). The extracts were eluted at 25°C using 1 ml min^{-1} as flow rate and 24% (v/v) acetonitrile, 3% (v/v) 2-propanol, and 72% (v/v) 0.05 M KH_2PO_4 as mobile phase adjusted to pH 2.5 with phosphoric acid. The calibration curve was made using a mix of LA and DHLA standards (Sigma, Steinheim, Germany) in the range of 4-100 ng.

2.8.3. Tocopherols (vitamin E)

Tocopherols (α-, β-, γ-, δ-) were extracted using chloroform/methanol (2:1, v/v) and, in order to remove the salts, washed three times with KCl 0.88% (w/v). Chloroform phases were taken to dry and then resuspended in chloroform/ethanol (1:5, v/v). Immediately, after resuspension the tocopherols were determined by isocratic RP-HPLC using the same apparatus, electrochemical detector and column as described for determination of lipoic acid. Detection was performed according to Sgherri et al. (2010) at 25°C with an applied oxidation potential of +0.6 V. The extracts were eluted with 95% methanol containing 20 mM $LiClO_4$ at a flow rate of 1 ml min^{-1}. For identification and quantification of peaks, a calibration curve was prepared using standard mixtures of α-, β-, γ- and δ-tocopherol (Sigma, Steinheim, Germany) in the range of 25-75 ng.

2.9. Statistical analysis

Statistical analysis was carried out with Costat version 6.400 (1998-2008 CoHort software). The errors bars represent the standard error (SE) of the mean of several independent experiments (see Figures for details) each analyzed twice. The significance of differences among means was determined by one-way

ANOVA. Comparisons between means were performed using LSD test at the significant level of $P \leq 0.05$.

3. Results

3.1. Seedling growth

No significant changes were observed in the growth of seedlings, estimated as dry weight (DW), exposed for 24 h to $CuSO_4$ or CuEDDS in comparison with the control (DW_{roots}: (3.75 ± 0.65) mg; $DW_{primary\ leaves}$: (10.90 ± 2.20) mg).

3.2. Root solute leakage

Free Cu ions caused a significant leakage of solutes compared to the control, whereas the leakage was not significantly different from the control after exposure to CuEDDS (Figure 1a).

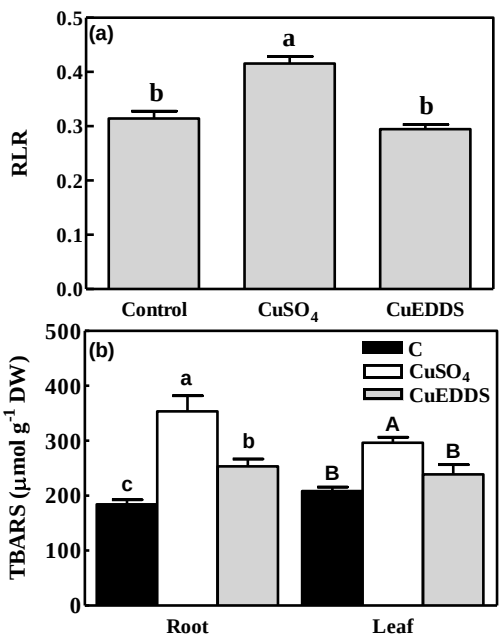


FIGURE 1. Root relative leakage ratio (RLR) in roots (**a**) and thiobarbituric acid reactive substances (TBARS) in roots and primary leaves (**b**) of 2-week-old-*B. carinata* after 24 h of treatment with 30 μM $CuSO_4$ or CuEDDS. Means ± SE (n = 7) followed by different letters are significantly different at $P \leq 0.05$ as determinated by one-way ANOVA, LSD test.

3.3. Thiobarbituric acid reactive substances

After 24 h exposure, the TBARS content in the roots was significantly increased for both treatments, resulting in 2- and 1.5-fold higher levels in $CuSO_4$

or CuEDDS-exposed seedlings, respectively (Figure 1b). In the primary leaves, the TBARS levels were only significantly different in $CuSO_4$-exposed seedlings (Figure 1b).

3.4. Elements

In comparison with control seedlings, exposure to $CuSO_4$ or CuEDDS caused significant increases in root Cu content (14- and 2-fold, respectively), whereas in primary leaves the enhancements were less pronounced (2- and 1.5-fold, respectively) (Figure 2).

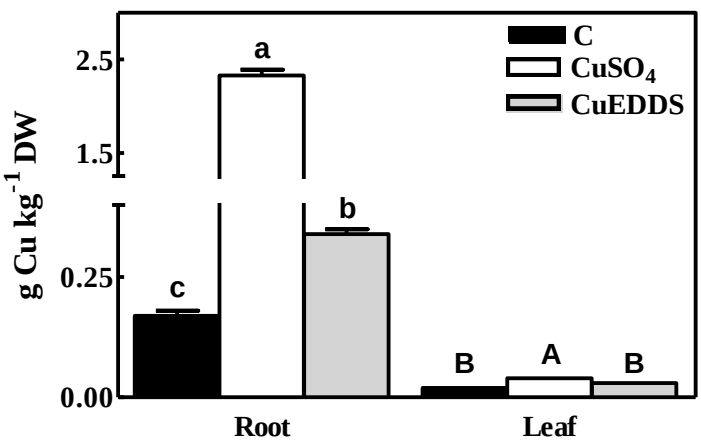


FIGURE 2. Copper concentration in roots and primary leaves of *B. carinata* seedlings incubated for 24 h in solutions containing 30 µM $CuSO_4$ or CuEDDS. Results are means ± SE (n = 5). For each plant organ means followed by the same letter are not significantly different by one-way ANOVA (LSD test, $P \leq 0.05$).

Except for Fe, Mn and Zn, which did not show significant changes in both plant organs, different trends were observed for the other elements after exposure to $CuSO_4$ or CuEDDS (Table 1). In the roots, significant decreases in K, S and P contents and an increased Ca content were observed in the seedlings exposed to $CuSO_4$. The CuEDDS exposure caused an increase only in S content. No changes were detected in Mg levels. In the primary leaves of the same

seedlings, there were significant increases in Ca (+14%), K (+7%) and Mg (+22%) contents in CuEDDS-exposed seedlings in comparison with the control. Like in the roots, the K content decreased significantly by about 30% in the seedlings exposed to $CuSO_4$ treatment. No effects were observed on S and P contents of the primary leaves of seedlings exposed to both treatments (Table 1).

TABLE 1. Element concentration (g Kg^{-1} DW) in roots and primary leaves of 2-week-old *B. carinata* seedlings after 24 h of exposure to 30 µM $CuSO_4$ or CuEDDS. Values are mean ± SE (n = 5). For each plant organ means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$).

Elements (g Kg^{-1} DW)	Control	$CuSO_4$	CuEDDS
Roots			
Ca	28.09 ± 0.85 b	32.12 ± 0.92 a	28.06 ± 1.30 b
Fe	7.67 ± 0.52 a	7.82 ± 0.38 a	7.70 ± 0.77 a
K	25.87 ± 0.99 a	17.86 ± 0.79 b	28.38 ± 0.53 a
Mg	6.92 ± 0.17 a	7.19 ± 0.08 a	7.28 ± 0.01 a
Mn	0.21 ± 0.02 a	0.21 ± 0.01 a	0.19 ± 0.03 a
P	2.95 ± 0.16 a	2.31 ± 0.08 b	3.09 ± 0.17 a
S	2.63 ± 0.04 b	2.28 ± 0.08 c	3.28 ± 0.15 a
Zn	0.03 ± 0.00 a	0.03 ± 0.00 a	0.03 ± 0.00 a
Leaves			
Ca	14.15 ± 0.20 b	13.68 ± 0.25 b	16.41 ± 0.35 a
Fe	0.07 ± 0.01 a	0.08 ± 0.01 a	0.07 ± 0.01 a
K	66.79 ± 0.48 b	47.93 ± 1.71 c	71.60 ± 0.91 a
Mg	2.49 ± 0.12 b	2.72 ± 0.10 b	3.18 ± 0.13 a
Mn	0.09 ± 0.01 a	0.09 ± 0.00 a	0.09 ± 0.01 a
P	3.20 ± 0.17 a	3.57 ± 0.18 a	3.36 ± 0.21 a
S	5.55 ± 0.12 a	5.37 ± 0.14 a	6.11 ± 0.36 a
Zn	0.02 ± 0.00 a	0.02 ± 0.00 a	0.02 ± 0.00 a

3.5. Gene expression

Transcriptional changes of different ROS-producing and antioxidative enzymes were determined in the roots and primary leaves of $CuSO_4$- and CuEDDS-exposed seedlings (Table 2). Significant increases in transcript levels of

ROS-producing enzymes, such as *LOX3*, *LOX4* and *RBOHF*, were observed in both plant tissues of the seedlings exposed to $CuSO_4$; *RBOHE*, which was present only in the leaves, also increased (Table 2).

TABLE 2. Transcript levels of ROS-producing and antioxidative enzymes, normalized relative to the control (100%), in roots and primary leaves of 2-week-old *B. carinata* seedlings exposed to 30 µM $CuSO_4$ or CuEDDS for 24 h. Values are mean ± SE (n = 4). For each point means followed by the same letter are not significantly different by ANOVA (LSD test, $P \leq 0.05$).

	ROOTS			**LEAVES**		
Gene	Control	$CuSO_4$	CuEDDS	Control	$CuSO_4$	CuEDDS
LOX3	1 ± 0.07 b	5.33 ± 0.68 a	0.38± 0.08 b	1 ± 0.04 b	2.56 ± 0.17 a	0.85 ± 0.16 b
LOX4	1 ± 0.06 b	21.77 ± 1.40 a	0.90 ± 0.16 b	1 ± 0.07 b	2.33 ± 0.23 a	0.72 ± 0.11 b
RBOHE	-	-	-	1 ± 0.09 b	1.99 ± 0.15 a	1.02 ± 0.07 b
RBOHF	1 ± 0.14 b	1.52 ± 0.16 a	0.70 ± 0.03 b	1 ± 0.19 b	2.60 ± 0.33 a	1.26 ± 0.32 b
CSD2	1 ± 0.12 b	0.74 ± 0.16 b	5.03 ± 0.52 a	1 ± 0.10 c	6.58 ± 0.32 a	3.96 ± 0.20 b
FSD1	1 ± 0.05 a	1.07 ± 0.09 a	1.17 ± 0.15 a	1 ± 0.04 b	1.93 ± 0.26 a	1.97 ± 0.16 a
MSD1	1 ± 0.13 a	1.11 ± 0.09 a	1.04 ± 0.15 a	1 ± 0.05 b	1.33 ± 0.08 a	1.30 ± 0.03 a
CAT2	1 ± 0.09 b	0.08 ± 0.01 c	1.91 ± 0.20 a	1 ± 0.13 a	0.28 ± 0.04 b	0.32 ± 0.06 b
CAT3	1 ± 0.20 b	4.91 ± 0.51 a	1.26 ± 0.12 b	1 ± 0.02 c	21.60 ± 0.39 a	2.14 ± 0.07 b
APX1	1 ± 0.18 b	2.72 ± 0.30 a	2.28 ± 0.07 a	1 ± 0.13 b	1.71 ± 0.05 a	0.16 ± 0.01 c
GR1	1 ± 0.03 a	1.34 ± 0.14 a	1.32 ± 0.23 a	1 ± 0.13 b	1.73 ± 0.10 a	1.11 ± 0.12 b
GR2	1 ± 0.28 b	1.85 ± 0.29 b	6.07 ± 0.57 a	1 ± 0.10 b	1.74 ± 0.23 a	1.34 ± 0.02 ab

In the roots, gene expression levels of antioxidative enzymes, such as *CSD2*, *CAT2* and *GR2*, were significantly up-regulated in CuEDDS-exposed seedlings, whereas *CAT2* decreased and *CAT3* increased significantly both in roots and primary leaves exposed to $CuSO_4$. In addition, *APX1* gene expression increased significantly in roots following both exposures, while no significant changes were observed in *FSD1*, *MSD1* and *GR1* expressions. In the primary leaves, $CuSO_4$ induced a significant up-regulation of the mRNA levels of the antioxidative

enzymes *CSD2*, *CAT3*, *APX1*, *GR1* and *GR2* and a down-regulation of *CAT2* gene expression. CuEDDS induced significant decreases in *CAT2* and *APX1* expressions and, on the contrary, increased the expression of *CSD2* and *CAT3* transcript levels, although to a lesser extent than in case of $CuSO_4$ exposure. *FSD1* and *MSD1* gene expressions were significantly up-regulated under both conditions.

3.6. Enzyme activities

Compared to the control, total SOD and SPX activities in roots increased significantly after exposure to $CuSO_4$ and CuEDDS, although SPX activity in roots exposed to CuEDDS was higher than in those exposed to $CuSO_4$ (Table 3).

TABLE 3. Enzyme activities (U mg^{-1} proteins) in roots and primary leaves of 2-week-old *B. carinata* seedlings exposed to 30 µM $CuSO_4$ or CuEDDS for 24 h. Results are mean ± SE (n = 4). For each point means followed by the same letter are not significantly different by one-way ANOVA (LSD test, $P \leq 0.05$).

Enzymes (U mg^{-1} proteins)	Control	$CuSO_4$	CuEDDS
Roots			
SOD	1.17 ± 0.07 b	1.96 ± 0.21 a	2.07 ± 0.16 a
CAT	0.09 ± 0.02 a	0.03 ± 0.00 b	0.10 ± 0.02 a
APX	15.73 ± 0.57 b	5.79 ± 0.26 c	35.90 ± 1.23 a
SPX	21.90 ± 0.79 c	31.63 ± 0.15 b	43.19 ± 3.06 a
GPX	4.92 ± 0.63 b	5.33 ± 0.49 b	9.56 ± 0.54 a
GR	0.41 ± 0.07 a	0.22 ± 0.03 b	0.50 ± 0.09 a
Leaves			
SOD	0.46 ± 0.03 a	0.46 ± 0.00 a	0.46 ± 0.02 a
CAT	0.12 ± 0.01 a	0.07 ± 0.00 b	0.09 ± 0.02 b
APX	6.09 ± 0.07 a	4.91 ± 0.04 b	3.75 ± 0.18 c
SPX	0.30 ± 0.01 a	0.23 ± 0.01 b	0.17 ± 0.01 c
GPX	0.07 ± 0.00 a	0.05 ± 0.00 b	0.05 ± 0.00 b
GR	0.22 ± 0.01 a	0.20 ± 0.02 a	0.17 ± 0.02 a

GPX increased significantly only in roots of CuEDDS-exposed seedlings. A marked increase in APX activity was noticed in the CuEDDS-exposed roots, while the activity of the same enzyme decreased in the $CuSO_4$-exposed roots. Exposure to $CuSO_4$ reduced CAT and GR activities in comparison with the control (Table 3). In the primary leaves, significant decreases of CAT and PX (APX, SPX and GPX) activities were observed following both exposures; the reductions of SPX and APX activities were more pronounced in primary leaves of CuEDDS-exposed seedlings. For the activities of SOD and GR no significant changes were observed for both exposures in the primary leaves (Table 3).

3.7. Glutathione and ascorbate

Total glutathione (GSH + GSSG, Figure 3a), GSH (Figure 3b) and GSH/GSSG ratio (Figure 3c) showed similar trends under both conditions. In roots, after exposure to $CuSO_4$ the content of GSH + GSSG, the GSH level and the GSH/GSSG ratio increased significantly by about +46%, +70% and +56%, respectively, as compared to the control (Figure 3a, b and c). In case of CuEDDS exposure, only the total glutathione increased significantly (+30%) (Figure 3a). In the primary leaves no differences were observed as compared to the control seedlings. In comparison with the control, the concentration of total ascorbate (AsA + DHA, Figure 4a) increased significantly in roots of seedlings exposed to $CuSO_4$ or CuEDDS (+86% and +49%, respectively). In roots of seedlings exposed to CuEDDS the AsA content decreased with a factor 2.5 (Figure 4b), whereas the AsA/DHA ratio decreased significantly by about 95% in both treatments (Figure 4c). In the aerial parts, no significant changes were observed due to the exposures.

3.8. Lipoic acid

DHLA was found in trace amounts only in the control. In comparison with the control, $CuSO_4$ exposure reduced LA levels by 73% in roots and 27% in primary

leaves, whereas no significant changes were detected in both plant tissues treated with CuEDDS (Figure 5a).

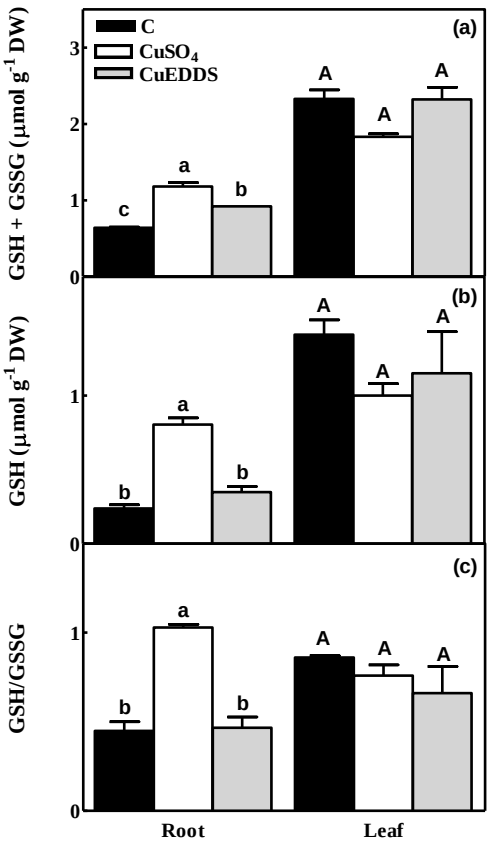


FIGURE 3. Total glutathione (GSH + GSSG, **a**), reduced glutathione (GSH, **b**) and reduced/oxidized glutathione ratio (GSH/GSSG, **c**) in root and primary leaves of *B. carinata* treated for 24 h with 30 µM $CuSO_4$ or CuEDDS. Values are means ± SE (n = 3). For each treatment means followed by the same letters are not significantly different by one-way ANOVA (LSD test, $P \leq 0.05$).

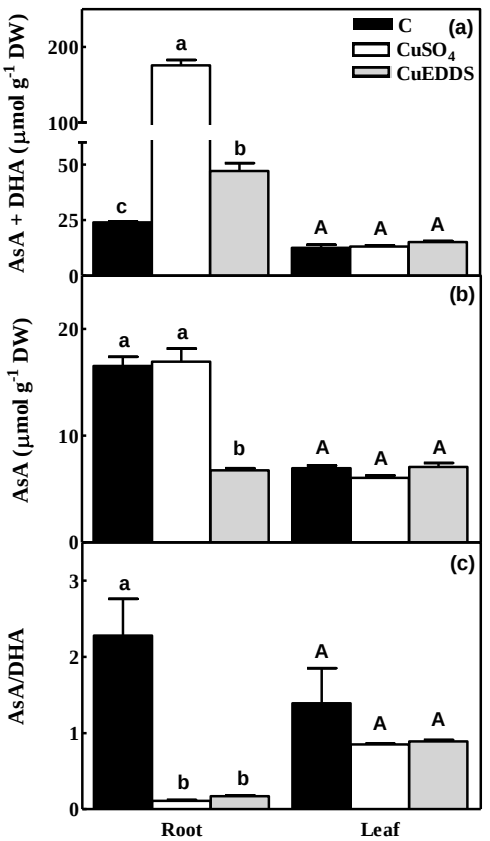


FIGURE 4. Total ascorbate (AsA + DHA, **a**), reduced ascorbate (AsA, **b**) and reduced/oxidized ascorbate ratio (AsA/DHA, **c**) in root and primary leaves of *B. carinata* treated for 24 h with 30 µM $CuSO_4$ or CuEDDS. Statistical analysis was as in Figure 3.

3.9. *Tocopherol*

In roots, only γ- and δ-tocopherol were detected (Figure 5b). Their concentrations increased after $CuSO_4$ exposure (+27% and +34%, respectively) and decreased in case of CuEDDS (-36% and -27%, respectively). In primary leaves, only α- and γ-tocopherol were detected (Figure 5b). Compared to the control they were both reduced by about 88% and 64%, respectively, following exposure to $CuSO_4$ and CuEDDS respectively.

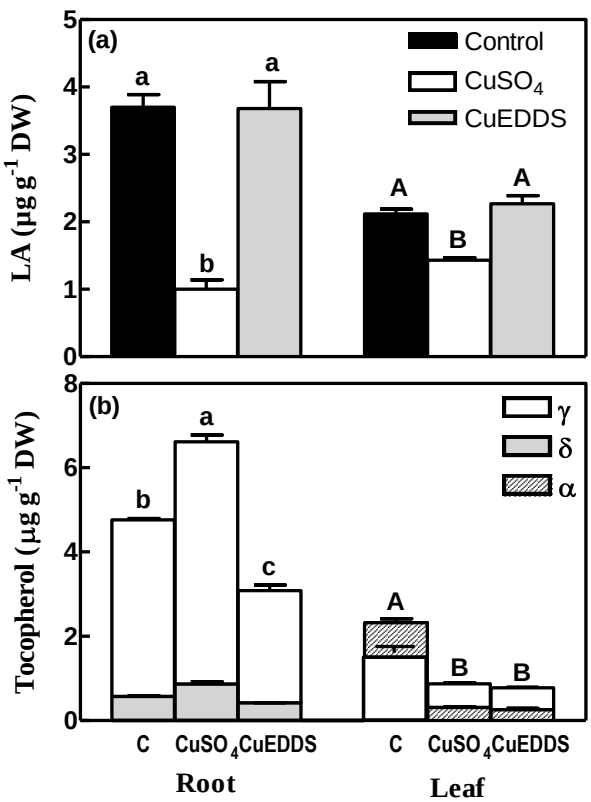


FIGURE 5 Lipoic acid (LA) (**a**) and tocopherols (α, γ, δ) (**b**) in root and primary leaves of *B. carinata* grown in hydroponics for 2 weeks and treated for 24 h with 30 µM $CuSO_4$ or CuEDDS. Statistical analysis was as in Figure 3.

4. Discussion

Brassica carinata seedlings were exposed to either $CuSO_4$ or CuEDDS to investigate whether the use of the Cu chelator EDDS improves the plant tolerance to Cu with respect to oxidative stress related parameters. This knowledge is important for developing chelator-based phytoextraction

strategies. The exposure concentration and exposure time were chosen based on previous studies (Cestone et al., 2010) and preliminary experiments. Under these circumstances neither visible symptoms of damage (data not shown) nor reduction in biomass (root and primary leaf) were detected at the end of the exposure period.

4.1. $CuSO_4$ is more cytotoxic to the roots than CuEDDS, hence affecting the Cu translocation factor

In roots of seedlings exposed to $CuSO_4$ increases in RLR (Figure 1a) and TBARS (Figure 1b) occurred, suggesting an effect at the cell membrane level. Roots are in direct contact with the hydroponic medium and therefore are more markedly affected by Cu than the aerial plant parts (Figure 1), especially in case of short-term exposure like in the present experiment. On the contrary, CuEDDS exposure did not cause changes in RLR (Figure 1a) and induced an increase in TBARS only in roots but to a significantly lesser extent than $CuSO_4$ (Figure 1b), suggesting the activation of an efficient free radical scavenging system, which minimizes the adverse effects of a general oxidation. In this case, CuEDDS might have been split before Cu uptake (Cestone et al., 2010) and, due to this, the effects of Cu toxicity could have been delayed or mitigated and the seedlings could have had the time to respond. In agreement with data published before (Cestone et al., 2010), CuEDDS appeared to be less toxic than the free metal ion (when applied as $CuSO_4$). In case of $CuSO_4$ exposure, a starting disorganization of the membrane is further supported by the observed decrease in K^+ concentration (Table 1). Similar results were also obtained by Smeets et al. (2009) and Cuypers et al. (2011) in *Arabidopsis* seedlings exposed to Cu. Previous studies (Quartacci et al., 2003, 2007; Tandy et al., 2006; Cestone et al., 2010) have shown that the presence of EDDS increased root to shoot translocation of Cu. This was confirmed in this study, where the metal translocation factor in case of exposure to CuEDDS was 5 times higher than when exposed to $CuSO_4$

(Figure 2). This might be related to the lower RLR values (Figure 1a) and the consequently more restricted damage to the root cells (Figure 1b).

A higher Cu concentration in roots exposed to $CuSO_4$ in comparison with roots exposed to CuEDDS (Figure 2) induced immediately a series of cellular responses as evidenced by transcripts of ROS-producing and antioxidative enzymes, enzyme activities and metabolites produced (Table 2, 3; Figure 3, 4, 5). Beside a direct production of ROS through Fenton reactions, the significant up-regulation of *LOX 3*/*4* and *RBOHE*/*F* transcript levels (Table 2) might indicate that enzyme-related ROS production occurs under $CuSO_4$ exposure leading to oxidative damage or signaling. The increased transcript levels may also indicate that these enzymes are involved in signal transduction pathways via the production of a large number of structurally different oxylipins and/or jasmonates and ROS under metal stress (Porta and Rocha-Sosa, 2002; Mithöfer et al., 2004; Cuypers et al., 2011). In addition, a complex interaction between both enzyme groups exists but this needs further investigation (Remans et al., 2010). Foreman et al. (2003) have demonstrated that ROS production mediated by plasma membrane-NADPH oxidases regulates plant cell growth and that this process is controlled by the activation of plasma membrane Ca^{2+}- and K^{+}-permeable channels in plant root cells. The increases in transcript levels of seedlings exposed to free Cu that are not observed after CuEDDS exposure could explain the different behavior of these elements in roots following both exposures (Table 1).

To counteract the increased ROS levels, several antioxidant defense systems come into play. SOD are known to contribute to tolerance against oxidative stress, but in case the production rate of the superoxide radical under abiotic stress exceeds the capacity of the SOD, oxidative damage will occur (Alscher et al., 2002). Elevation in total SOD activity was observed in roots exposed to both treatments (Table 3). However, only in the roots of CuEDDS exposed seedlings the transcriptional level of *CSD2* was significantly higher, whereas in roots of

seedlings exposed to $CuSO_4$ no changes were detected (Table 2). A much higher Cu content in roots of $CuSO_4$-exposed seedlings might induce a metal-catalyzed conversion of different ROS (H_2O_2 and $O_2^{\bullet-}$) directly into $OH^{\bullet}$ radicals so less detoxification of $O_2^{\bullet-}$ is necessary. Furthermore the $OH^{\bullet}$ radicals are harmful and cause oxidative damage as is seen for different parameters in these roots. The less toxic CuEDDS instead might have provoked a lower production of ROS, such as H_2O_2, which, in this case, could function as a signaling agent, activating increased *CSD2* transcript levels (Table 2). In future experiments, it is essential to unravel and analyze all the different SOD isoenzymes at both, transcriptional and enzymatic level, in order to be able to draw conclusive statements.

Plants have developed several enzymatic pathways to detoxify H_2O_2 produced by SOD activity and photorespiration, *e.g*. CAT and PX (such as APX). Wang et al. (2004) showed that CAT activity of roots of *Brassica juncea* L. exposed to excess Cu was suppressed, while Li et al. (2009) observed a different action of CAT activities in leaves of two cultivars of cabbage exposed to 10 µM $CuSO_4$. In the present study, CAT activity decreased significantly in roots treated with $CuSO_4$ (Table 3). This reduction may indicate that Cu weakens the antioxidative defense system directly. However, gene expressions of *CAT2* and *CAT3* showed an opposite behavior (Table 2), similar to that observed in *Arabidopsis thaliana* seedlings (Cuypers et al., 2011). Zimmermann et al. (2006) demonstrated that in *Arabidopsis CAT2* down-regulation is the initial step in producing a high H_2O_2 content during senescence, leading to the induction of *CAT3* expression and activity. This suggests that Cu induces early senescence like also reported by Cuypers et al. (2011) in roots of *Arabidopsis* exposed to Cd. The different transcriptional pathways and the variation of activity of CAT may be linked to the diverse actions of the enzyme in the cell, *i.e*. as H_2O_2 detoxifier or regulator of H_2O_2 as signaling molecule (Cuypers et al., 2011). Depending on the levels of free Cu in the tissues, *i.e.* stress intensity, the enzyme acts differently. Indeed, after exposure to $CuSO_4$, the degradation of H_2O_2 might have been

realized by APX and not by CAT, because Cu may have been bound to thiol groups or replaced cofactors present in the enzyme, such as Fe^{2+}, leading to an inactivation of CAT (Drążkiewicz et al., 2004; Wang et al., 2004; Srivastava et al., 2006). In case of the seedlings exposed to CuEDDS, the CAT activity is increased as an antioxidative defense system.

The difference in increases of PX (APX, GPX and SPX) (Tables 2 and 3) following $CuSO_4$ or CuEDDS exposure might therefore indicate different targets of oxidative damage (Sgherri et al., 2002). In roots, although *APX1* transcriptional levels were up-regulated following both exposures (Table 2), APX activity decreased when exposed to $CuSO_4$ and increased after CuEDDS exposure (Table 3). The changes in APX activity are strictly correlated with plant tolerance to oxidative stress and its reduction is likely correlated with the increase in stress intensity, supporting the hypothesis that the roots were more affected by $CuSO_4$ stress as compared to CuEDDS. In case of roots exposed to CuEDDS, the increases in GPX and SPX (Table 3) may be correlated with cell wall lignification (Sgherri et al., 2001; Cuypers et al., 2002) as the CuEDDS complex is split in the apoplast before Cu-uptake and hence a controlled sequestration of Cu in the lignified cell walls can be controlled as a detoxification strategy. An increase in APX activity however (Table 3) could be responsible for a tight regulation of H_2O_2 as a signaling molecule (Cuypers et al., 2011).

Copper affects the activities of the enzymes, such as APX and GR (Table 3)., involved in the AsA-GSH cycle as well as the metabolite content (Figure 3 and 4). The reduction in APX and GR activities in roots of *Brassica carinata* seedlings exposed to $CuSO_4$, which was also observed in red cabbage exposed to toxic concentrations of Cu (Posmyk et al., 2009), might suggest that in these conditions the involvement of the AsA-GSH pathway in H_2O_2 scavenging might be hampered. Regeneration of reduced glutathione by GR is a critical step in the ROS scavenging system (Navari-Izzo and Rascio, 2010). Despite the decrease in root GR activity (Table 3) and unchanged *GR1* and *GR2* (Table 2) transcriptional

levels in $CuSO_4$-exposed seedlings, an increase in the amount of GSH was observed (Figure 3b), suggesting that an activation of GSH synthesis and its accumulation occurred as a general feature of enhanced oxidation of the cytosol (Foyer and Noctor, 2005). The induction of GSH synthesis (Figure 3b) and the higher GSH/GSSG ratio (Figure 3c) observed in the roots of $CuSO_4$-exposed seedlings may be correlated with the impairment of the enzymatic defense mechanism and the lack of utilization of GSH in the reduced form, suggesting that an insufficient counterbalance of GSH oxidation took place. The CuEDDS application induced an enzymatic antioxidant defense response in the roots, but, at the same time, total glutathione increased (Figure 3a), whereas GSH (Figure 3b) and GSH/GSSH ratio (Figure 3c) remained constant because the newly synthesized glutathione might have been used for phytochelatin synthesis (Lomonte et al., 2010).

In the roots, $CuSO_4$ exposure increased the level of AsA + DHA (Figure 4a) without any significant change in AsA content (Figure 4b), suggesting an enhanced synthesis of ascorbate to overcome the oxidation of AsA that is apparent under these conditions. On the contrary, the decrease in AsA content (Figure 4b) after CuEDDS-exposure could be explained with its utilization by APX, which activity increased (Table 3) for detoxification of H_2O_2. This also suggests that AsA contents were sufficient for maintaining APX activity that can be irreversibly inhibited when AsA levels decrease.

Although DHLA has been found in plant species in other studies (Navari-Izzo et al., 2002; Sgherri et al., 2002, 2010), in *Brassica carinata* it was only detected in trace amounts in the control seedlings. This might indicate that DHLA as well as LA contents depend on plant species and, most likely, in *B. carinata* the low amount of DHLA was immediately used to regenerate LA. Decreases of LA were observed in roots of seedlings exposed to $CuSO_4$ (Figure 5a), suggesting the involvement of LA to prevent oxidative processes. In addition, the consumption of LA without any regenerations could have been due to its chelation with other

metals different from Cu (Navari-Izzo et al., 2002) and a possible inhibition of its reduction to DHLA as demonstrated by Taylor et al. (2002) in mitochondria isolated from stressed pea plants. However, following CuEDDS exposure LA content was not affected in both plant tissues (Figure 5a), suggesting that the seedlings adapted better to stress conditions with a positive influence on redox homeostasis.

During normal metabolism AsA reacts directly with ROS as a primary antioxidant and acts as a secondary antioxidant reducing the oxidized form of lipophilic tocopherol and preventing membrane injury, while GSH predominantly works as a preventive antioxidant, demonstrating its efficiency at low tocopherol concentrations (Navari-Izzo et al., 1997). AsA and GSH should act as radical scavengers only when there is an efficient removal of tocopherol forms (Navari-Izzo et al., 1997). Exposure to $CuSO_4$ increased both γ- and δ-tocopherol contents (Figure 5b), indicating that the reaction to membrane damage, supported by enhanced RLR and TBARS levels (Figure 1), was in progress. A protective role of AsA and tocopherols in the tolerance of roots exposed to $CuSO_4$ was also observed by Collin et al. (2008) in *Arabidopsis* exposed to high concentrations of Cu. A constant AsA content (Figure 4b) might suggest that newly synthesized AsA was consumed to regenerate tocopherol (Figure 5b). A reduction of both tocopherols (Figure 5b), instead, in CuEDDS-exposed roots could be correlated with a lower membrane injury (Figure 1), due to a well functioning tocopherol cycle, counteracting the oxidative pressure (Navari-Izzo and Rascio, 2010).

4.2. Copper translocated to the primary leaves differently affects the oxidative stress response

Although the Cu translocation factor is much higher in CuEDDS exposed plants in comparison with $CuSO_4$ exposed seedlings, the net result of Cu content is lower in the former (Figure 2) for this short exposure time. Therefore, the increase in TBARS observed in the primary leaves of seedlings exposed to $CuSO_4$

(Figure 1b) can be mediated either by ROS produced as a direct consequence of Cu redox properties or by enhanced LOX or NADPH oxidase activities as suggested by the increased transcript levels (Table 2). Although a clear stimulation of gene transcripts of antioxidant enzymes was noticed (stronger increase in case of $CuSO_4$ than in CuEDDS exposed seedlings), the opposite was observed for the enzyme activities (stronger decrease in case of CuEDDS than in $CuSO_4$ exposed seedlings) (Table 2 and 3). This might indicate that other factors are important for the detoxification of ROS. Although no alterations are observed in GSH and AsA content (Figure 3 and 4), tocopherols and LA are suggested as a primary antioxidant defence (Figure 5), indicating that the cellular redox balance can be maintained under these conditions.

5. Conclusions

Antioxidants together with antioxidative enzymes have a role in reducing the highly harmful potential of ROS. Copper clearly induced oxidative stress in seedlings of *B. carinata*, but, at the same exposure concentration, $CuSO_4$ showed to be more cytotoxic than CuEDDS. In fact, plants respond positively to CuEDDS exposure in contrast with $CuSO_4$, developing adaptive mechanisms to the stress by adjusting gene expression. This is probably connected with the different signaling routes in Cu stress.

These results further indicate that the use of EDDS to facilitate root uptake and the increased root to shoot translocation of Cu could limit phytotoxic responses of plants growing on a contaminated soil. In the current experimental set-up, the clear adaptive responses in CuEDDS exposed seedlings after a short exposure are important in the long run, when plants are exposed for a longer period and can accumulate more Cu in a controlled way. Moreover, the use of *Brassica carinata* in an EDDS-enhanced phytoextraction system allowing at the same time an improved metal extraction combined with the production of biofuel seems promising.

Acknowledgements

Seeds of *Brassica carinata* cv. 180 were kindly provided by Prof. M. Mazzoncini (Dipartimento di Agronomia e Gestione dell'Agrosistema, University of Pisa). This study was supported by Hasselt University (Methusalem project 08M03VGRJ) and the University of Pisa.

References

Alscher, R.G., Erturk, N., Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53, 1331-1341.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Cestone, B., Quartacci, M.F., Navari-Izzo, F., 2010. Uptake and translocation of CuEDDS complexes by *Brassica carinata*. Environ. Sci. Technol. 44, 6403-6408.

Chaoui, A., El Ferjani, E., 2005. Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L.) seedlings. C. R. Biol. 328, 23-31.

Collin, V.C., Eymery, F., Genty, B., Rey, P., Havaux, M., 2008. Vitamin E is essential for the tolerance of *Arabidopsis thaliana* to metal-induced oxidative stress. Plant Cell Environ. 31, 244-257.

Cuypers, A., Vangronsveld, J., Clijsters, H., 2002. Peroxidases in roots and primary leaves of *Phaseolus vulgaris*. Copper and Zinc Phytotoxicity: a comparison. J. Plant Physiol. 159, 869-876.

Cuypers, A., Smeets, K., Ruytinx, J., Opdenakker, K., Keunen, E., Remans, T., Horemans, N., Vanhoudt, N., Van Sanden, S., Van Belleghem, F., Guisez, Y., Colpaert, J., Vangronsveld, J., 2011. The cellular redox state as a modulator

in cadmium and copper responses in *Arabidopsis thaliana* seedlings. J. Plant Physiol. 168, 309-316.

Drążkiewicz, M., Skórzyńska-Polit, E., Krupa, Z., 2004. Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana.* Biometals 17, 379-387.

Foreman, J, Demidchik, V, Bothwell, J.H.F., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D.G., Davies, J.M., Dolan, L., 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422, 442–446.

Foyer, C.H., Noctor G., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28, 1056-1071.

Jahangir, M., Abdel-Farid, I.B., Choi, Y.H., Verpoorte, R., 2008. Metal ion-inducing metabolite accumulation. J. Plant Physiol. 165, 1429-1437.

Krieger-Liszkay, A., Trebst, A., 2006. Tocopherol is the scavenger of singlet oxygen produced by the triplet states of chlorophyll in the PSII reaction centre. J. Exp. Bot. 57, 1677-1684.

Li, Y., Song, Y., Shi, G., Wang, J., Hou, X., 2009. Response of antioxidant activity to excess copper in two cultivars of *Brassica campestris* ssp. *chinensis* Makino. *Acta* Physiol. Plant. 31, 155-162.

Lomonte, C., Sgherri, C., Baker, A.J.M., Kolev, S.D., Navari-Izzo, F., 2010. Antioxidative response of *Atriplex codonocarpa* to mercury. Environ. Exp. Bot. 69, 9-16.

Mithöfer, A., Schulze, B., Boland, W., 2004. Biotic and heavy metal stress response in plants: evidence for common signals. FEBS Letters 566, 1-5.

Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490-498.

Navari-Izzo, F., Quartacci, M.F., Sgherri, C.L.M., 1997. Desiccation tolerance in Higher Plants Related to Free Radical Defences. Phyton (Austria), Special issue Free Radicals 37, 203-214.

Navari-Izzo, F., Quartacci, M.F., Sgherri, C., 2002. Lipoic acid: a unique antioxidant in the detoxification of activated oxygen species. *Plant Physiol. Biochem. 40, 463-470.*

Navari-Izzo, F., Cestone, B., Cavallini, A., Natali, L., Giordani, T., Quartacci, M.F., 2006. Copper excess triggers phospholipase D activity in wheat roots. Phytochemistry 67, 1232–1242.

Navari-Izzo, F., Rascio, N., 2010. Heavy metal pollution: damage and defense strategies in plants. In: Pessarakli, M. (Ed.), *Handbook of Plant and Crop Stress*. Taylor & Francis Publishing Company, Florida, pp. 635-674.

Palma, J.M., Gómez, M., Yáñez, J., Del Río, L.A., 1987. Increased levels of peroxisomal active oxygen-related enzymes in copper-tolerant pea plants. Plant Physiol. 85, 570-574.

Porta, H., Rocha-Sosa, M., 2002. Plant lipoxygenases. Physiological and molecular features. Plant Physiol. 130, 15-21.

Posmyk, M.M., Kontek, R., Janas, K.M., 2009. Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicol. Environ. Saf. 72, 596-602.

Quartacci, M.F., Cosi, E., Navari-Izzo, F., 2001. Lipids and NADPH-dependent superoxide production in plasma membrane vesicles from roots of wheat grown under copper deficiency or excess. J. Exp. Bot. 52, 77-84.

Quartacci, M.F., Cosi, E., Meneguzzo, S., Sgherri, C., Navari-Izzo, F., 2003. Uptake and translocation of copper in *Brassicaceae*. J. Plant Nutr. 26, 1065-1083.

Quartacci, M.F., Irtelli, B., Baker, A.M.J., Navari-Izzo, F., 2007. The use of NTA and EDDS for enhanced phytoextraction of metals from multiply contaminated soil by *Brassica carinata*. Chemosphere 68, 1920-1928.

Quartacci, M.F., Irtelli, B., Gonnelli, C., Gabbrielli, R., Navari-Izzo, F., 2009. Naturally-assisted metal phytoextraction by *Brassica carinata*: Role of root exudates. Environ. Pollut. 157, 2697-2703

Remans, T., Smeets, K., Opdenakker, K., Mathijsen, D., Vangronsveld, J., Cuypers, A., 2008. Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentration. Planta 227, 1343-1349.

Remans, T., Opdenakker, K., Smeets, K., Mathijsen, D., Vangronsveld, J., Cuypers, A., 2010. Metal-specific and NADPH oxidase dependent changes in lipoxygenase and NADPH oxidase gene expression in *Arabidopsis thaliana* exposed to cadmium or excess copper. Funct. Plant Biol. 37, 532-544.

Russo, M., Sgherri, C., Izzo, R., Navari-Izzo, F., 2008. *Brassica napus* subjected to copper excess: Phospholipases C and D and glutathione system in signalling. Environ. Exp. Bot. 62, 238-246.

Sagi, M., Fluhr, R., 2006. Production of Reactive Oxygen Species by Plant NADPH Oxidases. Plant Physiol. 141, 336-340.

Sgherri, C., Milone, M.T.M., Clijsters, H., Navari-Izzo F., 2001. Antioxidative enzymes in two wheat cultivars, differently sensitive to drought and subjected to subsymptomatic copper doses. J. Plant Physiol. 158, 1439-1447.

Sgherri, C., Quartacci, M.F., Izzo, R., Navari-Izzo, F., 2002. Relation between lipoic acid and cell redox status in wheat grown in excess copper. *Plant Physiol. Biochem. 40, 591-597.*

Sgherri, C., Quartacci, M.F., Navari-Izzo, F., 2007. Early production of activated oxygen species in root apoplast of wheat following copper excess. J. Plant Physiol. 164, 1152-1160.

Sgherri, C., Cecconami, S., Pinzino, C., Navari-Izzo, F., Izzo, R., 2010. Levels of antioxidants and nutraceuticals in basil grown in hydroponics and soil. Food Chem. 123, 416-422.

Smeets, K., Opdenakker, K., Remans, T., Van Sanden, S., Van Belleghem, F., Semane, B., Horemans, N., Guisez, Y., Vangronsveld, J., Cuypers A., 2009. Oxidative stress-related responses at transcriptional and enzymatic levels after exposure to Cd or Cu in a multipollution context. J. Plant Physiol. 166, 1982-1992.

Srivastava, S., Mishra, S., Tripathi, R.D., Dwivedi, S., Gupta, D.K., 2006. Copper-induced oxidative stress and responses of antioxidants and phytochelatins in *Hydrilla verticillata* (L.f.) *Royle. Aquat. Toxicol. 80, 405-415.*

Tandy, S., Schulin, R., Nowack, B., 2006. The influence of EDDS on the uptake of heavy metals in hydroponically grown sunflower. Chemosphere 62, 1454-1463.

Taylor, N.L., Day, D.A., Millar, A.H., 2002. Environmental Stress Causes Oxidative Damage to Plant Mitochondria Leading to Inhibition of Glycine Decarboxylase. J. Biol. Chem. 277, 42663-42668.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1-11.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., Van Der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ. Sci. Pollut. Res. Int. 16, 765-794.

Wang, S.H., Yang, Z.M., Yang, H., Lu, B., Li, S.Q., Lu, Y.P., 2004. Copper-induced stress and antioxidative responses in roots of *Brassica juncea* L. Bot. Bull. Acad. Sin. 45, 203-212.

Yruela, I. (2009) Copper in plants: acquisition, transport and interactions. Funct. Plant Biol. 36, 409-430.

Zimmermann, P., Heinlein, C., Orendi, C., Zentgraf, U., 2006. Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant Cell Environ. 29, 1049-1060.

CONCLUSIONS

In order to improve chelant-assisted metal phytoextraction in the cleaning up of a multiple metal-polluted area, the present study was carried out to investigate the potential use of (S,S)-N,N'-ethylenediamine disuccinic acid (EDDS). The aim was to increase the availability of metals in the site of interest (Torviscosa, Udine) and to enhance their translocation in the two accumulator crops *B. carinata* and *R. sativus*. The single moderate dose of EDDS (2.5x1 mmol kg^{-1}) resulted to be more effective than the splitting of the chelant application in more doses. This amendment applied one week before harvest positively affected uptake and translocation of metals implying a higher metal removal. Indeed, no biomass reduction occurred when *B. carinata* was treated with the lowest single application, demonstrating its ability to survive and tolerate the presence of more metals in their harvestable tissues. To further increase EDDS-assisted phytoextraction effectiveness and, therefore, the capacity of *B. carinata* to accumulate more metals at the same time, a better knowledge of the mechanisms and of the biological processes concerning both uptake and translocation of Cu as free ion or in its complexed form is needed. This may allow a better understanding of metal accumulation and represent the starting point for the development of new strategies for enhancing toxic elements uptake by plants. The results obtained from the study of the EDDS and Cu influx kinetics and translocation showed that EDDS and Cu did not enter the roots in their complexed form, but mainly by two different routes confirming that chelant influx was due to a nonselective passive apoplastic pathway. In addition, the presence of EDDS demonstrated to increase Cu translocation after passing into the xylem through the formation of electro-neutral complexes between free Cu ions and EDDS, probably because of a weaker affinity of the metal for binding to the negatively charged cell wall components (as carboxylic and hydroxylic groups). Cu was then transported to the shoots via transpiration

stream as the main driving force. At the same time, EDDS-assisted Cu uptake and transport resulted in preserved root endodermal barrier indicating that CuEDDS complexes were much less harmful to root membranes than free Cu ions as also supported by micro-PIXE analysis. The complex had to be split before Cu uptake and, in this way, CuEDDS induced a lower Cu uptake and toxicity. Since metals are translocated to mesophyll cells only after all binding sites in leaf veins are saturated, micro-PIXE Cu localization maps showed that Cu was sequestered within the leaf vascular tissues thus preventing Cu mobilization into the photosynthetically active tissues and consequentially increasing tolerance of *B. carinata* to Cu. The responses at metabolic and transcriptional levels induced in *B. carinata* by Cu (as free or complexed form) were studied because plants which have higher antioxidative response can cope better with metal toxicity and may uptake for a longer time significant amounts of the toxic elements. In Cu-exposed roots the up-regulation of lipoxygenases 3/4 (*LOX*) and respiratory burst oxidase homolog F (*RBOHF*) transcripts indicated a higher toxicity of Cu alone, a higher reactive oxygen species (ROS) production and a higher membrane damage as confirmed by the higher root solute leakage and peroxidation values. On the contrary, the general activation of the antioxidative enzymes and their higher gene expression in CuEDDS-treated roots evidenced that the seedlings when exposed to the complex were able to contrast the Cu damaging effects. In this case, the antioxidant contents together with the antioxidative enzymes demonstrated their role in reducing the highly harmful potential of ROS. In fact, seedlings exposed to CuEDDS adapted better to the stress conditions counteracting the oxidative pressure as evidenced by the ascorbate (AsA)-reduced glutathione (GSH) cycle and the levels of lipoic acid (LA) and tocopherols. On the contrary, the impairment of the defence mechanism caused by $CuSO_4$ exposure was also confirmed by the lack of utilization of GSH. In addition, the LA decrease and the tocopherols increase indicate that the plants tried to prevent oxidative stress, notwithstanding the membrane damage

was in progress. In the primary leaves, a general depression of transcripts and enzyme activities was evidenced to indicate that the cellular redox balance was maintained under stress conditions. Even in this case, CuEDDS demonstrated to induce more tolerance of *B. carinata* to Cu.

The use of accumulator and tolerant plants like *B. carinata* and of a highly biodegradable chelant like EDDS in an EDDS-enhanced phytoextraction programme could become a reliable phytoextraction strategy in extreme pollution conditions as those found in pyrite wastes.

ABBREVIATIONS

APX, ascorbate peroxidase;

AsA, ascorbate;

CAT, catalase;

CSD, copper/zinc superoxide dismutase;

DHA, dehydroascorbic acid;

DHLA, dihydrolipoic acid;

DTPA, diethylenetriaminepentaacetic acid;

DW, dry weight;

EDDS, (S,S)-N,N'-ethylenediaminedisuccinic acid;

EDTA, ethylenediaminetetraacetic acid;

FSD, iron superoxide dismutase;

GPX, guaiacol peroxidase;

GSH, reduced glutathione;

GSSG, glutathione disulphide;

GR, glutathione reductase;

H^+-ATPase, proton-pumping ATPase;

H_2O_2, hydrogen peroxide;

ICQ, intensity correlation quotient;

I, H^+ -ATPase inhibitor sodium orthovanadate;

IGV, italian guideline values;

LA, lipoic acid;

LOX, lipoxygenase;

Micro-PIXE, micro-proton induced X-ray emission;

MSD, manganese superoxide dismutase;

MT_s, metallothioneins;

$O_2^{\bullet-}$, superoxide radical;

$OH^{\bullet}$, hydroxyl radical;

PC_s, phytochelatins;

PIXE, particle induced X-ray emission;

PM, plasma membrane;

PX, peroxidase;

QAES, quantitative analysis of environmental samples,

RBOH, respiratory burst oxidase homolog;

RLR, relative leakage ratio;

ROS, reactive oxygen species;

RP-HPLC, reverse-phase high performance liquid chromatography;

SOD, superoxide dismutase;

SPX, syringaldazine peroxidase;

STIM, scanning transmission ion microscopy;

TBARS, thiobarbituric acid reactive substances;

XRF, X-ray fluorescence.

ACKNOWLEDGEMENTS

Thanks are due to people who helped me to reach this goal: Prof.dr. Flavia Navari-Izzo, Dr. Mike Frank Quartacci, Dr. Cristina Sgherri (University of Pisa, Italy); Dr. Marianna Bandiera, Prof.dr. Teofilo Vamerali, Prof.dr. Nicoletta Rascio (University of Padova, Italy); Prof.dr. Jaco Vangronsveld, Prof.dr. Ann Cuypers, Dr. Panos Gkorezis, Dr. Els Keunen, Dr. Michiel Op De Beeck, Dr. Sarah Croes, Dr. Marijke Jozefczak, Dr. Tony Remans (Hasselt University, Belgium); Dr. Katarina Vogel-Mikuš, Dr. Primož Pelicon, Dr. Paula Pongrac (University of Ljubljana and Jožef Stefan Institute, Slovenia).

Special thanks to Antonio, my family and my friends for their endless love and support.