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Zinc Distribution and Speciation within Rocket Plants (*Eruca vesicaria* L. *Cavalieri*) Grown on a Polluted Soil Amended with Compost as Determined by XRF Microtomography and Micro-XANES

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Zinc distribution and speciation within different organs (root, petiole, and leaf) of the edible plant *Eruca vesicaria* L. *Cavalieri* were determined using synchrotron microbeam X-ray techniques (XRF microtomography and μ-XANES) for plants grown in polluted soil with or without compost amendment. Data on soil derived from different extraction procedures and using μ-XANES analyses on rhizospheric soil indicated that compost amendment did not significantly influence the Zn speciation and availability in soil. However, major differences were observed within the plants. Plants grown in the presence of compost were able to partly block zinc immediately outside the root endodermis in the form of zinc-phytate, while a smaller Zn fraction was allowed to xylem transport as zinc-citrate. In the leaves, zinc was largely excluded from leaf cells, and about ~50% was in the form of phosphate precipitates, and the other 50% was complexed by cysteine and histidine residues. The reported data provide new information concerning the mechanisms of zinc tolerance in *E. vesicaria* L. *Cavalieri*, a very common edible plant in Mediterranean regions, and on the role of compost in influencing the molecular strategies involved in zinc uptake and detoxification.

KEYWORDS: Compost; *Eruca vesicaria* L. *Cavalieri*; micro-XANES; polluted soil; XRF microtomography; zinc

INTRODUCTION

Large amounts of metals in soil may be toxic in cultivated plants and can pose a serious risk for human health, especially in the case of vegetables intended for alimentary use. Organic matter (OM) amendments can modify metal bioavailability in contaminated soils, thus reducing the amount of metals that could be uptaken by plants. The effects of OM depend not only on the particular metal of concern and on the type of soil being treated but also on the OM characteristics with respect to its degree of humification, original metal content, pH, and its potential influence on soil physicochemical properties (structure, cation exchange capacity (CEC), porosity, water-holding capacity, etc.) and fertility.

The addition of highly humified OM in the form of compost can induce the formation of stable, insoluble complexes with humic substances that reduces metal mobility and availability for plant uptake (*1*). Compost is considered to be a good amendment agent for remediating metal-polluted soils (*2, 3*) and, at the same time, is known to enhance soil fertility by modifying soil chemical, physical, and biological properties (*4*).

Zinc, although an essential element for plants, when present at high concentrations in soil can cause toxicity effects on growing vegetables. One of the main routes of exposure to zinc for humans is via the consumption of vegetables. Agricultural soils can be contaminated by zinc, mainly as a result of sewage sludge disposal, application of fertilizers and pesticides, along with atmospheric depositions. Zinc is usually required at low levels in plants, and its average concentration in plant tissues ranges from 15 to 30 mg kg^{-1} (*5*). Many reports describe detrimental effects and phytotoxicity phenomena caused by excessive zinc concentrations in soil (*6–8*). Concentrations above 200 mg kg^{-1} may be considered to be toxic for most of the plants (*9*). Supra-optimal zinc concentrations may inhibit root elongation and shoot growth (*10*) and may reduce the rate of photosynthesis and leaf chlorophyll content (*11*).

To reduce the amount of zinc that can be uptaken by plants, soil can be amended with mature compost. However, such a

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Table 1. Soil Characteristics

physicochemical parameters		mineralogical composition[a]	
pH (H_2O)	7.25	illite	53%
pH (KCl)	5.95	kaolinite	21%
CEC	29.7 $cmol_{(+)}$ g^{-1}	illite/smectite	19%
organic carbon	3.2 g kg^{-1}	quartz	5%
total N	0.67 g kg^{-1}	plagioclase	1%
total Zn	114 mg kg^{-1}	hematite	1%
available P	17.5 mg kg^{-1}	calcite	traces
total Fe	46 g kg^{-1}		

[a] Determined by XRD following the method reported in ref *35*.

treatment can not only modify the bioavailability of the metal in soil but also influences the processes involved in metal uptake, translocation, and detoxification within the plant. In this research, plants of rocket (*Eruca vesicaria* L. *Cavalieri*), a common edible vegetable in Mediterranean regions, were used as a bioindicator to study the effects of compost amendment on zinc uptake, translocation, and accumulation forms within the plant. To reach this goal, two microanalytical tools based on the use of a microfocused synchrotron generated X-ray beam were employed: X-ray fluorescence (XRF) microtomography and micro-X-ray absorption near-edge structure spectroscopy (μ-XANES).

Most of the studies employing such analytical techniques have addressed the study of metal hyperaccumulating plants (*12–16*), in attempts to unravel as to which plant tissues store metals and in what chemical form. Most of the analytical approaches for determining the distribution and speciation of metals in plant organs are limited by low sensitivity as well as by considerable sample handling and pretreatment requirements that can potentially alter metal allocation and speciation. In addition, our knowledge on organic binding forms of metals in the rhizosphere and in plant tissues is mainly based on correlations of separate concentration measurements of organic ligands and metal ions. Synchrotron X-ray based techniques can overcome many of these limitations by providing direct, highly sensitive, spatially resolved information on the distribution of the metal as well as on its speciation within plant organs and only require a limited level of sample manipulation (*15*). Recent studies on the forms of zinc in different plant species have profited from the use of synchrotron generated X-rays to better understand the mechanisms of zinc accumulation and detoxification (*12, 17–20*).

In this research, XRF microtomography and conventional 2-D scanning μ-XRF were used to visualize the distribution of Zn within different plant organs (root, petiole, and leaf) of rocket plants grown on an artificially polluted soil, amended or not with compost. In addition, μ-XANES spectra were recorded from different plant parts and from the rhizospheric soil to determine the speciation of zinc at the root−soil interface and within the plant. All these data provide new and useful information concerning the role of compost in promoting and enhancing the mechanisms involved in Zn uptake and accumulation in *E. vesicaria* L. *Cavalieri* plants.

MATERIALS AND METHODS

Soil. In pot experiments, a silty clay loam material (USDA) originated from an Ap horizon of a Rhodoxeralf soil (USDA) from the area of Locorotondo (Bari, Italy) was selected. Soil physicochemical and mineralogical characteristics are reported in **Table 1**.

Soil Contamination. The soil, dry sieved at 2 mm, was artificially contaminated with a 0.1 M $ZnCl_2$ (Sigma Aldrich) solution to reach an average total zinc concentration of 820 mg kg^{-1}. The soil was then left to stabilize for 1 month. After this period, the soil was extensively washed with deionized water to eliminate the excesses of free zinc and chloride. The final total zinc concentration remaining in the soil after soil washing was determined by microwave assisted acid sample digestion and ICP-AES analysis (Tracescan, Thermo Jarrel Ash), following U.S. EPA method 3051. The determined final average concentration of zinc was 665 mg kg^{-1}.

Table 2. Compost Characteristics

pH (H_2O)	8.6	total N	20 g kg^{-1}
organic carbon	218 g kg^{-1}	total P	8.6 g kg^{-1}
organic matter	375.5 g kg^{-1}	total K	13.8 g kg^{-1}
H_2O	145 g kg^{-1}	Zn	245 mg kg^{-1}
ash	580 g kg^{-1}	Cu	134 mg kg^{-1}
humification index	0.2	Ni	29 mg kg^{-1}
C/N ratio	10.8	Cd	1.7 mg kg^{-1}
total Fe	11 g kg^{-1}		

Compost. The compost employed in this study was purchased from the Fertil Company (Bergamo, Italy) and was produced from green wastes in agreement with the European regulations (EU Ecolabel, 668/2001/CE). Some compost characteristics are reported in **Table 2**.

The compost clearly exhibited no phytotoxicity, as evidenced by seed germination and plant growth bioassays (data not shown). Prior to its application to soil, the compost was air-dried and ground to pass through a 1 mm sieve.

Soil Treatment. The artificially polluted soil was carefully homogenized and then distributed in 4 L plastic pots. Each pot was filled with 3 kg of soil.

Five pots were filled only with the polluted soil (no compost), while to other five pots also was added 210 g of compost (compost), corresponding to a hypothetical field amendment of 60 t ha^{-1}. The soil amended with compost was left to stabilize for 1 month before seeding.

In addition, five more pots were amended with a mineral fertilizer NPK (NPK: KNO_3, NH_4NO_3, and Na_2HPO_4), providing the following amount of elements: N (0.1 g kg^{-1}, corresponding to 97 kg ha^{-1}), P (0.05 g kg^{-1}, corresponding to 43 kg ha^{-1}), and K (0.09 g kg^{-1}, corresponding to 41 kg ha^{-1}).

Plant Growth. *E. vesicaria* L. *Cavalieri* seeds were purchased from Fuscello Sementi (Andria, Italy). Ten seeds of rocket were sown in each pot to ensure at least four sprouts per pot. Four plants were left in each pot after thinning at the first leaf stages, whereas all other sprouts were discarded, and therefore, 20 plants for each treatment were left. The experiment was carried out in a greenhouse, with natural light and a temperature range from 15 to 35 °C. During the growing cycle, pots were regularly watered with tap water to keep them close to the water field capacity. After 2 months, plants were harvested and prepared for further analyses.

Soil Analyses. Zinc distribution among operationally defined soil fractions was assessed by using the BCR standardized sequential (three-step) extraction procedure (*21*). According to this method, the first acid-soluble fraction (0.11 M acetic acid) comprises the water-soluble, exchangeable, carbonate-bound metal as well as soluble metallo-organic complexes with low bonding forces. The second fraction is reducible (0.1 M hydroxilamine chloride, pH 2) and is generally attributed to metals bound to iron and manganese oxides and hydroxides. The third step releases the oxidizable fraction (hydrogen peroxide at 85 °C and pH 2; 1 M ammonium acetate), which usually contains metals bound to organic matter and sulfide compounds. At the end, a residual fraction was extracted using aqua regia (*21*). Potentially bioavailable forms of zinc were evaluated by using 0.005 M diethylenetriaminepentaacetic acid (DTPA) (*22*). The reported results (**Table 3**) are the average of three replicate analyses performed on composite samples collected from five different pots for each soil treatment.

Plant Analyses. Before harvesting, the leaf chlorophyll content was assessed by using a portable chlorophyll meter (Minolta SPAD-502). The SPAD-502 determines the relative amount of chlorophyll present by measuring the absorbance of the leaf at two wavelengths in the red and near-infrared regions. Using these values, the meter calculates a numerical SPAD value (0–99.9) that is proportional to the amount of chlorophyll. All the measurements were taken in the morning on the first top three expanded leaves. Each reading was the mean of three measurements: three readings were taken for each leaf, three leaves

Table 3. Amount of Zinc (mg kg^{-1}) Extractable from Different Pools in Treated (Compost and NPK) and Untreated (No Compost) Soils According to the BCR Sequential Extraction Procedure and DTPA Method

	soil (BCR): [Zn] (mg kg^{-1})				DTPA
	acid-soluble	reducible	oxidizable	residual	extractable
no compost	230 ± 5	179 ± 6	30 ± 2	150 ± 17	191 ± 21
compost	227 ± 13	175 ± 18	30 ± 5	140 ± 3	199 ± 16
NPK	232 ± 8	175 ± 6	25 ± 3	143 ± 19	193 ± 12

per plant, and four plants per pot. After harvesting, plant growth parameters such as shoots and roots dry weight and shoots and roots length were determined.

In addition, five plants for each treatment, collected from different pots, were separated into roots and shoots, thoroughly washed with deionized water to remove soil particles, and oven-dried at 50 °C until a constant weight was achieved. Then, the shoots and roots were ground to a powder using a Retsch MM200 Mixer Mill and digested under reflux with HNO_3 and H_2O_2, and the resulting solutions (properly filtered and diluted) were finally analyzed to determine the zinc concentration using ICP-AES (Tracescan, Thermo Jarrel Ash).

The accumulation factor (AF) and translocation factor (TF) were calculated as follows:

$$AF = [Zn]_{plant}/[Zn]_{soil} \text{ and } TF = [Zn]_{shoots}/[Zn]_{roots} \quad (1)$$

Other plants were prepared for analyses with synchrotron radiation as described in the next section.

XRF Microtomography on Plant Samples. Plants to be analyzed by XRF microtomography were thoroughly washed with deionized water to completely remove the attached soil particles, divided into shoots and roots, and immediately frozen under liquid nitrogen. Subsequently, the plant parts were freeze-dried under vacuum (*15*). Shock freezing and drying were required because the high power density of the microfocused X-ray beam may cause motion associated with partial dehydration of fresh living plants, thus compromising the quality of the images obtainable by μ-XRF tomography. For tomographic analyses, the petioles were separated from the leaves, and part of the roots were excised to properly fit into a rotating stage. Only petioles and roots could be analyzed by tomography, while the leaves were analyzed by conventional 2-D scanning μ-XRF.

Microtomography experiments were carried out at Beamline L (HASYLAB, Hamburg, Germany) focusing the X-rays at 15–20 μm using a single bounce capillary (*23*) and using an energy of 13 keV. A multilayer monochromator was adopted for defining the energy of the monochromatic excitation. A Vortex-EX detector (Radiant Detector Technologies) was used to detect the fluorescence radiation. Roots and petioles were mounted on a goniometer head and rotated over 360° inside the beam with step increments of 3° and a collection time of 1 s per location. The resulting sinograms were then converted into the corresponding images of the petiole and root sections.

Two plants were analyzed for each soil treatment by XRF microtomography. Within each treatment, the plants showed similar elemental distribution maps. For each treatment, one representative set of elemental distribution images is reported.

***μ*-XANES Analyses on Soil and Plant Samples.** For μ-XANES measurements, fresh living plants were analyzed to preserve the chemical speciation of Zn within the plant. Problems connected to partial dehydration are less important for μ-XANES since the total time the sample is inside the X-ray beam is much shorter than in tomography and because no image reconstruction is needed. Small movements of the sample do not influence the analyses, provided that the analyzed microscopic area and its immediate surroundings are characterized by a homogeneous Zn speciation.

Plant samples were shipped to HASYLAB (DESY, Hamburg, Germany) in their pots and were removed from the soil just immediately before analysis. Before being placed in the X-ray beam, plants were thoroughly washed with deionized water, and all the remaining soil particles were carefully removed.

A few milligrams of rhizospheric soil was sampled using a sharp scalpel, by collecting the soil particles remaining attached to the root after vigorous shaking and included within 2 mm from the root surface (*24*). Rhizospheric soil was then homogeneously distributed over a metal-free adhesive tape for μ-XANES analyses. For each soil sample, three spectra were collected at different locations and then averaged. μ-XANES data collection was carried out using a Si (111) double crystal monochromator and a polycapillary lens (X-ray Optical System, Albany, NY) to focus the beam down to ca. 10–20 μm.

For plant analyses, spectra were collected from the middle part of the root, the main vein of the leaf, and areas included within the secondary veins of the leaf, comprising the parenchyma cells (henceforth called leaf cells). For each plant part, three spectra were collected and then averaged.

The energy was scanned through the absorption edge of Zn (9630–9850 eV) and was calibrated by recording the absorption edge of a zinc foil. μ-XANES spectra were collected in fluorescence yield mode using a Vortex-EX detector. Reduction of the XANES data was carried out using the WinXAS 3.1 software package (*25*). The edge spectrum collected from the plant material was fit using a least-squares algorithm to a linear combination (LC) of edge spectra from a library of Zn model compounds. The fractional contribution of each model spectrum to the fit is then directly proportional to the percentage of Zn present in that form in the plant material (*26*). The quality of the fit was estimated by calculating the residual $R = [\Sigma|y_{exp}(i) - y_{fit}(i)|/\Sigma|y_{exp}(i)|]100$ (*12, 27*). A lower R value represents a better match between the fitted model spectra and the experimental spectrum. The library of model compounds was experimentally created by collecting spectra for Zn compounds most likely to be present in plant samples (*12, 17, 19, 28, 29*).

Zinc standards were freshly prepared to have the following characteristics: free zinc solution, 7.0 mM $ZnCl_2$ solution, pH 7.0; zinc-malate solution, 7.0 mM $ZnCl_2$ + 70 mM malic acid, pH 6.5; zinc-oxalate solution (saturated), 7.0 mM $ZnCl_2$ + 70 mM oxalic acid, pH 7.0; zinc-citrate solution, 6.7 mM $ZnCl_2$ + 27 mM citric acid, pH 6.5; zinc-succinate solution, 7.0 mM $ZnCl_2$ + 70 mM succinic acid, pH 7.0; and zinc-histidine solution, 6.7 mM $ZnCl_2$ + 80 mM histidine, pH 7.0. Zinc-cysteine and zinc-glutathione spectra were digitized (*19*) for the following solutions: zinc-cysteine solution, 7.0 mM $ZnCl_2$ + 70 mM cysteine, pH 7.0; zinc-glutathione solution, 7.0 mM $ZnCl_2$ + 70 mM glutathione, pH 7.0. Zinc-sulfide (ZnS, 99% purity) and zinc-phosphate ($Zn_3(PO_4)_2$, 99.999% purity) were purchased from Sigma Aldrich as powders. Zinc-phytate was prepared by precipitation from 7.0 mM $ZnCl_2$ + 70 mM phytic acid solution (Sigma Aldrich), pH 7.0. The precipitate was then lyophilized. Standard solutions were wrapped in Ultralene foil (SPEX Certipure, Metuchen, NJ) and mounted for analysis on an aluminum support while the powder standards were ground, homogenized, and distributed on metal-free adhesive tape.

Table 4. Agronomical Parameters, Zinc Concentration, and Accumulation (AF) and Translocation Factors (TF) for Plants Grown without Soil Amendment (No Compost), Compost Amendment (Compost), and NPK Fertilization (NPK)

	dry wt (g)		length (cm)		chlorophyll content	[Zn] (mg kg^{-1})			indexes	
	roots	shoots	roots	shoots	(SPAD units)	roots	shoots	total	AF	TF
no compost	0.032 ± 0.017	0.10 ± 0.05	13.7 ± 2.3	6.3 ± 1.2	30 ± 8	460 ± 130	860 ± 230	720 ± 160	1.1	1.9
compost	0.51 ± 0.30	2.4 ± 1.5	21.5 ± 5.1	20.5 ± 5.4	53 ± 4	460 ± 80	530 ± 70	510 ± 70	0.8	1.2
NPK	0.35 ± 0.20	2.2 ± 1.5	20.3 ± 5.1	20.2 ± 6.6	46 ± 6	470 ± 90	690 ± 90	660 ± 80	1.0	1.5

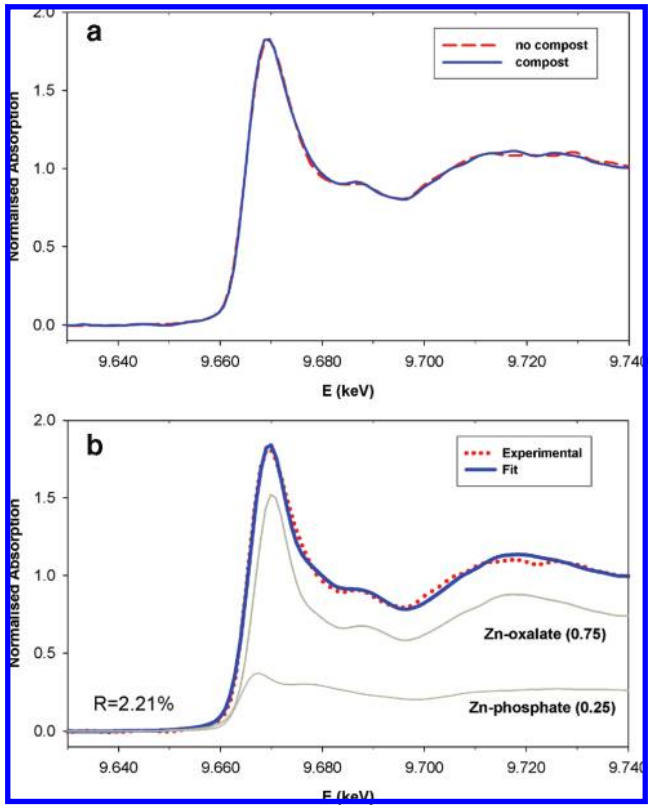

Figure 1. Zn K-edge μ-XANES spectra for the rhizospheric soil of the compost-treated (compost) and untreated (no compost) plants (**a**) and LC fit of the experimental data (**b**). The fractional contribution of the principal components making up the fitted spectra also is reported together with the evaluation parameter of the fit (*R*).

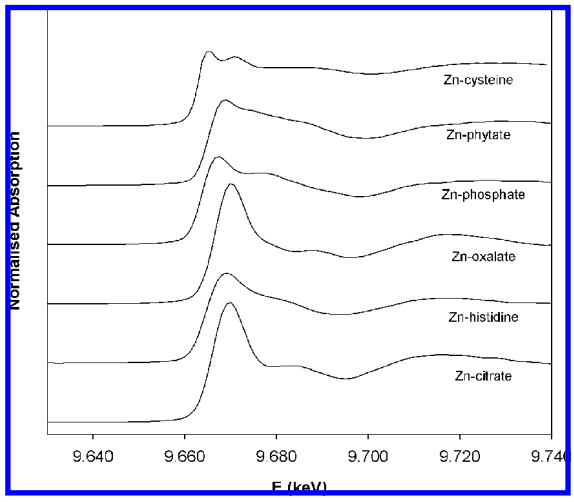

Figure 2. Zn K-edge spectra of zinc model compounds used in the fitting of the experimental data.

RESULTS

Zinc, although an essential element for plant growth, in excess amounts caused toxicity effects in plants grown in nonamended soil, inhibiting root and shoot growth and reducing the leaf chlorophyll content (**Table 4**). For the plants grown in soil amended with compost, the shoot and root length as well as biomass were remarkably higher (**Table 4**). Similar agronomical data as those observed for the compost amendment also were observed when an inorganic NPK fertilizer was added to soil. However, plants grown in the presence of compost showed a larger root system and more healthy leaves (as chlorophyll content) (**Table 4**).

As reported in **Table 4**, the total concentration of zinc in roots was identical for plants grown in compost-treated and untreated soil (460 mg kg^{-1}), while significant differences were observed for shoots (530 mg kg^{-1} in the compost as opposed to 860 mg kg^{-1} in the no compost samples). The plants grown in the soil amended with compost showed a significant lower zinc concentration in the aerial parts. For comparison, the zinc concentration in roots and shoots of plants grown in the same soil but not artificially polluted by zinc was similar for plants grown with or without compost and ranged from 30 to 50 mg kg^{-1}.

From our observations, the compost amendment did not seem to significantly affect zinc speciation and availability in bulk soil, at least as what could be assessed by using the BCR sequential extraction scheme and the DTPA extraction method. In fact, the amount of zinc distributed among the different BCR operationally defined fractions as well as the DTPA extractable fraction was very similar for the soil treated with compost or untreated (**Table 3**).

Nevertheless, the effect of compost on the growth and general health of the plants was clearly visible, as can be inferred from the data reported in **Table 4**. Therefore, these large differences must be derived from other factors (e.g., directly involving the plant rather than the behavior of zinc in the soil).

Another confirmation of this assumption is represented by the speciation of zinc determined by interpreting the μ-XANES spectra collected for the rhizosphere of compost-treated and untreated soil. As can be seen from **Figure 1a**, the two spectra are almost identical, suggesting the same speciation for zinc also in this soil fraction. μ-XANES spectra were interpreted by using linear combinations of spectra from selected Zn standards that are shown in **Figure 2**. By fitting the experimental data obtained for the rhizospheric soil with different combinations of these spectra, we found that the best fit was obtained by employing a linear combination of zinc-oxalate (75%) and zinc-phosphate (25%) (**Figure 1b**).

Also, NPK treatment did not seem to modify zinc speciation in soil (**Table 3**) and the total Zn concentration in roots (**Table 4**). However, the zinc concentration in shoots of plants grown with the NPK fertilization was higher than with the compost amendment. In addition, plants grown in the presence of compost showed the lowest degree of zinc accumulation (AF) and translocation to shoots (TF) (**Table 4**).

Significant differences between rocket plants grown on compost-treated and nonamended soils were observed in the data obtained by XRF microtomography and μ-XANES analyses on plants. For plant roots, different zinc distributions were imaged by XRF microtomography (**Figure 3a**). In plants grown in the presence of compost, a well-defined compartmentalization is visible, while in plants grown on the untreated soil, the zinc distribution is much more homogeneous. In particular, in the plants grown in the compost-amended soil, Zn is predominantly concentrated immediately outside the endodermis and inside the xylem vessels, while in the plants grown in the untreated soil, even if a higher concentration is still visible on the endodermis, the zinc distribution appears to be the same inside the stele as well as in the cortex. Also, the distribution observed for some other essential elements (K, Ca, and S) is much more heterogeneous in the plants grown with the compost amendment than in those grown without amendment; in the latter, these elements appear to be distributed predominantly outside the root. Iron, in both cases, is located only at the root surface (**Figure 4a**).

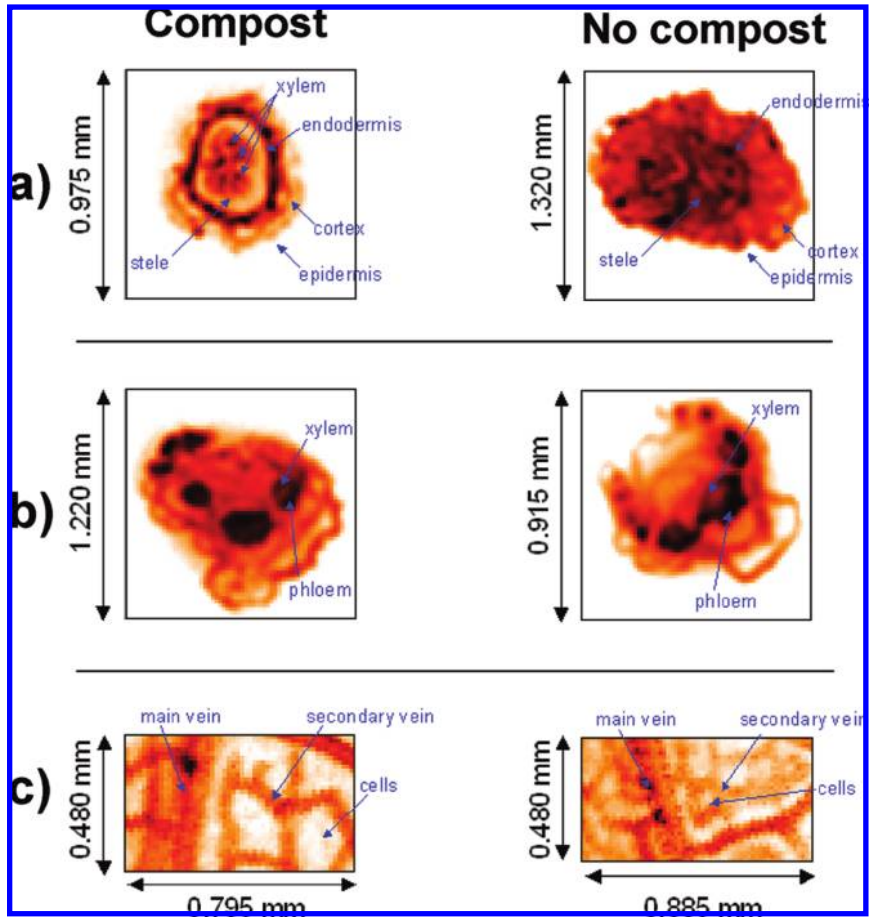

Figure 3. XRF microtomography zinc distribution in roots (**a**) and petioles (**b**) of plants grown in a polluted soil treated with compost (compost) or nonamended (no compost). Zinc distribution in leaves (**c**) was imaged by 2-D scanning micro-XRF. Darker pixels correspond to areas with a relatively higher zinc concentration.

Slight but still significant differences in zinc distribution also are visible in the petiole and in the leaf (**Figure 3b**,**c**). The zinc concentration in the xylem and in the phloem vessels of the petiole is similar for the plants grown with compost addition to soil, while those grown without compost show a lower concentration in the xylem vessels (**Figure 3b**). The other elements show an almost similar distribution with the exception of Ca and Fe, whose concentrations appear to be higher in the petioles of the plants grown without compost amendment (**Figure 4b**).

In the leaf of the plants grown with compost amendment, zinc is highly concentrated in the main vein as well as in the secondary veins, while its concentration is very low in the area corresponding to the leaf cells. On the other hand, the zinc distribution is less defined in the secondary veins of the leaves of plants grown in the untreated soil since the concentration in the cells is also quite high, and therefore, a more diffuse Zn distribution is apparent (**Figure 3c**). As for other elements (K, Ca, Fe, S, and Cl), their distribution is similar for both plants with the exception of sulfur that, in general, appears to be more concentrated in the untreated plants (**Figure 4c**). However, areas within the leaf characterized by a higher density or thickness can provide a stronger XRF signal, thus suggesting apparent higher concentrations.

Information on the chemical forms in which zinc is present in the plants was extracted using Zn μ-XANES. Within the plant, μ-XANES spectra were collected from the middle part of the root, the main vein of the leaf, and the leaf cells. The spectra recorded from the different plant parts appear to be different from one another, suggesting a marked variability in Zn speciation also among the different parts of the same plant (**Figure 5a**,**b**). In addition, the speciation of zinc determined for plants grown in the soil amended with compost was significantly different from that of plants grown in the non-amended soil. As for the root, zinc was found in the form of zinc-phosphate (66%) and zinc-oxalate (34%) in the plants grown in the untreated soil (**Figure 6a**), while its speciation was completely different in those grown in compost-treated soils: zinc-phytate (76%) and zinc-citrate (24%) (**Figure 6b**). Zinc forms were also different in the leaf. In the leaf cells, fitting of μ-XANES data to reference spectra showed the presence of zinc-phosphate (57%) and zinc-oxalate (43%) in the plants grown without amendment (**Figure 7a**), whereas zinc-phosphate (54%) together with zinc-cysteine (25%) and zinc-histidine (21%) contributed to the spectrum collected for the plants grown on the compost-treated soil (**Figure 7b**). The spectra collected on the main vein of the leaves also differ between the treated and the untreated specimens, even though they hardly could be interpreted on the basis of the model compounds we used in this study. Probably, other chemical forms of zinc, at present difficult to contemplate on the basis of the available literature, should be taken into consideration to correctly fit these experimental data.

DISCUSSION

From our results, soil amendment with compost did not appear to influence the amount of potentially available zinc in soil as

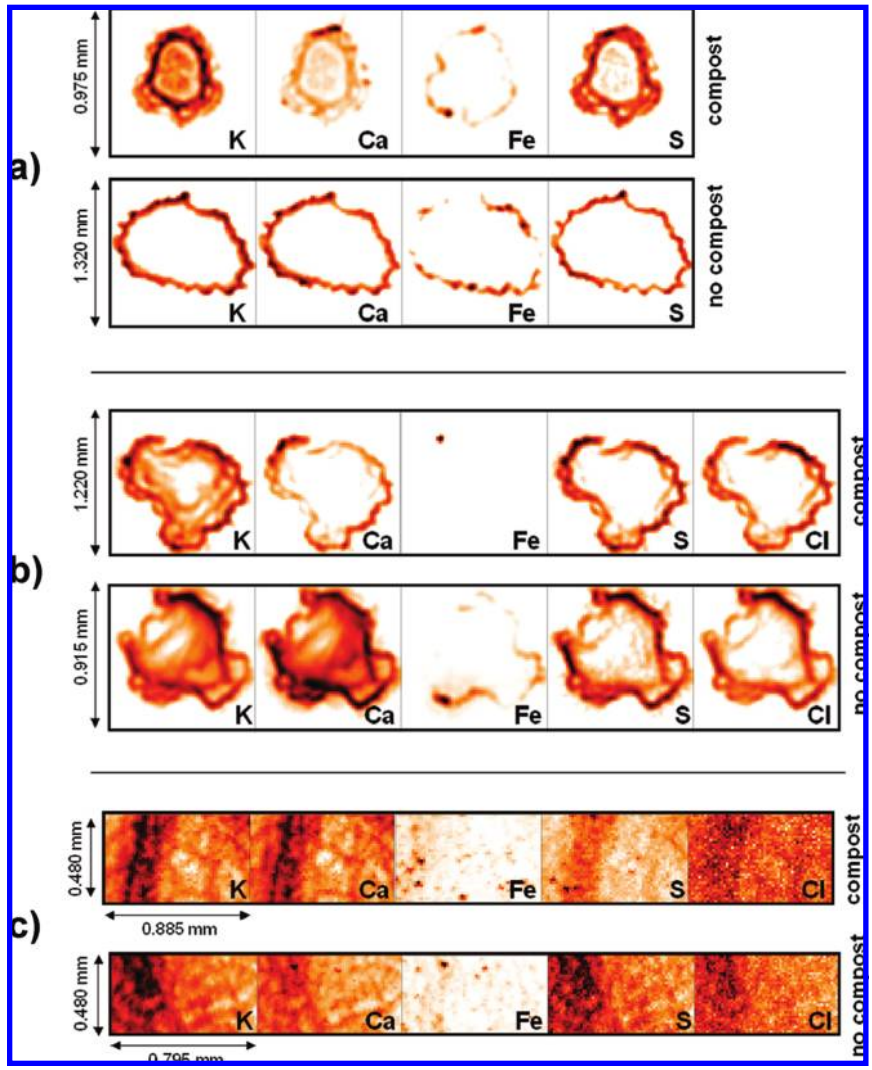

Figure 4. XRF microtomography distribution of K, Ca, Fe, S, and Cl in roots (**a**) and petioles (**b**) of plants grown in a polluted soil treated with compost (compost) or nonamended (no compost). Elemental distribution in leaves (**c**) was imaged by 2-D scanning micro-XRF. Darker pixels correspond to areas with a relatively higher zinc concentration.

much as zinc uptake and translocation by plants. It cannot be excluded that, in the case of the compost amended soil, the extraction procedures adopted may have overestimated the amount of available zinc. In fact, the addition of compost to soil caused an increase in the soil pH from 7.3 to 7.8. This increase could have partly reduced the amount of zinc in solution available for the uptake by the treated plants. Nevertheless, the amount of Zn that was found in the root was exactly the same for the treated and untreated plants (**Table 4**). Therefore, as will be discussed later, it is at the root level rather than in the soil that the major differences were observed.

It is rather evident that compost strongly influenced plant growth by providing important elements for plant nutrition, thus helping the plant to fully develop and put into action several biochemical mechanisms to efficiently resist zinc toxicity. These mechanisms may involve processes such as preventing zinc from entering the stele, reducing Zn translocation to shoots, restricting Zn access inside leaf cells, and stabilizing the metal inside the cells by forming less toxic complexes with organic molecules. On the contrary, plants grown without any amendment showed clear symptoms of toxicity.

In soil, high zinc concentrations can lead to the formation of zinc-phosphate precipitates, thus reducing the amount of available phosphorus. As a consequence of phosphorus deficiency, dicotyledonous plants can release in the rhizosphere root exudates (e.g., oxalic acid) that can acidify the soil in the proximity of the root and form stable complexes with zinc and remobilize phosphorus (*30*). In agreement with these processes, both in the compost-treated and in the untreated rhizospheric soils, we found a lower amount of zinc still in the form of insoluble zinc-phosphate (25%), whereas most of it was complexed as zinc-oxalate (75%). The presence of other zinc mineral forms, such as Zn sorbed on phyllosilicates or iron-oxides, in the rhizospheric soil cannot be excluded. However, in a mixture, it is difficult to ascertain by XANES the presence of components whose contribution is lower than 15%. In addition, plants can strongly alter Zn speciation in soils and sediments, even mobilizing Zn from very stable minerals [e.g., ZnS (*31*)]. The type of clay minerals composing the soil adopted in this study (mainly illite and kaolinite, see **Table 1**) are characterized by a limited cation exchange capacity as well as the fact that iron-oxides are mainly amorphous (only 1% of crystalline hematite was detected in soil, against 6.5% of Fe_2O_3) and therefore easily dissolved by chelating agents such as oxalate.

Data from XRF microtomography showed that, at the root level, plants grown in the polluted soil without amendment evidenced a reduced capability to restrict zinc access toward

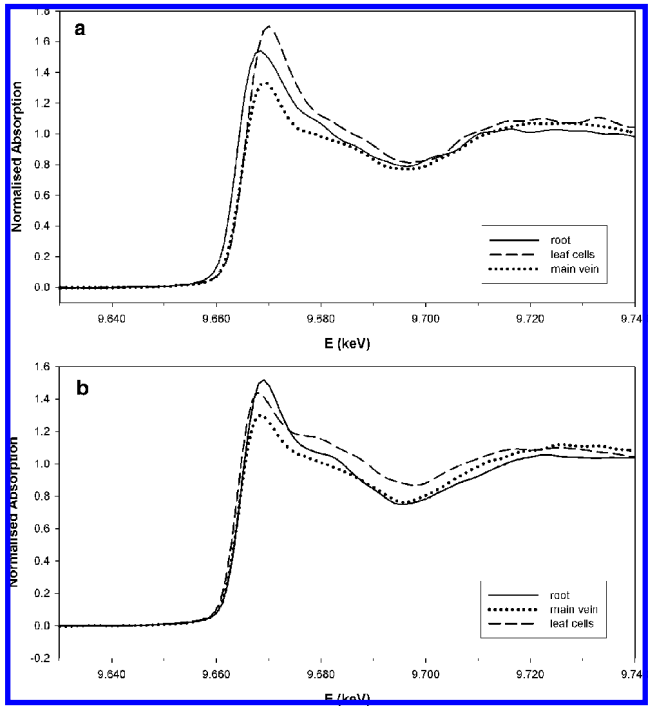

Figure 5. μ-XANES Zn K-edge spectra collected in different parts (root, main vein, and leaf cells) of plants grown in a zinc-polluted soil (no compost) (**a**) or in the polluted soil treated with compost (compost) (**b**).

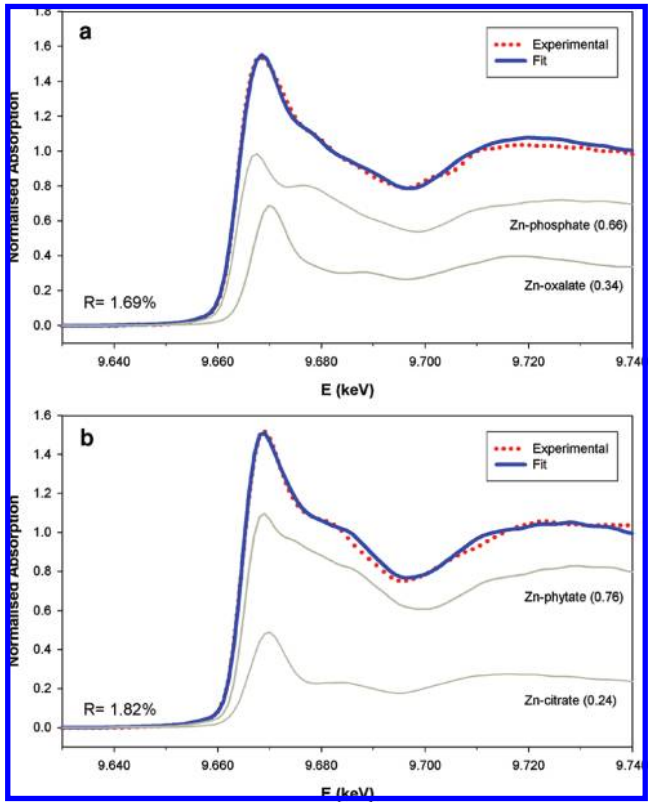

Figure 6. Results of the fitting of Zn K-edge μ-X-ray absorption spectra of roots of plants grown in the nonamended soil (**a**) or in the soil treated with compost (**b**). The fractional contribution of the principal components making up the fitted spectra also is reported together with the evaluation parameter of the fit (*R*).

the xylem sap and therefore toward the aerial parts. In fact, in these plants, zinc is uniformly distributed inside the root, and

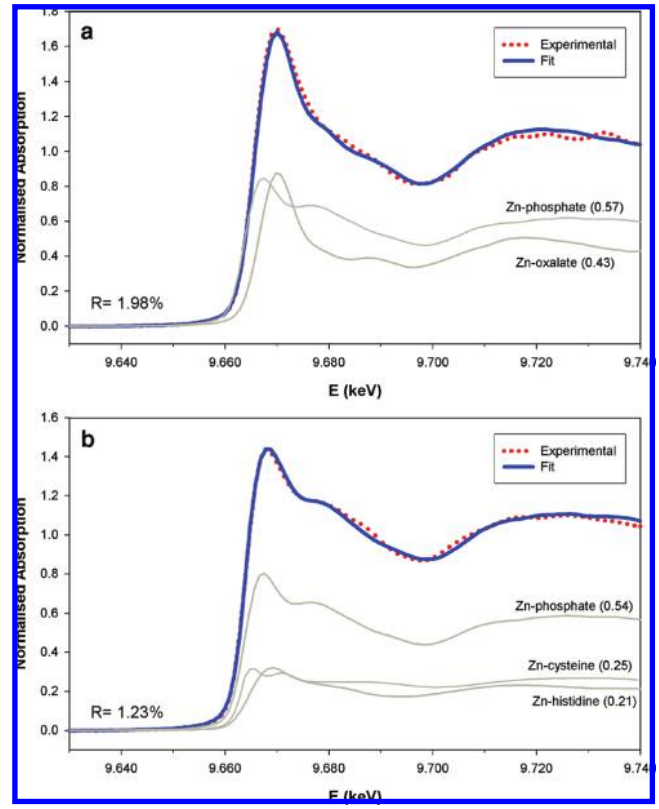

Figure 7. Results of the fitting of Zn K-edge μ-X-ray absorption spectra collected on the leaf cells area of plants grown in the nonamended soil (**a**) or in the soil treated with compost (**b**). The fractional contribution of the principal components making up the fitted spectra also is reported together with the evaluation parameter of the fit (*R*).

the same degree of accumulation is visible in the stele as well as in the cortex. This observation probably implies that zinc exclusion mechanisms were no longer working, as also suggested by the higher AF and TF (**Table 4**). In addition, chemical detoxification processes were likely to be altered since zinc in the root was almost in the same forms as in soil, even though different abundances of the species (the phosphate form in the root was higher: 66%). Further, nutrients such as K, Ca, and S appeared to be hardly uptaken and translocated by the plant. On the other hand, in plants grown in the compost-treated soil, zinc was partly blocked just outside the stele, most probably in the form of zinc-phytate and, for a lower fraction, was allowed to xylem transport as zinc-citrate.

The formation of zinc-phosphate or zinc-phytate precipitates are well-known processes for zinc immobilization in roots and leaves (*18*). From a chemical point of view, phytate (myo-inositol kis-hexaphosphate) is a more complex phosphate-containing molecule than inorganic phosphate. Küpper et al. (*32*) observed precipitates attributed to zinc-phosphate on the outer cell walls of the root of the hyperaccumulator *Arabidopsis halleri*. Differently, in *E. vesicaria* L. *Cavalieri*, zinc-phosphate or -phytate precipitates accumulated inside the root, and in plants grown in the presence of compost, zinc-phytate was concentrated just outside the endodermis. Similar observations were reported by Van Steveninck et al. (*28*) for different dicotyledoneous edible plants (radish, cabbage, soybean, lupins, etc.). Our data confirm the hypothesis made by Van Steveninck et al. (*28*) that the formation of insoluble zinc-phytate that deposits in the specialized cells of the endodermis can function as an effective screen against the transport of excessive Zn to the shoots.

Citrate has been found also in other plants [e.g., *Thlaspi caerulescens* (*17*)] to complex zinc in the xylem sap during its translocation to the shoots. In general, chelation with low molecular weight organic acids is a common mechanism for metal transportation within the plant vascular system. The presence of zinc-oxalate rather than zinc-citrate complexes in the root of the untreated plant possibly could be ascribed to a different complexing strategy by using a simpler molecule, as might have occurred also in the case of phosphate. In conditions of stress, under nutritional deficiency and reduced photosynthetic activity (as observed by the reduced chlorophyll content in plants grown in the untreated soil), plants may synthesize simpler molecules to save carbon atoms to be used in primary vital processes rather than in secondary defensive pathways. In this sense, compost amendment might have favored the formation of more complex and effective metal-immobilizing agents such as phytate and citrate. In addition, in the plants grown in the compost-treated soil, the distribution observed for K, Ca, and S inside the root seems to indicate that nutrients were normally uptaken and translocated by the plant.

With compost addition, several important nutrients are provided to the plant, including large amounts of N, P, and K (**Table 2**). It has been suggested that an increase in plant available P may have some relationship to an observed decrease in the amount of Zn in plants (*33*). In fact, deficiencies in P or other essential nutrients may result in the breakdown of plant metabolic processes and in the integrity of root uptake mechanisms (*33*). As a confirmation of this, rocket plants grown on the same soil amended with an inorganic NPK fertilizer showed very good growth, almost in the same way as with compost amendment. However, the plants grown in the compost-amended soil presented a better root development, a slightly higher chlorophyll content, and, in particular, lower accumulation and translocation factors. Therefore, compost, in comparison with NPK treatment, besides providing important nutrients also might have improved soil physical characteristics (e.g., structure and porosity), thus enhancing root growth and penetration into the soil, and influenced the processes responsible for Zn uptake and translocation to shoots, better promoting exclusion detoxification strategies.

Plants have developed different tolerance strategies to grow in soils rich in potentially toxic metals. A large number of them are called excluders since they are able to restrict root uptake and, in particular, root-to-shoot translocation of metals. Some plants, on the other hand, have developed a strategy to accumulate toxic metals in the aboveground parts and are called hyperaccumulators. In hyperaccumulator plants, at the basis of a number of detoxification strategies, there is the translocation process of metals from the root, where they are uptaken from soil, up to the leaves, where they can be stored and accumulated in harmless chemical forms, mostly in the vacuoles of leaf cells. On the basis of the data presented in this paper, rocket plants seem to follow an exclusion strategy.

As a further confirmation of this, the higher concentration of zinc in the leaf veins as opposed to the very low concentration observed in the leaf cells area of the plants grown in the presence of compost suggests that, similarly as in the root, zinc was somehow excluded from accessing the cells. In addition, in the leaf cells, zinc was precipitated for ca. 50% as phosphate and, for the other 50%, complexed by cysteine and histidine. Zinc-phosphate has been found also in the leaves of *Phaseolus vulgaris* (*18*), while histidine is considered to be an ideal chelator for zinc at the pH values found in cytoplasm (*17*). Cysteine residues as well as S-containing proteins and molecules (e.g., phytochelatins) often have been advocated to complex metals in plants, but even if zinc has been shown to induce the synthesis of phytochelatins, direct evidence of the formation of such complexes had not been demonstrated yet (*18, 19*).

Differently from the hyperaccumulating plants *T. caerulescens* (*34*) and *A. halleri* (*29*), from our data it seems that *E. vesicaria* L. *Cavalieri* prefers to detoxify zinc in the leaves by forming zinc-phosphate precipitates and complexes with S- and N-containing ligands rather than with organic acids (e.g., citric acid).

As for the leaves of the plants grown in the nonamended soil, zinc appears to be diffusely distributed also inside the cell area, thus suggesting a reduced capacity of the plant to exclude high amounts of zinc from entering the cells. Also, zinc speciation is different from that of the plants grown on the compost-treated soil: zinc was found again in the same simple chemical forms as in the roots. No complexation with more complex molecules such as histidine and cysteine was observed.

In *T. caerulescens*, it was found (*34*) that Zn accumulated predominantly in the epidermal cells of mature leaves, while the mesophyll cells appeared to have lower concentrations of zinc. However, the scanning 2-D μ-XRF technique used in the present study to visualize zinc distribution in leaves cannot discriminate if zinc accumulated in the epidermal cells or in the mesophyll since the high penetration capacity of X-rays into matter provides information from the full sample depth.

In general, toxicity caused by the high amount of zinc in the untreated plants altered primary metabolic processes (e.g., photosynthesis), thus leading to a reduced growth and development of the plant, and inhibited a number of metal exclusion/detoxification mechanisms that, on the other hand, markedly have been expressed in the compost-treated plants.

All the reported data provide new information on the mechanisms of zinc tolerance of *E. vesicaria* L. *Cavalieri*, a largely diffuse edible plant in Mediterranean regions, and on the role of compost in influencing the mechanisms involved in zinc uptake and detoxification. From our results, it can be concluded that compost amendment did not appear to significantly influence the amount of potentially available zinc in soil. However, major differences were observed within the plants. Compost, by providing the plants with important elements for nutrition, allowed the plant to fully develop and to activate several biochemical mechanisms to efficiently resist zinc toxicity. In particular, even if the Zn concentration in the roots was exactly the same for plants grown in the soil amended with compost or in the nonamended soil, plants grown in the presence of compost were able to partly block zinc immediately outside the endodermis in the form of zinc-phytate and, for a lower fraction, to allow zinc to xylem transport as zinc-citrate. Also, in the leaves, zinc mostly was excluded from accessing the leaf cells and was ca. 50% precipitated as phosphate and 50% complexed by cysteine and histidine residues. In the plants grown on the nonamended soil, all these mechanisms were no longer operative, and only very simple zinc complexes were detected within the plant (mainly phosphates and oxalates).

Therefore, compost amendment, also in comparison to inorganic NPK fertilization, seems to promote exclusion processes in rocket plants to better tolerate high zinc concentrations in soil. The excluder disposition of this plant also was confirmed by the different mechanisms adopted by the plant in comparison with the strategies adopted by hyperaccumulating plants. However, most of the data available in literature dealing with detoxification and hyperaccumulating strategies in plants are based on plant models growing under hydroponic conditions

and not on real soils, as was reported in this study. The proposed analytical approach also can be adopted to study the distribution and speciation of metals, either as pollutants or as micronutrients, within many other plant species including hyperaccumulators or plants of particular interest as food sources.

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