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Food chain

Antioxidant responses of edible and model plant species subjected to subtoxic zinc concentrations

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ABSTRACT

Zinc (Zn) is a common heavy metal in polluted soils, as it is a widespread pollutant deriving both from natural sources and anthropogenic activities. The antioxidant tolerance/defence mechanisms against oxidative stress induced by subtoxic concentrations of Zn (50 and 150 μM $ZnSO_4$) were studied in a widespread edible plant (lettuce; *Lactuca sativa* L.) and in an important model plant (*Arabidopsis thaliana* (L.) Heynh.). After 10 days (Arabidopsis) and 20 days (lettuce) of Zn exposure, Zn uptake/translocation was evaluated in both roots and shoots, while indicators of oxidative stress and stress intensity, total antioxidant capacity, and enzymatic and non-enzymatic antioxidative defence were measured in leaves. From an overall comparison of the two species, Zn root uptake in Arabidopsis subjected to 50 and 150 μM $ZnSO_4$ was approximately 3- and 5-fold lower than in lettuce, while Zn translocation from roots to apical leaves was more efficient in Arabidopsis (23.7 vs 21.3% at 50 μM $ZnSO_4$ and 19.3 vs 12.9% at 150 μM $ZnSO_4$). Generally, a higher degree of Zn-induced oxidative stress (863.8 vs 21.3 μg g^{-1} FW H_2O_2 and 1.33 vs 0.75 μM g^{-1} FW MDA_{eq} at 150 μM $ZnSO_4$) and antioxidant response (441.2 vs 258.5 mM g^{-1} FW TEAC and 91.0 vs 54.9% RSA at 150 μM $ZnSO_4$) were found in lettuce. The aim of this study is understanding (a) if subtoxic Zn levels can affect Zn uptake and translocation in the studied species and (b) if this eventual Zn absorption can influence plant oxidative status/antioxidant response. Considering that soil contamination by Zn can affect crop production and quality, the results of this research could be important for environmental, nutritional and human health issues.

1. Introduction

Zinc (Zn) is a common heavy metal in soil and is essential for plant nutrition being part of numerous biomolecules and acting as cofactor in many enzymatic processes [1]. However, in soil subjected to high anthropogenic pressure, Zn and other heavy metals may be present at concentrations exceeding the background values and induce, as a consequence, potential stress to plants and other living organisms [2]. Some plants use avoidance strategies to reduce Zn assimilation, while others adopt internal defence mechanisms to cope with the excessive Zn levels in tissues [3,4]. Zinc homeostatic concentrations in plant tissues range from 30 to 200 μg g^{-1} dry weight (DW) [5]. Conversely, concentrations higher than 300–400 μg g^{-1} DW may induce stress effects in plant species [5], such as growth inhibition, alteration of mineral and chlorophyll contents, and disruption of enzyme activities [6,7]. The excessive Zn accumulation may cause also the abnormal production of reactive oxygen species (ROS), which are responsible for the oxidative stress [8,9]. The accumulation of ROS leads to lipid peroxidation, protein oxidation, membrane and DNA/RNA damage, and metabolism imbalance [10]. Together with ROS production, plants exposed to Zn often activate an antioxidant response aimed to scavenge ROS and reduce antioxidant damage. Some of these defences are enzymatic (such as superoxide dismutase, catalase, peroxidases and enzymes of the ascorbate-glutathione cycle) while other ones are non-enzymatic (e.g., ascorbate, glutathione and tocopherols) [6,8,11,12]. These responses can already be observed at low levels of phytotoxicity (subtoxic concentrations) before visible symptoms become evident [13,14], therefore they may be used as physiological and metabolic biomarkers of early stress indicators [13].

Strategies adopted by plants to cope with Zn excess in soil should be

Abbreviations: APX, ascorbate peroxidase; Arabidopsis, *Arabidopsis thaliana*; ASA, reduced ascorbate; ASC, total ascorbate; CAT, catalase; CAE, chlorogenic equivalents; Chl*a*, chlorophyll *a*; Chl*b*, chlorophyll *b*; DHA, dehydroascorbate (oxidised ascorbate); DW, dry weight; FW, fresh weight; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSS, Goxidized glutathione; MDA_{eq}, malondialdehyde equivalents; PAL, phenylalanine ammonia lyase; POD, peroxidase; RSA, radical scavenging activity; ROS, reactive oxygen species; SOD, superoxide dismutase; TEAC, Trolox equivalent antioxidant capacity; TGSH, total glutathione; Zn, zinc

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investigated under subtoxic concentrations of the metal in the soil, namely in a more realistic situation than many *in vitro* experiments done on plants [13,15]. Physiological, biochemical and molecular mechanisms that allow certain plants to grow in Zn-contaminated soils are nowadays still poorly understood [3,13,16]. Therefore, this study aims to understand (a) if subtoxic Zn levels commonly found in metal-polluted soils affect Zn uptake and translocation in an edible plant (lettuce; *Lactuca sativa* L.) and a model plant (*Arabidopsis thaliana* (L.) Heynh.), and (b) if this eventual Zn absorption influences plant oxidative status/antioxidant response. The study on a model plant can allow to better understand the basic mechanisms of antioxidant defences, as Arabidopsis is genetically and biochemically well-known and thus comparable with other plant species [5]. On the other side, lettuce is agronomically important for food purposes and human health, due to its polyphenolic profile, antioxidant capacity and mineral composition [17].

Zinc accumulation in edible parts of plants grown in Zn-rich soils is of paramount importance for medical issues. Human body has no specialized Zn storage system [18], thus a daily dietary intake of Zn is required to maintain the normal cellular metabolism. Zinc is essential for growth, development and reproduction as it is required for the catalytic activity of approximately 100 enzymes, and plays a role in immune function, protein synthesis, wound healing, DNA synthesis and cell division [19]. However, Zn daily intake for an adult should not exceed 40 mg day^{-1} [20], thus high Zn content in vegetable products and fruits may be harmful to humans. On the other side, defence mechanisms adopted by Zn-polluted plants could increase the amounts of plant phenolics or other antioxidants, that act as free radical scavengers and protectants against oxidative stress in human body [13,21].

2. Materials and methods

2.1. Experimental design and plant material

Seeds of *Arabidopsis thaliana* (L.) Heynh. (Columbia ecotype; Col-0) were sterilized by soaking in 50% (v/v) ethanol for 5 min followed by 5 min of 1% Na-hypochlorite, and finally rinsed with sterile distilled water. After 24-h imbibition on moist filter paper at 4 °C in the dark, seeds were placed in polyethylene containers (36 seeds per container) filled with sterilized sand (mean particle diameter = 0.25 mm, in order to allow an efficacious and rapid root extraction from the sand, without tissue damage), and moistened with 300 mL of one-quarter strength Hoagland liquid medium. Throughout the experiment, the Hoagland solution was continuously replaced in order to maintain a constant volume of 300 mL and to keep the roots moistened. Seedlings were grown in a growth chamber at 20 °C and relative humidity of 65%, with a 16-h photoperiod and an irradiance (in terms of photosynthetic photon flux density) of 300 μmol $m^{-2} s^{-1}$ at leaf level. One-week-old seedlings were subsequently exposed to 50 and 150 μM $ZnSO_4$, simulating the concentrations possibly present in soils contaminated by Zn [2,5]. Plants were sampled 10 days after Zn application. All the experimental determinations were repeated at least three times. All the reagents used in this experimental work were purchased from Sigma-Aldrich S.r.l., Milan, Italy.

In the experiment with lettuce (*Lactuca sativa* L., var. capitata), seeds of a red-leaf type (*Four Seasons*) were germinated in vermiculite for 16 days. After germination, the seedlings were planted on perforated polystyrene plates, floating on an aerated Hoagland nutrient solution, which was renewed every week. The plants were grown hydroponically for 21 days in a growth chamber at 20–22 °C, relative humidity of 65%, and 12-h photoperiod with an irradiance of 250 μmol $m^{-2} s^{-1}$. After that, plants were treated with nutrient solutions containing different Zn concentrations: 0 μM (control), 50 and 150 μM, supplied as Zn sulphate ($ZnSO_4 \cdot 7H_2O$). Plants were sampled at 20 days after Zn application.

For both the species, at the end of the period of Zn exposure, fresh weights were recorded on some plants randomly collected, and samples of apical leaves were immediately frozen in liquid nitrogen and then used for the following determinations.

2.2. Zinc content

Aliquots of shoot and root samples were digested in a HNO_3:H_2O_2 solution (5:1 v/v) using a high-performance microwave digestion unit (MLS-1200 Mega, Milestone Inc., CT, USA). Zinc concentration was determined by means of quadrupole inductively coupled plasma mass spectrometry, ICP-QMS (Elan DRC II, Perkin-Elmer SCIEX, CT, USA). High purity He (99.9999%) and H_2 (99.9995%) were used, in order to minimize the potential problems caused by unidentified reactive contaminant species in the cell. Blanks and a Zn standard stock solution at the concentration of 50 mg L^{-1} were analyzed for reference purposes. Reagent blanks containing ultra-pure water were additionally analyzed in order to control the purity of the reagents and the laboratory equipment. All glassware was soaked overnight in 10% ultra-pure grade HNO_3 and then rinsed copiously with ultra-pure water before use. Results were expressed as μg of Zn for g of plant DW. Dry weights were determined after drying plant tissues at 105 °C for 48 h.

2.3. Lipid peroxidation and H_2O_2

The level of lipid peroxidation was determined spectrophotometrically using a Jasco V-530 UV–vis spectrophotometer (Jasco Corp., Tokyo, Japan) in terms of thiobarbituric acid-reactive substances (TBARS) concentration as described by Hodges et al. [22]. Leaf samples of 0.5 g were homogenized in 4 mL of 1% (w/v) trichloroacetic acid (TCA), and then centrifuged at 10,000*g* for 10 min. To a 1.5 mL aliquot of the supernatant, 1.5 mL of 20% (w/v) TCA containing 0.5% (w/v) TBA was added. The mixture was heated at 95 °C for 30 min and then quickly cooled in an ice bath. After centrifugation at 10,000*g* for 5 min, the values of absorbance measured at 532, 600 and 440 nm of the supernatant were recorded. The value for a specific absorption at 600 nm was subtracted and a standard curve of sucrose (from 2.5 to 10 μmol mL^{-1}) was used to correct the results from the interference of soluble sugars in samples. The concentration of TBARS was calculated using an absorption coefficient of 156 $mmol^{-1} cm^{-1}$, and the results expressed as MDA equivalents (MDA_{eq}).

The determination of H_2O_2 was carried out according to the method of Lee and Lee [23] with some modifications. A 0.5 g aliquot of leaves was homogenized in 2 mL of 100 mM sodium-phosphate buffer, pH 6.8. The homogenate was filtered through three layers of cheesecloth and then centrifuged at 20,000*g* for 15 min at 4 °C. A 0.5 mL aliquot of supernatant was mixed with 2.5 mL of peroxide reagent, containing of 83 mM sodium phosphate, pH 7.0, 0.005% (w/v) o-dianisidine, and 40 μg mL^{-1} peroxidase. The mixture was thermostated at 30 °C for 10 min in a water bath and then the reaction was stopped by adding 0.5 mL of 1 N $HClO_4$. After centrifugation at 10,000*g* for 5 min, the absorbance at 436 nm of the supernatant was compared to the extinction of a H_2O_2 standard.

2.4. Chlorophyll and carotenoids

Pigment extraction was carried out at 4 °C in dark conditions. Samples of leaves (25 mg) were homogenized in 1.5 mL of 80% acetone (v/v) using mortar and pestle. The extracts were centrifuged in sealed tubes at 15,000*g* for 5 min.

The supernatant was collected and the absorbance was read at 663 and 647 nm for chlorophyll *a* (Chl*a*) and chlorophyll *b* (Chl*b*), respectively, and at 470 nm for carotenoid content. The concentrations of total chlorophyll (Chl*a* + Chl*b*), and the total carotenoids (xanthophylls + carotenes) were calculated according to the equations of Lichtenthaler and Buschmann [24].

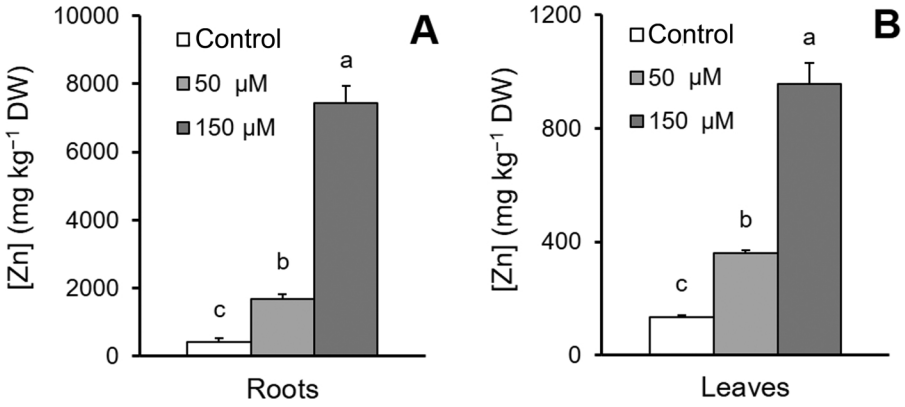

Fig. 1. Concentration of Zn in (A) roots and (B) leaves of lettuce after 20 days of exposure to two Zn concentrations (50 µM, light grey columns, and 150 µM, dark grey columns) and in leaves of control plants without Zn addition (white columns). Mean values (n = 25) ± standard deviation with different lower-case letters are significantly different ($P \leq 0.05$) between the three treatments.

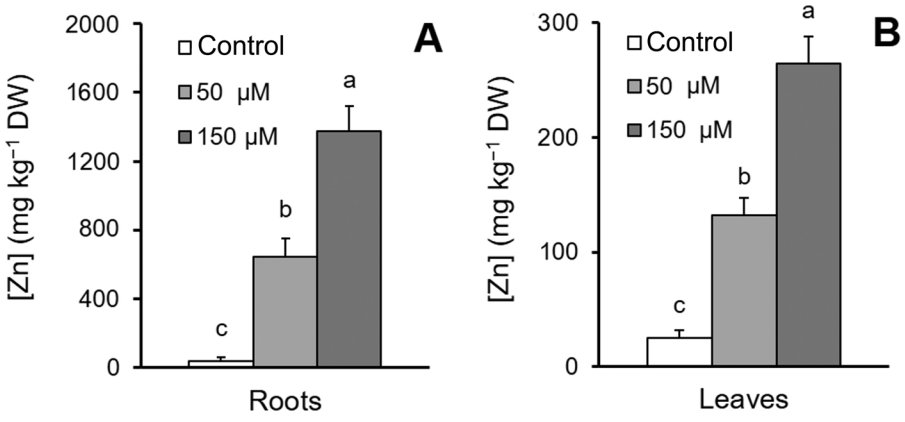

Fig. 2. Concentration of Zn in (A) roots and (B) leaves of Arabidopsis after 10 days of exposure to two Zn concentrations (50 µM, light grey columns, and 150 µM, dark grey columns) and in leaves of control plants without Zn addition (white columns). Mean values (n = 25) ± standard deviation with different lower-case letters are significantly different ($P \leq 0.05$) between the three treatments.

2.5. Anthocyanins, total phenols and PAL activity

Aliquots of milled leaves (2.5 g) were shaken in 15 mL of acidified methanol (1% HCl) for 2 h at room temperature in the dark and then centrifuged at 1000 × *g* for 15 min.

Anthocyanin levels in the methanolic extracts were calculated with the formula [A530 − (0.24 × A653)] [25], where A530 and A653 are the absorbances determined at 530 nm and 653 nm, respectively. Total anthocyanin content was determined as µmol cyaniding 3-glucoside equivalents (CGE) per g of fresh weight (FW).

Total phenolic content was determined by the Folin-Ciocalteu colorimetric method using chlorogenic acid as a standard. Each reaction mixture contained 20 µL of 1:1 sample extract:distilled water and 100 µL of freshly prepared 4% Na_2CO_3 solution. The sample was vortexed and let stand for 5 min. Successively, 20 µL of Folin-Ciocalteu reagent were added and, after 30 min, absorbance was measured at 750 nm. Total phenolic content was expressed as mg chlorogenic equivalents (CAE) per g of FW.

Phenylalanine ammonia lyase (PAL) was extracted and its activity measured spectrophotometrically at 275 nm, according to [26]. One unit of PAL deaminates 1.0 mmol of L-tyrosine to *p*-coumarate and NH_3 h^{-1}.

2.6. Tocopherols

For the determination of α-, γ- and δ-tocopherol, fresh samples of leaves (0.5 g) were homogenized in 4 mL of ethanol, and then the mixture was centrifuged at 10,000*g* for 5 min at 4 °C. The supernatant was recovered and filtered through a Whatman no. 1 filter paper, and added with 5 mL of *n*-hexane. Tocopherols were extracted twice in the hexane phase, and the collected extract dried under a stream of liquid nitrogen. The dried extract was solubilised in 1 mL of HPLC-quality methanol. The quantification was carried out according to Miller et al. [27] by detection using a Shimadzu Nexera HPLC system (Shimadzu, Kyoto, Japan). A Hypersil ODS C_{18} reverse phase column (5-mm particle size, 250 mm × 4.6 ID Themohypersil GmbH, Germany) fitted with a C_{18} guard column was used. As mobile phase, methanol:acetonitrile:chloroform (47:42:11 v/v) was adopted at a flow rate of 1 mL min^{-1}. Total tocopherols were calculated by summing the single contributions of α-, γ- and δ-tocopherol.

2.7. Total antioxidant activity assays

The Trolox Equivalent Antioxidant Capacity (TEAC), based on the scavenging of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical (ABTS•) converting it into a colourless product, was determined and calculated according to Arts et al. [28].

The ability of the sample extracts to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured as described previously [29], with some modifications. Aliquots (100 µL) of extracts were added to 3 mL of DPPH solution (6×10^{-5} mol L^{-1}) and the absorbance was measured at 515 nm every 5 min until the steady state. Percent radical scavenging activity (% RSA) of samples was determined, while dimethyl sulfoxide (DMSO) was used as control, using the following formula: % RSA = 100 − [Absorbance of test compounds/Absorbance of control × 100].

2.8. Extraction and activity of antioxidant enzymes

2.8.1. SOD, POD, CAT

All procedures for enzyme extraction and determination of enzyme activities were carried out at 0 °C on ice bath unless otherwise stated. Leaf samples were prepared for the analyses by homogenizing 0.5 g of fresh leaves in 4 mL of ice cold 50 mM K-phosphate buffer (pH 7.0) containing 2 mM Na-EDTA and 1% (w/v) polyvinyl-polypirrolidone (PVPP). The homogenate was centrifuged at 10,000*g* at 4 °C for 10 min.

Table 1
Oxidative stress parameters measured in leaves of lettuce after 20 days of exposure to two Zn concentrations (50 and 150 µM Zn) and in leaves of control plants without Zn addition. Mean values ($n = 10$) ± standard deviation with different lower-case letters are significantly different ($P \leq 0.05$) between the three treatments. Light grey shading indicates a significant increase ($P \leq 0.05$) and dark grey shading a significant decrease ($P \leq 0.05$) in relation to the control plants.

Parameter	Unit	Control	50 µM Zn	150 µM Zn
Growth indicators				
FW of shoots	g plant^{-1}	46.0 ± 2.7 **a**	35.5 ± 4.7 **b**	8.1 ± 1.7 **c**
Oxidative stress indicators				
H_2O_2	µg g^{-1} FW	176.7 ± 43.7 **b**	296.8 ± 62.4 **b**	863.8 ± 160.2 **a**
MDA_{eq}	µM g^{-1} FW	0.37 ± 0.02 **b**	0.40 ± 0.03 **b**	1.33 ± 0.20 **a**
Stress intensity indicators				
Chl*a*	µmol g^{-1} FW	0.53 ± 0.01 **ab**	0.58 ± 0.02 **a**	0.48 ± 0.04 **b**
Chl*b*	µmol g^{-1} FW	0.13 ± 0.01 **a**	0.16 ± 0.02 **a**	0.13 ± 0.01 **a**
Ratio Chl*a*/Chl*b*		4.1 **a**	3.7 **a**	3.7 **a**
Total Chl	µmol g^{-1} FW	0.66 ± 0.02 **ab**	0.74 ± 0.03 **a**	0.61 ± 0.05 **b**
Anthocyanins	µmol g^{-1} FW	0.04 ± 0.00 **b**	0.12 ± 0.03 **ab**	0.22 ± 0.08 **a**
Total phenols	mg g^{-1} FW	0.12 ± 0.03 **b**	0.30 ± 0.12 **b**	1.00 ± 0.27 **a**
PAL	U g^{-1} protein^{-1}	0.23 ± 0.04 **b**	0.28 ± 0.04 **b**	0.56 ± 0.13 **a**
Antioxidant defense				
Antioxidant enzymes				
SOD	U g^{-1} protein^{-1}	117.9 ± 24.5 **b**	122.1 ± 24.0 **b**	365.8 ± 55.4 **a**
CAT	U g^{-1} protein^{-1}	0.22 ± 0.06 **a**	0.10 ± 0.02 **b**	0.27 ± 0.07 **a**
POD	U g^{-1} protein^{-1}	0.31 ± 0.03 **b**	0.34 ± 0.01 **b**	1.51 ± 0.10 **a**
APX	U g^{-1} protein^{-1}	0.87 ± 0.08 **b**	1.14 ± 0.10 **b**	4.45 ± 0.38 **a**
GPX	U g^{-1} protein^{-1}	82.7 ± 2.1 **b**	126.0 ± 6.6 **a**	124.1 ± 8.4 **a**
GR	U g^{-1} protein^{-1}	0.13 ± 0.01 **a**	0.09 ± 0.01 **b**	0.04 ± 0.01 **c**
Antioxidant metabolites				
ASA	µmol g^{-1} FW	0.64 ± 0.07 **b**	0.90 ± 0.08 **b**	1.47 ± 0.26 **a**
DHA	µmol g^{-1} FW	0.27 ± 0.01 **c**	0.65 ± 0.10 **b**	1.41 ± 0.22 **a**
ASC	µmol g^{-1} FW	0.91 ± 0.21 **b**	1.55 ± 0.56 **a**	2.89 ± 0.37 **a**
% reduced ASC	%	69.2 ± 3.3 **a**	58.1 ± 4.3 **b**	50.9 ± 5.2 **b**
GSH	µmol g^{-1} FW	0.22 ± 0.04 **b**	0.34 ± 0.08 **b**	1.97 ± 0.35 **a**
GSSG	µmol g^{-1} FW	0.31 ± 0.05 **a**	0.28 ± 0.05 **a**	0.33 ± 0.06 **a**
TGSH	µmol g^{-1} FW	0.53 ± 0.10 **b**	0.62 ± 0.12 **b**	2.31 ± 0.22 **a**
% reduced TGSH	%	41.6 ± 1.3 **b**	54.8 ± 15.8 **b**	85.3 ± 11.7 **a**
Total antioxidant capacity				
TEAC	mM g^{-1} FW	121.3 ± 19.0 **b**	247.8 ± 73.0 **b**	441.2 ± 92.7 **a**
% RSA	%	32.9 ± 0.4 **c**	54.3 ± 11.1 **b**	91.0 ± 0.2 **a**

The supernatant was used for determining the activities of superoxide dismutase (SOD; EC 1.15.1.1), peroxidase (POD; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6), according to Sorkheh et al. [30] with some modifications. For the measurement of SOD activity, the reaction mixture (3 mL) contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 4 mM riboflavin, 0.1 mM EDTA, and 0.25 mL enzyme extract. The test tubes were shaken then placed in a light box consisting of six fluorescent lamps (15 W preheat, daylight 6500 °K; light intensity = 900 µmol m^{-2} s^{-1}). The reaction was based on the reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin in light, and it was stopped by switching off the light and placing the test tubes into dark. One unit of enzyme activity was determined as the amount of the enzyme to reach an inhibition of 50% NBT reduction measured at 560 nm. For CAT, the decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The reaction was initiated by adding enzyme extract to 3 mL of reaction mixture containing 50 mM K-phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µL of enzyme extract. CAT activity was determined as the consumption of H_2O_2 at 240 nm over a 2-min interval. For POD, the activity was determined by measuring the oxidation of guaiacol in the presence of H_2O_2, and following an increase in absorbance at 470 nm over a 2-min interval. The assay mixture (3 mL) contained 0.33 mM guaiacol, 10 mM K-phosphate buffer (pH 7.0), and 50 µL of enzyme extract. The reaction was initiated by adding 40 mM H_2O_2. All the enzyme activities were expressed on protein basis.

2.8.2. APX, MDHAR, DHAR, GPX, GR

An aliquot of 1 g of leaves was homogenized in 10 mL of 50 mM K-phosphate buffer, pH 7.6, containing 1% (w/v) polyvinylpolypyrrolidone (PVPP) and 1 mM Na-EDTA. Only for the estimation of the activities of ascorbate peroxidase (APX; EC 1.11.1.11) and dehydroascorbate reductase (DHAR, EC 1.8.5.1), 1 mM ascorbate and 2 mM 2-mercaptoethanol were added to the homogenizing buffer to prevent inactivation of the respective enzymes. The homogenate was filtered through three layers of cheesecloth (Miracloth) and then centrifuged at 15,000*g* for 30 min, at 4 °C.

The obtained supernatant was recovered, desalted on a Sephadex™ G–25 M column and subjected to the determination of the activities of APX, monodehydroascorbate reductase (MDHAR, EC 1.6.5.4), DHAR, glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione reductase (GR, EC 1.6.4.2), according to Sofo et al. [31] with some modifications. The activities of APX and GPX were measured spectrophotometrically

Table 2
Oxidative stress parameters measured in leaves of Arabidopsis after 10 days of exposure two Zn concentrations (50 and 150 µM Zn) and in leaves of control plants without Zn addition. Mean values ($n = 10$) ± standard deviation with different lower-case letters are significantly different ($P \leq 0.05$) between the three treatments. Light grey shading indicates a significant increase ($P \leq 0.05$) and dark grey shading a significant decrease ($P \leq 0.05$) in relation to the control plants.

Parameter	Unit	Control	50 µM Zn	150 µM Zn
Growth indicators				
FW of shoots	mg plant^{-1}	14.5 ± 5.4 **a**	14.0 ± 3.2 **a**	13.6 ± 4.7 **a**
FW of roots	mg plant^{-1}	3.5 ± 1.0 **a**	3.0 ± 0.5 **a**	3.1 ± 0.5 **a**
Oxidative stress indicators				
H_2O_2	µg g^{-1} FW	71.0 ± 13.3 **a**	79.4 ± 20.0 **a**	130.3 ± 36.9 **b**
MDA_{eq}	µM g^{-1} FW	0.25 ± 0.05 **a**	0.31 ± 0.06 **a**	0.75 ± 0.14 **b**
Stress intensity indicators				
Chl*a*	µmol g^{-1} FW	0.30 ± 0.05 **a**	0.38 ± 0.10 **a**	0.38 ± 0.07 **a**
Chl*b*	µmol g^{-1} FW	0.08 ± 0.03 **a**	0.09 ± 0.02 **a**	0.10 ± 0.05 **a**
Ratio Chl*a*/Chl*b*		3.9 **a**	4.1 **a**	3.9 **a**
Total Chl	µmol g^{-1} FW	0.38 ± 0.04 **a**	0.47 ± 0.05 **a**	0.47 ± 0.10 **a**
Carotenoids	µg g^{-1} FW	1.15 ± 0.23 **c**	1.47 ± 0.07 **b**	1.94 ± 0.45 **a**
Anthocyanins	µmol g^{-1} FW	0.07 ± 0.01 **c**	0.20 ± 0.07 **b**	0.34 ± 0.09 **a**
Tocopherols	µg g^{-1} FW	0.32 ± 0.06 **b**	0.47 ± 0.03 **a**	0.52 ± 0.12 **a**
Total phenols	mg g^{-1} FW	0.44 ± 0.08 **a**	0.25 ± 0.08 **b**	0.26 ± 0.07 **b**
Antioxidant defense				
Antioxidant enzymes				
SOD	U g^{-1} protein^{-1}	66.7 ± 11.7 **b**	148.3 ± 33.6 **a**	168.8 ± 20.9 **a**
CAT	U g^{-1} protein^{-1}	0.10 ± 0.02 **b**	0.09 ± 0.02 **b**	0.17 ± 0.05 **a**
POD	U g^{-1} protein^{-1}	0.20 ± 0.05 **a**	0.23 ± 0.04 **a**	0.39 ± 0.18 **a**
APX	U g^{-1} protein^{-1}	0.40 ± 0.06 **a**	0.54 ± 0.12 **a**	0.45 ± 0.09 **a**
Antioxidant metabolites				
ASA	µmol g^{-1} FW	0.38 ± 0.10 **c**	0.60 ± 0.10 **b**	1.30 ± 0.31 **a**
DHA	µmol g^{-1} FW	0.15 ± 0.09 **c**	0.47 ± 0.05 **b**	1.04 ± 0.32 **a**
ASC	µmol g^{-1} FW	0.53 ± 0.12 **c**	1.07 ± 0.56 **b**	2.34 ± 0.37 **a**
% reduced ASC	%	71.7 ± 8.4 **a**	56.0 ± 12.0 **b**	55.6 ± 13.2 **b**
GSH	µmol g^{-1} FW	0.46 ± 0.09 **c**	0.70 ± 0.13 **b**	1.42 ± 0.25 **a**
GSSG	µmol g^{-1} FW	0.59 ± 0.10 **a**	0.62 ± 0.16 **a**	0.64 ± 0.15 **a**
TGSH	µmol g^{-1} FW	1.05 ± 0.23 **b**	1.32 ± 0.29 **b**	2.04 ± 0.45 **a**
% reduced TGSH	%	44.8 ± 5.1 **b**	53.0 ± 4.5 **b**	69.6 ± 7.6 **a**
Total antioxidant capacity				
TEAC	mM g^{-1} FW	85.2 ± 8.2 **c**	132.1 ± 24.7 **b**	258.5 ± 56.6 **a**
% RSA	%	20.4 ± 4.7 **c**	32.5 ± 4.1 **b**	54.9 ± 3.7 **a**

by recording the decrease in ascorbate content at 290 nm. A 1.0 mL aliquot of enzyme extract was treated with 100 µL of 10 mM H_2O_2 and 100 µL of 80 mM hydroxylamine, a selective inhibitor of APX, for 5 min. The same procedure, but with 200 µL distilled water and without hydroxylamine, was used to determine total (APX + GPX) activity in a second 1.0 mL aliquot of the same extract. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 500 µM ascorbate, 100 µM EDTA, 100 µM H_2O_2 and 100 µL of enzyme extract, in a final volume of 3.0 mL. The reaction started with the addition of H_2O_2 and an absorption coefficient of 2.8 mM^{-1} cm^{-1} was used for calculations. One unit of APX activity was defined as the amount of enzyme that oxidizes 1 µmol of ascorbate per min at 20 °C, while one unit of GPX was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per min at 25 °C. The activity of MDHAR was determined by following the decrease in absorbance at 340 nm due to NADH oxidation. A 1.0 mL aliquot of enzyme extract was added to a reaction mixture containing 50 mM potassium phosphate, pH 7.6, 0.3 mM NADH and 2.5 mM AsA. The reaction was started by adding AsA oxidase (EC 1.10.3.3) (Sigma-Aldrich A0157) to produce monodehydroascorbate (MDHA) and an absorption coefficient of 6.2 mM^{-1} cm^{-1} was used for calculations. One unit of MDHAR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADH per min at 25 °C. The activity of DHAR was determined by monitoring the increase in absorbance at 265 nm due to AsA production. A 1.0 mL aliquot of enzyme extract was added to a reaction mixture containing 50 mM K-P buffer, pH 6.5, 0.1 mM EDTA, 0.5 mM DHA, and 2.5 mM GSH. The non-enzymatic reduction of DHA by GSH was subtracted. An absorption coefficient of 14.6 mM^{-1} cm^{-1} was used for calculations. One unit of DHAR activity was defined as the amount of enzyme that produces 1 nmol of AsA per min at 25 °C. The activity of GR was measured by following the decrease in absorbance at 340 nm due to NADPH oxidation. A 200 µL aliquot of enzyme extract was added to a reaction mixture containing 1.5 mL of 0.1 M K-P buffer, pH 7, 150 µL of 20 mM GSSG, 1 mL of distilled water and 150 µL of 2 mM NADPH (dissolved in Tris-HCl buffer, pH 7), in a final volume of 3.0 mL. An absorption coefficient of 6.2 mM^{-1} cm^{-1} was used for calculations. One unit of GR activity was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per min at 25 °C.

2.8.3. Total protein

The activity of each antioxidant enzyme was expressed on protein basis. For total protein determination, leaves (0.5 g) were ground with 10 mL of 50 mM cooled K-phosphate buffer (pH 7.8) placed in an ice bath. The homogenate was centrifuged at 6000 × *g* for 20 min at 4 °C and the soluble proteins content of the extract was determined by the Coomassie blue dye binding method, using bovine serum albumin as a standard.

2.9. Ascorbate and glutathione forms

A 0.5 g aliquot of leaves was homogenized in 1.0 mL of ice-cold 2.5 N $HClO_4$. The homogenate was filtered through three layers of cheesecloth (Miracloth) and then centrifuged at 15,000*g* for 5 min. The supernatant was pH-adjusted with 5 M K_2CO_3 up to pH 4.5 for ascorbate determination and to pH 6.5 for glutathione determination.

Ascorbic acid was measured spectrophotometrically by reading absorbance at 265 nm due to ascorbate oxidation by ascorbate oxidase, according to Foyer et al. [32]. The concentration of dehydroascorbate (DHA) was calculated as the difference between ASC and reduced ascorbate (ASA). The levels of glutathione were measured spectrophotometrically by monitoring the reduction of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 412 nm, once applied the method of Griffith [33]. The concentration of reduced glutathione (GSH) was calculated as the difference between total glutathione (TGSH) and reduced glutathione (GSSG).

2.10. Statistics

The statistical analysis was performed by Sigmastat 3.1 SPSS Inc. software (SPSS Inc., Quarry Bay, Hong Kong). One-way analysis of variance (ANOVA) for each parameter was carried out with metal treatment as factor. Means were statistically analyzed by Tukey HSD Test at $P \leq 0.05$. The number of replicates (n) for each measured parameter is specified in the table and figure captions.

3. Results and discussion

3.1. Zinc uptake and translocation

In lettuce roots, Zn accumulation was 4.0 and 17.8 times higher for plants growing under 50 μM and 150 μM Zn, respectively, compared to the control (Fig. 1A). In lettuce plants exposed to 50 μM Zn, the concentration of this element in leaves increased 2.6 times in relation to the control (plants grown in the absence of Zn), while for plants exposed to 150 μM Zn, the increase was 7-fold higher (Fig. 1B). Zinc uptake and translocation in Arabidopsis resulted to be more efficient than in lettuce, as root Zn in plants exposed to 50 and 150 μM Zn was 21.3- and 45.6-fold higher, compared to the control, and foliar Zn was 5.2- and 13.0-fold higher, compared to the control, respectively (Fig. 2).

Considering the absolute values of Zn concentration in plant tissues, Zn accumulation in Arabidopsis was much lower than in lettuce. Indeed, Zn root level in Arabidopsis grown at 50 and 150 μM $ZnSO_4$ was approximately 3- and 5-fold lower than that of lettuce, respectively, and Zn foliar level in Arabidopsis was approximately 3- and 3.5-fold lower than that measured in lettuce (Figs. 1 and 2). For both species, Zn was accumulated more in roots than in shoots, similarly to what found by Barrameda-Medina et al. [34]. Finally, Zn translocation from roots to apical leaves was more efficient in Arabidopsis (23.7 vs 21.3% at 50 μM $ZnSO_4$, and 19.3 vs 12.9% at 150 μM $ZnSO_4$, considering a value of 100% Zn in roots), as depicted in the graphical abstract.

3.2. Growth and oxidative stress

The results of the different parameters involved in the antioxidant defence mechanism of lettuce and Arabidopsis leaves are reported in Tables 1 and 2, respectively. Results revealed strong significant reduction in FW of lettuce leaves at both Zn concentrations, in agreement to the reduced size of Zn-treated plants, compared to control plants. At 150 μM Zn, lettuce leaves appeared very small and withered. The reduction in biomass (Table 1) is a common effect of metal toxicity, and similar results were observed by Barrameda-Medina et al. [34] in other lettuce cultivars treated with higher Zn concentrations (up to 500 μM). Arabidopsis growth was not significantly affected by the two Zn treatments (Table 2), similarly to what observed by Sofo et al. [13] in plants exposed to 100–200 μM Zn in agar medium. This result may be ascribed to the lower levels of Zn accumulated in Arabidopsis tissues (Table 2).

The levels of H_2O_2 and MDA_{eq} were significantly higher than the control only in lettuce (Table 1) and Arabidopsis (Table 2) plants exposed to 150 μM Zn, revealing that this Zn concentration was able to induce oxidative stress. Conversely, the lower concentration of Zn was not sufficient to cause oxidative stress in both the species (Tables 1 and 2) likely because, as Zn is an essential element for plants, relatively higher concentrations are needed to provoke oxidative stress. Studies on *Brassica rapa* plants also revealed increased levels of H_2O_2 and MDA but at much higher concentrations of Zn (500 μM) [7].

Visible signs of reddening of leaves characterized lettuce plants exposed to 150 μM Zn. This reddening was not only due to the decrease in total Chl (Table 1), that is also an important indicator of metal stress in plants [35], but mainly to significantly increased levels of anthocyanins in plants grown at 150 μM Zn (Table 1), confirming that the synthesis of these phenolics is a response to Zn-induced stress. The synthesis and accumulation of anthocyanins and other phenols containing conjugated double bonds, able to protect cell macromolecules from oxidative stress, is known to occur in lettuce [21]. Although anthocyanins have been reported to be involved in detoxification mechanisms against heavy metal induced stress [36], the increase in anthocyanin content is probably plant-specific, as studies in *Cistus monspeliensis*, a species highly tolerant to Zn, showed a decrease in the content of these pigments at Zn concentrations up to 2000 μM [37]. The same decrease in anthocyanins was recently observed in olive plants subjected to airborne metal pollution [35]. Concomitantly with anthocyanin increase, lettuce plants treated with the highest Zn dose presented the highest PAL activity (Table 1). This enzyme catalyses the biosynthesis of the phenylpropanoid skeleton in higher plants (i.e., the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia), being involved in the biosynthesis of phenols, that were significantly higher at 150 μM Zn, compared to control (Table 1).

Shoot colour in Arabidopsis turned from light green in the control to reddish-green in Zn-exposed seedlings, as also observed by Sofo et al. [14] in Arabidopsis seedling exposed to 150 μM Zn for 12 days. This physiological response was likely due to the significant increase in anthocyanins and carotenoids (both also acting as ROS-protectant pigments) at 50 and 150 μM Zn (Table 2), as chlorophyll parameters did not show any significant changes (Table 2). In Arabidopsis, phenolics and tocopherols, known to be scavengers of ROS and lipid radicals [30], had two opposite trends, as tocopherols significantly increased in the presence of added Zn, while total phenols decreased (Table 2). The decrease of phenols in Zn-treated Arabidopsis could be due to their secretion outside the roots, where, according to Mourato et al. [8], they can act as metal chelators and/or directly as ROS scavengers.

3.3. Enzymatic and non-enzymatic antioxidant response

The activities of antioxidant enzymes confirm the Zn-related oxidative stress, as a significant increase in SOD, POD, APX and GPX occurred in lettuce plants exposed to the higher Zn dose (Table 1). However, the stimulation of plant antioxidative system was not enough to compensate the increase in ROS. The observed increase in SOD is a commonly detected effect of heavy-metal induced oxidative stress [8,12], which leads to the production of H_2O_2, that is then scavenged by the activities of other enzymes, such as POD, APX and GPX, but not

CAT. In a study with other lettuce cultivars, a decrease in SOD activity was observed at 500 μM Zn [34]. In the present study, it appeared that CAT in lettuce was not involved in the antioxidant defence at the highest Zn level but other enzymes, like POD, were (Table 1). Thus, the observed increase in total phenolic compounds in 150 μM Zn-treated lettuce plants (Table 1) may be useful to maintain the activity of this important enzyme, although phenolics may also play a direct role in the antioxidative machinery of lettuce [11]. Moreover, APX activity that uses ASA as a substrate for detoxifying H_2O_2, appeared significantly increased in plants treated with the highest Zn concentration, and this intense activity may be maintained by the higher ASA levels measured in these plants, as observed by Sbartai et al. [38] in Zn-stressed tomato. Generally, the enzymatic antioxidant mechanism of lettuce was not significantly induced at 50 μM Zn, except for GPX activity (Table 1). Nevertheless, the increase of GPX activity possibly balanced the reduced CAT activity in 50 μM Zn-treated plants (Table 1). Most likely, the observed increase in DHA (oxidised ascorbate) in 50 μM Zn-treated plants (Table 1), and the corresponding decrease in percentage of reduced ASC, allowed to compensate the lower levels of Zn-induced oxidative stress. At 150 μM Zn, a general increase in ASA and GSH was measured in lettuce (Table 1), proving that the full range of enzymatic and non-enzymatic defence was activated by this higher Zn concentration. It thus seems that ASA is a first line of defence of lettuce plants against Zn-induced oxidative stress, followed by the activation of the enzymatic system when this line is overwhelmed by a more intense stress, as observed by Cuypers et al. [6]. In contrast with the other antioxidant enzymes, GR activity appeared to be significantly depressed in metal-exposed lettuce plants both at 50 and 150 μM Zn (Table 1). Therefore, the observed GSH increase (Table 1) should be caused partially by GR activity, enough for reducing GSSG, but likely also by the depolymerization of Zn-induced phytochelatins [14].

In Arabidopsis, the antioxidant enzymatic system appeared less enhanced than in lettuce. The activity of SOD increased in the presence of Zn at both the used concentrations (Table 2), proving that SOD is a key-enzyme to hinder the oxidative stress in Arabidospsis. Also CAT activity was enhanced by the highest Zn concentration. The ascorbate and glutathione pools were significantly activated in the presence of Zn, particularly at concentrations of 150 μM (Table 2), in accordance with outcomes reported by Remans et al. [39]. Data obtained confirmed that, also in Arabidopsis, the non-enzymatic antioxidant system is the first line of defence against oxidative stress.

At 50 μM Zn, a moderate increase in the antioxidant capacity (TEAC) was detected only in Arabidopsis, compared to the control, whereas, at 150 μM Zn, a strong significant increase in TEAC was detected in both the species (Tables 1 and 2). This was expected, as it was accompanied by general increase in ASA and GSH (Tables 1 and 2). On the other side, % RSA in lettuce leaves, being a more sensitive method than TEAC [29], was significantly higher at both Zn concentrations, compared to control plants (Table 1), so demonstrating the presence in lettuce of a certain degree of oxidative stress already at 50 μM Zn. Also, in Arabidopsis the value of% RSA at both Zn concentrations resulted significantly higher than the control (Table 2), especially at the highest Zn dose.

4. Conclusion

From an overall comparison of the two species, lettuce resulted to be a better Zn accumulator and, at the same time, a species with well-developed antioxidant response, compared to Arabidopsis. It appears clear that Zn-induced oxidative stress, particularly at the highest Zn concentration (150 μM) affected the metabolism of both lettuce and Arabidopsis, decreasing growth (only in lettuce) and enhancing antioxidant defences in both the species. This is particularly important for lettuce, as it is primarily consumed as whole heads or fresh-cut product and its worldwide consumption has steadily increased in the last decades [13]. Considering an average DW/FW ratio of 10 ($n = 25$) of lettuce shoots and the maximum recommended dietary Zn intake level for humans (40 mg day^{-1}), it can be concluded that approximately 400 g (for plants grown at 150 μM Zn) and 1 kg (for plants grown at 50 μM Zn) of lettuce fresh leaves could be eaten every day, that is a conspicuous amount for a vegetable. The dietary Zn intake for lettuce could be also supplemented/compensated by the higher amounts of ROS-scavenging antioxidants found in Zn-treated plants.

Conflict of interest

None.

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