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Copper and Zinc Accumulation in Young Leaves of *Eruca sativa* (L.) Grown in Soilless Culture

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Abstract: Heavy metals are environmental pollutants that cause toxicity in plants and represent a risk for human health, linked to bioaccumulation through the food chain. However, excess accumulation may not occur in young plants in the early stages of exposure to the toxic element. In the present work, rocket (*Eruca sativa* L.) plants grown in hydroponics were exposed for three weeks to excess concentrations (25, 50, or 100 µM) of Cu or Zn in the nutrient solution and were more sensitive to Cu than Zn toxicity. However, a significant decrease in the leaf biomass production as compared with the control was observed only after two or three weeks, and only minor signals of metal-induced adverse effects were evidenced concerning photosynthesis, oxidative stress indicators, antioxidant metabolites, and macronutrients. After two or three weeks, the leaf level of Cu occasionally approached the upper value associated with the recommended limits of dietary intake for human adults. However, as rocket leaves are commercialized when they achieve a 10–15 cm length, after one week of cultivation in perlite, the plants had an adequate size without metal contamination and could be considered suitable for the food market, even after exposure to Cu or Zn concentrations up to 100 µM.

Keywords: rocket; baby leaf; heavy metals; toxicity; nutrient uptake; dietary intake

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1. Introduction

Heavy metals are persistent pollutants that are spread in the environment from anthropogenic sources, such as mining, industrial processing, and agricultural practices [1], which have contributed to the contamination of soils and water bodies [2–4]. From a chemical point of view, heavy metals are elements with high atomic weights or high densities; however, the term is commonly used to indicate either metals or metalloids that have toxic effects on both the environment and living organisms and that are generally bioaccumulated through the food chain [1]. In this respect, a major health risk could be associated with vegetables, which are an essential part of the human diet. Furthermore, the problem could involve especially leafy vegetables, as heavy metals are generally accumulated more in root and leaf tissues than fruits [5].

In plants, heavy metals can cause physiological and morphological modifications by inducing the production of reactive oxygen species (ROS), inhibiting enzyme activity, reducing photosynthesis, and activating detoxification mechanisms [6]. However, some heavy metals, including copper (Cu) and zinc (Zn), are also essential trace elements (or micronutrients) involved in biochemical reactions or in functional molecules, such as enzymes or structural proteins [7]. Essential plant micronutrients participate in cell functions that regulate the circadian rhythm and cell senescence and apoptosis [8]. At metal concentrations below the functional requirement of plants, visible deficiency symptoms may occur consisting in leaf chlorosis or necrosis [9]. At concentrations above the physiological range, generally, photosynthesis is inhibited, mineral nutrition is impaired, and oxidative stress occurs. Under these conditions, plant growth is limited, and specific mechanisms

are activated to preserve the tissues from oxidative damage and maintain homeostasis. The detoxification process may take place by excluding or sequestering the excess element through decreased absorption, chelation by organic anions, the regulation of translocation to the leaves, or compartmentalization in the vacuoles [10]. These mechanisms generally involve the antioxidant system with the activation of antioxidant enzymes and the stimulation of the synthesis of antioxidant secondary metabolites. The capacity of the plant to tolerate element concentrations above the optimal level without tissue damage is the basis for sustainable biotechnologies, like plant food biofortification or the phytoremediation of marginal soils. However, the plant tolerance mechanisms could be ineffective at excessive concentrations and toxicity may occur, often with symptoms similar to those caused by element deficiency, especially chlorosis and necrosis [11].

Both Cu and Zn have been reported among the most hazardous environmentally relevant heavy metals [1]. The former is a widespread contaminant in agricultural soils and intensive crop systems (greenhouse crops, soilless culture) for the extensive use of copper-based pesticides in many annual (i.e., vegetables, flower culture, herbs) and perennial crops, particularly in vineyards [12]. Copper is a cofactor in enzymes that regulate photosynthesis and activate ROS scavenging. Along with the cell damage caused by ROS production, Cu poisoning causes physiological and morphological alterations, such as limited plant growth and photosynthetic activity, chlorosis, and a reduced nutrient content in root tissues [6]. Similarly, Zn is a widespread pollutant and is the second most abundant transition metal in living organisms after iron [13]. Zn is involved in enzyme activation, photosynthesis, nitrogen (N) metabolism, and cell multiplication [14]. Excess Zn can be toxic to plants, with effects similar to those caused by excess Cu: chlorosis, the deficiency of other micronutrients, the inactivation of enzymes involved in ROS scavenging (e.g., peroxidase), and the inhibition of root development [6]. It has been reported that Zn can cause toxicity in plants at tissue concentrations above 300 μg/g of dry matter [10], provoking nutritional and photosynthetic impairment. Both the threshold concentration causing toxicity and the mechanisms to counteract the cytotoxic effects depend mainly on the plant species and on the chemical properties of the metal element. Also, the time required for the adverse effects of metal poisoning to become apparent depends mainly on the genotype and chemical behaviour of the toxic agent. It has been found that heavy-metal accumulation increases with the plant age and is higher in the reproductive phase than the vegetative phase [15]. Therefore, young plants at the vegetative stage may not show significant toxicity symptoms after a short time of exposure to heavy metals, even when the toxic elements can cause severe long-term effects.

Rocket is one of the most popular crops in the Mediterranean areas and is mainly consumed as a ready-to-eat product [16]. Many fresh-cut leafy vegetables for the food market, including rocket, are often grown hydroponically in floating raft systems or other soilless systems that involve the growth of plants in inert material, such as rockwool, perlite, pumice, sand, etc., and organic-based material, such as peatmoss, cocosoil, etc. [17–19]. In hydroponics, plant roots are in direct contact with the nutrient solution during the whole growing cycle; therefore, they are exposed to the dissolved nutrients and non-nutrient ions. In this study, we tested the hypothesis that the short-term contamination by excess heavy metals is not relevant in young plants, regardless of the mechanisms of element uptake and detoxification. With this aim, rocket plantlets grown in pots with perlite were exposed to supra-optimal concentrations (25, 50, and 100 μM) of Cu or Zn in the nutrient solution. The plants were sampled after one week, at the commercial stage of baby leaf [20]. In addition, two further samplings were carried out after two and three weeks of treatment to verify that the phytotoxic effects were not evident, even with a slightly longer cultivation period. Two parallel experiments were conducted at the same time in the same greenhouse, using a common control treatment with 4.00 μM of Cu and 4.10 μM of Zn in the nutrient solution. The accumulation and tolerance toward Cu or Zn in the early stages of cultivation were evaluated and compared.

2. Materials and Methods

2.1. Plant Material and Cultivation Conditions

Rocket (*Eruca sativa* L.) seedlings at the stage of two cotyledons were used at the experimental greenhouse of the Cyprus University of Technology in Limassol, Cyprus. Plants were growing during January and February 2020, for a seven-week period. Seedlings were purchased from a commercial nursery, grown in a peat-based cubic (3 cm × 3 cm × 3 cm) medium, and each cube had approximately 30–35 young seedlings. Young seedlings were grown for six more days, irrigated with water, and their number was periodically reduced to seven per cube within that period. Each cube was transplanted in pots (one cube per pot; 1.5 L capacity) filled with expanded perlite and placed on plastic trays to achieve proper drainage [21]. Perlite properties have been described previously [22]. Plants were grown in an open (free-drainage) hydroponic system, and the drainage nutrient solution was delivered to plants through capillary suction.

Plants were sampled at three different growth stages, on a weekly basis. Plants were initially grown with the application of a half-strength nutrient solution (electrical conductivity (EC) and pH of 2.2 dS/m and 5.8, respectively). The nutrient solution composition was as follows: N-NO_3^-: 15.00 mM; K: 9.50 mM; P-PO_4^{3-}: 1.80 mM; Ca: 4.20 mM; Mg: 1.63 mM; S-SO_4^{2-}: 1.55 mM; Na: 1.85 mM; B: 30.00 µM; Fe: 35.05 µM; Mn: 6.10 µM; Cu: 4.00 µM; Zn: 4.10 µM; Mo: 0.52 µM. These concentrations were obtained using mineral salts, with the exception of iron, which was used in chelated form with ethylenediamine-N-N'bis(2-hydroxy-4-methylphenylacetic) acid (6.5% Fe EDDHMA). After 14 days, the plants were subjected to different Zn and Cu levels (seven treatments in total) in the nutrient solution: (i) 4.00 µM of Cu and 4.10 µM of Zn (control); (ii) 25 µM of Zn; (iii) 50 µM of Zn; (iv) 100 µM of Zn (in the form of $ZnSO_4$); (v) 25 µM of Cu; (vi) 50 µM of Cu; and (vii) 100 µM of Cu (in the form of $CuSO_4$). Plants were grown under Zn or Cu excess for an additional 21 days (in total, 49 days after transplanting). A total of 189 plants were used (7 Zn–Cu levels × 3 sampling periods × 9 replicates).

2.2. Plant Growth and Photosynthesis

Plant growth and physiological parameters were measured at three sampling periods (35, 42, and 49 days after transplanting, corresponding to 7, 14, and 21 days of treatment (DOT), respectively) considering six replicates per treatment and the growth period for measurements. Each replicate consisted of one individual plant. Plant height and leaf number were recorded. After harvest, the fresh weights (FWs) and dry weights (DWs) of leaves and roots were measured. Individual samples were collected and placed at 65 °C in a forced-air oven until constant weight to determine their DWs. Leaf stomatal conductance was measured with a ΔT-Porometer AP4 (Delta-T Devices-Cambridge, Burwell, Cambridge, UK), according to [23]. Leaf chlorophyll fluorescence (chlorophyll fluorometer, opti-sciences OS-30p, Hudson, NY, USA) was measured on two fully developed, light-exposed leaves per plant. Following leaf incubation in the dark for 20 min, the Fv/Fm ratio was measured. Leaf chlorophyll was extracted with dimethyl sulfoxide (DMSO) and chlorophyll a (Chl a) and chlorophyll b (Chl b), and total chlorophylls (total Chl) were assayed and expressed as µg/g FW.

2.3. Plant Stress Indicators

The cell damage index of lipid peroxidation in leaves was assessed in terms of the concentration of malondialdehyde (MDA), which was determined via the thiobarbituric acid reaction [24]. The concentration of hydrogen peroxide (H_2O_2) was measured according to [25]. The results were expressed as nmol MDA or µmol H_2O_2 per g FW. Four replicates were analysed for each treatment and sampling date.

2.4. Antioxidant Activity and Concentration of Total Phenols

The antioxidant activity of the methanolic leaf plant extracts was determined with four replicates per treatment and sampling date by the assays of 2,2-diphenyl-1-picrylhydrazyl

(DPPH) and ferric-reducing antioxidant power (FRAP), as previously described [26], and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay according to [27]. The Folin–Ciocalteu method was used for the determination of total phenols, as previously described [28], and the results were expressed as gallic acid equivalents (mg GAEs/g FW).

2.5. Mineral Concentration

Dried tissue (0.5 g) from leaves and roots from each treatment (4 biological replications; each replication was a pool of 2 individual plants) at the three sampling dates was subjected to dry ashing at 450 °C and acid extraction (2N HCl). The extracts were used for the determination of sodium (Na) and potassium (K) via flame photometry (Lasany Model 1832, Lasany International, Haryana, India), phosphorus (P) with the molybdate/vanadate method (yellow method) via spectrophotometry (Multiskan GO, Thermo Fischer Scientific, Waltham, MA, USA), and Zn and Cu via atomic absorption spectrometry (PG Instruments AA500FG, Leicestershire, UK). Nitrogen (N) was determined using the Kjeldahl method (BUCHI, Digest automat K-439 and Distillation Kjelflex K-360, Flawil, Switzerland) following [19]. Plant nutrient content was expressed in g/kg and mg/kg DW for macronutrients and micronutrients, respectively.

2.6. Statistical Analysis

Six samples per treatment were used for measurements concerning plant growth and photosynthesis, whereas mineral and antioxidant compositions were determined from four samples per treatment. Each sample consisted of one individual plant. The results were expressed as mean values and standard deviations (SDs). Normal distribution and homoscedasticity of the data were verified through the tests of Kolmogorov–Smirnov and Levene, respectively. In order to fulfil the requirements of ANOVA, the following datasets were subjected to the reciprocal transformation: stomatal resistance, DPPH, leaf N, and root Cu for the Cu treatments; stomatal resistance, leaf K, root Zn, and root Cu for the Zn treatments. The values of leaf numbers of Cu-treated plants were not normally distributed, even after data transformation, and were not subjected to further statistical analysis. For all the other datasets, the two-way ANOVA was performed with either the Zn or Cu concentration and the duration of exposure as the sources of variation; when their interaction was significant, means were compared using the LSD post-test at $p < 0.05$. Correlation and regression analyses of the data were also performed. The statistical analysis was accomplished with Statgraphics Centurion Version 17 software (Statpoint Technologies, Warrenton, VA, USA).

3. Results

3.1. Plant Growth

At the first two sampling dates, the DWs and FWs of both the roots and leaves were scarcely affected by the concentrations of Cu (Table 1A) or Zn (Table 1B) in the nutrient solution. With 50 µM of Cu, a significant decrease in both the FWs and DWs of the leaves as compared to the control was observed at each sampling time, and the same effect was observed also for the roots at 21 days of treatment (DOT), although, to a lesser extent, the Zn-treated plants showed a similar behaviour at the last two sampling dates. However, at 21 DOT, the values of the root or shoot DWs did not significantly differ in the control plants and in those grown with the 100 µM treatments. The concentration of either Cu or Zn did not significantly affect the leaf number or FW (Table 1 and Table S2 of Supplementary Materials). However, the leaf FW was generally lower for the Cu-treated plants than the Zn-treated plants (Table 1A,B). A higher root DW was observed for the Cu-treated plants than the Zn-treated plants only at element concentrations above 50 µM and at the first sampling. After 21 DOT, the root FWs and DWs did not differ significantly across any of the Cu and Zn treatments.

3.2. Photosynthesis

For both Cu and Zn, the effect of the concentration on either the chlorophyll fluorescence (Fv/Fm) or the stomatal resistance was not significant (Table 2A,B). Moreover, the highest values of the stomatal resistance were observed after two weeks exposure, and the values of the chlorophyll level were generally lower at 21 DOT in comparison with the previous sampling dates.

3.3. Plant Stress Indicators

The concentration of Cu or Zn in the nutrient solution, the sampling date, and their interaction significantly affected the plant response to oxidative damage (Figure 1). The content of H_2O_2 increased with time in the control plants and with 25 µM of Zn in the nutrient solution. Conversely, in all the other Cu or Zn treatments, the highest values of H_2O_2 content were found at 14 DOT. In the control plants, the lowest MDA content was found at 14 DOT, and the same behaviour was observed in all the treatments except for 100 µM of Cu. The highest values of MDA were found at 21 DOT for all the treatments, except for 25 and 100 µM of Cu.

Table 1. Growth parameters of rocket plants grown hydroponically in perlite, exposed to different concentrations of (A) copper (Cu) or (B) zinc (Zn) in the nutrient solution and sampled at different times. FW: fresh weight; DW: dry weight.

(A)

Cu	**Leaf FW (g)**		**Root FW (g)**		**Leaf DW (g)**		**Root DW (g)**	
GRAND MEAN	23.53		4.245		2.285		0.3220	
Time (days of treatment)								
7	12.48		2.893	c	1.048		0.2147	
14	23.92		4.546	b	2.226		0.3216	
21	34.19		5.296	a	3.582		0.4292	
Concentration (µM)								
4.00 (Control)	25.42		3.942	c	2.396		0.3038	
25	23.89		4.394	ab	2.221		0.3090	
50	19.82		3.993	bc	1.917		0.2933	
100	25.01		4.651	a	2.608		0.3811	
Time by Concentration								
7, Control	13.20	ab	2.708		1.076	ab	0.1788	b
7, 25 µM	10.94	b	2.627		0.920	b	0.1702	b
7, 50 µM	11.36	b	3.026		1.014	b	0.2466	a
7, 100 µM	14.44	a	3.210		1.183	a	0.2632	a
14, Control	25.91	a	4.015		2.345	a	0.3094	b
14, 25 µM	25.05	a	4.761		2.214	a	0.3034	b
14, 50 µM	20.77	b	4.065		1.819	b	0.2666	b
14, 100 µM	23.95	ab	5.344		2.524	a	0.4068	a
21, Control	37.14	a	5.103		3.767	a	0.4233	a
21, 25 µM	35.67	a	5.793		3.530	a	0.4534	a
21, 50 µM	27.33	b	4.887		2.917	b	0.3668	b
21, 100 µM	36.63	a	5.400		4.116	a	0.4734	a
MAIN EFFECTS								
A: *Time*	***		***		***		***	
B: *Concentration*	***		**		***		***	
INTERACTION								
A × B	*		ns		*		**	

Table 1. *Cont.*

(B)								
Zn	**Leaf FW (g)**		**Root FW (g)**		**Leaf DW (g)**		**Root DW (g)**	
GRAND MEAN	26.41		4.173		2.462		0.3086	
Time (days of treatment)								
7	14.36		2.881	c	1.140		0.1924	
14	25.22		4.264	b	2.185		0.2899	
21	39.67		5.375	a	4.060		0.4434	
Concentration (µM)								
4.10 (Control)	25.42		3.942	bc	2.396		0.3038	
25	29.23		4.624	a	2.777		0.3456	
50	21.94		3.756	c	2.008		0.2767	
100	29.07		4.373	ab	2.665		0.3038	
Time by Concentration								
7, Control	13.20	b	2.708		1.076	a	0.1788	b
7, 25 µM	16.04	a	3.047		1.220	a	0.2200	a
7, 50 µM	13.89	ab	2.727		1.120	a	0.1934	a
7, 100 µM	14.33	ab	3.041		1.145	a	0.1774	b
14, Control	25.91	ab	4.015		2.345	ab	0.3094	a
14, 25 µM	29.09	a	4.833		2.500	a	0.3168	a
14, 50 µM	21.86	b	3.868		1.871	b	0.2634	a
14, 100 µM	23.99	b	4.342		2.023	ab	0.2700	a
21, Control	37.14	ab	5.103		3.767	bc	0.4233	bc
21, 25 µM	42.56	ab	5.991		4.613	ab	0.5000	a
21, 50 µM	30.06	b	4.673		3.034	c	0.3734	c
21, 100 µM	45.84	a	5.734		4.828	a	0.4768	ab
MAIN EFFECTS								
A: *Time*	***		***		***		***	
B: *Concentration*	***		**		***		**	
INTERACTION								
A × B	***		ns		***		*	

Mean values of six replicates. ***: significant at $p < 0.001$; **: significant at $p < 0.01$; *: significant at $p < 0.05$; ns: not significant, according to two-way ANOVA. For each parameter, different letters indicate significant difference at $p < 0.05$ according to the LSD post-test. FW: fresh weight; DW: dry weight.

3.4. Antioxidant Activity and Total Phenol Concentration

In the leaf tissues, the markers linked to the antioxidant activity (that is, the content of total phenols and the antioxidant capacity as determined by three different assays) were significantly affected by the duration of the exposure to the toxic metal (Figure 2). For both Cu and Zn, slightly higher values of the antioxidant markers were generally observed at 14 and 21 DOT than in younger plants. Overall, Zn did not impact the antioxidant response of the rocket plants, regardless of the concentration; at 21 DOT, the values of all the markers were higher in the control than in the other treatments. Conversely, at 21 DOT, only the highest Cu concentration caused a significant decrease in all the markers as compared to the control. Unlike Zn, the Cu concentration significantly affected the antioxidant behaviour of the rocket plants. The highest values for both the content of total phenols and the antioxidant power were found at 14 DOT with 25 and 50 µM of Cu, whereas at 100 µM of Cu, the values of both parameters were similar to those of the control.

3.5. Mineral Concentration

The concentration of Cu or Zn, the time of exposure, and their interaction generally had a strong impact on the leaf mineral content (Table S5A,B of Supplementary Materials). For example, at 7 and 14 DOT, the leaves of the Zn-treated plants contained more N, K, and P than the Cu-treated ones. However, the concentration of either Cu or Zn in the nutrient solution did not have any significant effect on the root level of P. Apart from these observations, no common trends or systematic differences were evidenced between the Cu-

and Zn-treated plants concerning the leaf content of macronutrients and Na. For example, the roots of the plants exposed to excess Cu contained more Na at 7 DOT and more K at 21 DOT than those exposed to excess Zn. The leaf tissues invariably contained more Cu than Zn, regardless of the composition of the nutrient solution or the sampling date (Figure 3).

Table 2. Chlorophyll fluorescence (Fv/Fm), stomatal resistance, and concentration of total chlorophylls (Total Chl) in rocket plants grown hydroponically in perlite, exposed to different copper (Cu) or zinc (Zn) concentrations in the nutrient solution and sampled at different times.

(A)						
Cu	**Fv/Fm**		**Stomatal Resistance (s/cm)**		**Total Chl (µg/g FW)**	
GRAND MEAN	0.832		1.052		176	
Time (days of treatment)						
7	0.825	b	0.868		204	a
14	0.846	a	1.310		191	a
21	0.826	b	0.978		133	a
Concentration (µM)						
4.00 (Control)	0.835	a	1.288		165	b
25	0.830	a	0.959		176	ab
50	0.834	a	0.915		167	b
100	0.829	a	1.044		196	a
Time by Concentration						
7, Control	0.823		0.947	a	188	
7, 25 µM	0.820		0.773	a	195	
7, 50 µM	0.827		0.815	a	198	
7, 100 µM	0.828		0.935	a	233	
14, Control	0.854		1.925	a	184	
14, 25 µM	0.841		1.227	ab	209	
14, 50 µM	0.848		1.252	ab	173	
14, 100 µM	0.841		0.837	b	199	
21, Control	0.828		0.993	b	123	
21, 25 µM	0.829		0.878	b	124	
21, 50 µM	0.829		0.678	b	129	
21, 100 µM	0.819		1.360	a	156	
MAIN EFFECTS						
A: *Time*	***		*		***	
B: *Concentration*	ns		ns		*	
INTERACTION						
A × B	ns		*		ns	
(B)						
Zn	**Fv/Fm**		**Stomatal Resistance (s/cm)**		**Total Chl (µg/g FW)**	
GRAND MEAN	0.831		1.395		177	
Time (days of treatment)						
7	0.832	ab	0.928	b	189	
14	0.835	a	2.259	a	203	
21	0.824	b	0.996	b	138	
Concentration (µM)						
4.10 (Control)	0.835		1.288	b	165	
25	0.832		1.793	a	189	
50	0.826		1.000	b	175	
100	0.829		1.497	ab	178	
GRAND MEAN	0.831		1.395		177	

Table 2. *Cont.*

(B)			
Zn	**Fv/Fm**	**Stomatal Resistance (s/cm)**	**Total Chl (μg/g FW)**
Time (days of treatment)			
7	0.832 ab	0.928 b	189
14	0.835 a	2.259 a	203
21	0.824 b	0.996 b	138
Concentration (μM)			
4.10 (Control)	0.835	1.288 b	165
25	0.832	1.793 a	189
50	0.826	1.000 b	175
100	0.829	1.497 ab	178
Time by Concentration			
7, Control	0.823	0.947	188 bc
7, 25 μM	0.840	1.318	148 cd
7, 50 μM	0.831	0.600	200 ab
7, 100 μM	0.836	0.848	222 a
14, Control	0.854	1.925	184 bc
14, 25 μM	0.827	2.777	243 a
14, 50 μM	0.828	1.637	177 bc
14, 100 μM	0.832	2.698	207 ab
21, Control	0.828	0.993	123 b
21, 25 μM	0.831	1.283	177 a
21, 50 μM	0.819	0.763	148 ab
21, 100 μM	0.820	0.944	106 b
MAIN EFFECTS			
A: *Time*	ns	***	***
B: *Concentration*	ns	**	ns
INTERACTION			
A × B	ns	ns	***

Mean values of six replicates. Asterisks denote statistical significance according to two-way ANOVA: ***: significant at $p < 0.001$; **: significant at $p < 0.01$ *: significant at $p < 0.05$; ns: not significant. For each parameter, different letters indicate significant difference at $p < 0.05$ according to the LSD post-test. FW: fresh weight.

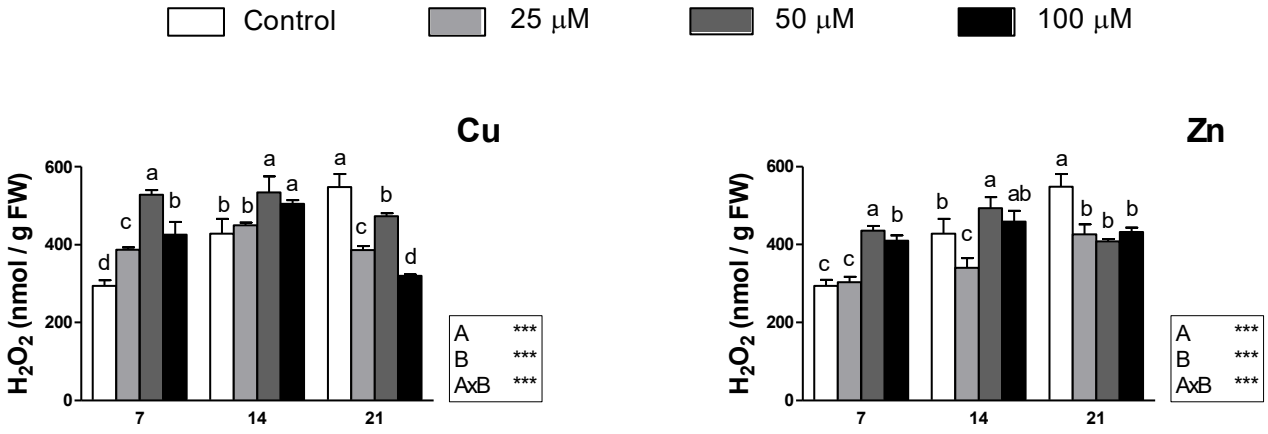

Figure 1. *Cont.*

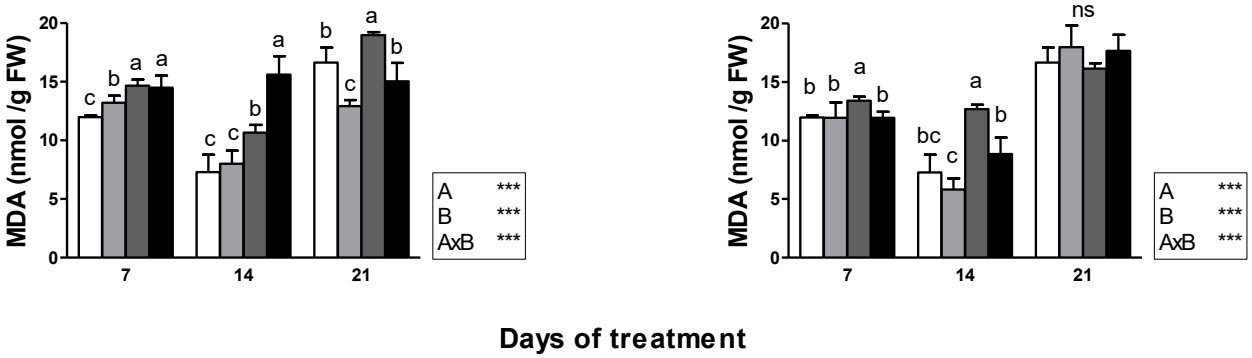

Figure 1. Leaf content of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in rocket plants grown hydroponically in perlite, exposed to different concentrations of copper (Cu; **left**) or zinc (Zn; **right**) in the nutrient solution and sampled at different times. The concentrations of Cu and Zn in the control solution were 4.00 and 4.10 μM, respectively. The data were subjected to two-way ANOVA (right-hand box of each graph; A: time; B: concentration; A × B: interaction; ***: significant at $p > 0.001$), followed by LSD post-test for means separation. Different letters indicate significant differences at $p < 0.05$; ns: not significant. Error bars show standard deviations (n = 4).

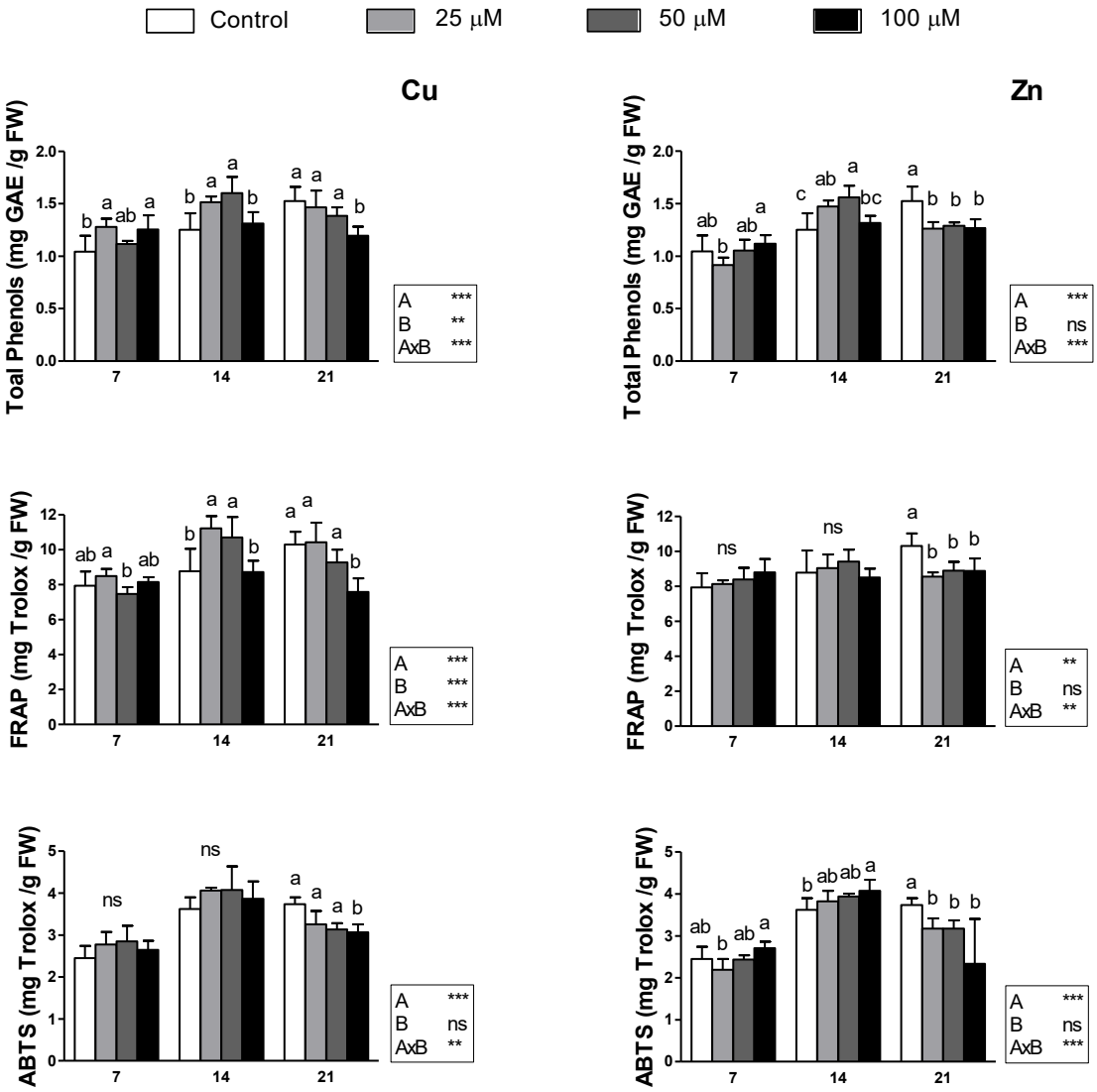

Figure 2. *Cont.*

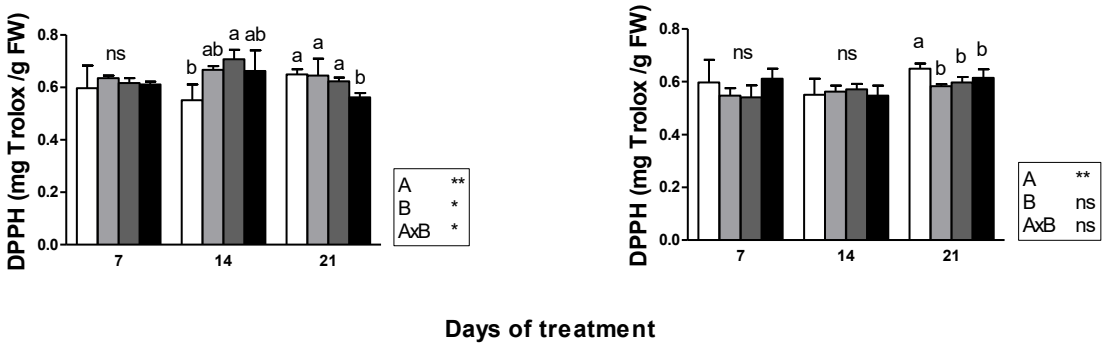

Figure 2. Content of total phenols, antioxidant capacity (FRAP), and radical scavenging activity (DPPH and ABTS) in the leaf tissues of rocket plants grown hydroponically in perlite, exposed to different concentrations of copper (Cu; **left**) or zinc (Zn; **right**) in the nutrient solution and sampled at different times. The concentrations of Cu and Zn in the control solution were 4.00 and 4.10 µM, respectively. The data were subjected to two-way ANOVA (right-hand box of each graph; A: time; B: concentration; A × B: interaction; ***: statistical significance at $p < 0.001$; **: statistical significance at $p < 0.01$; *: statistical significance at $p < 0.05$; ns: not significant), followed by LSD post-test for means separation. Different letters indicate significant differences at $p < 0.05$. Error bars show standard deviations (n = 4). GAEs: gallic acid equivalents; FW: fresh weight.

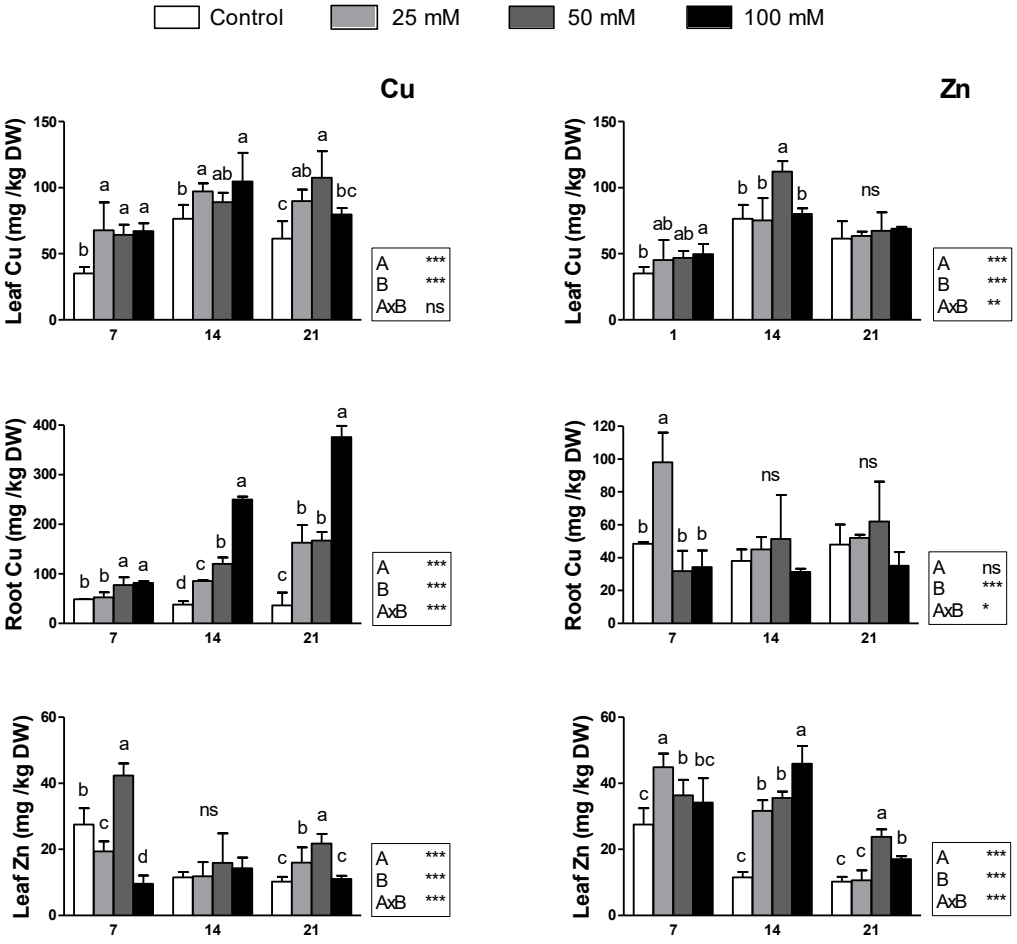

Figure 3. *Cont.*

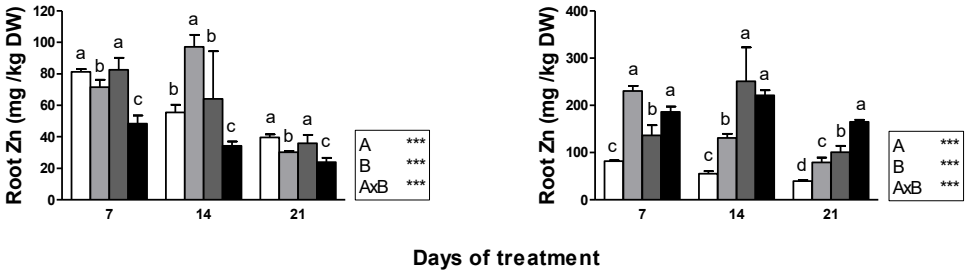

Figure 3. Leaf and root contents (mg/kg dry weight) of copper (Cu) or zinc (Zn) in rocket plants grown in perlite, exposed to different concentrations of Cu (**left**) or Zn (**right**) in the nutrient solution and sampled at different times. The concentrations of Cu and Zn in the control solution were 4.00 and 4.10 μM, respectively. The data were subjected to two-way ANOVA (right-hand box of each graph; A: time; B: concentration; A × B: interaction; ***: statistical significance at $p < 0.001$; **: statistical significance at $p < 0.01$; * statistical significance at $p < 0.05$; ns: not significant), followed by LSD post-test for means separation (n = 4; different letters: significant difference at $p < 0.05$; error bars: standard deviations).

4. Discussion

Rocket plants are generally harvested when they reach a height of about 15 cm [29]. Therefore, the experimental time adopted in the present work encompassed the typical growing cycle of commercial production, and the plants had already achieved a marketable size after one week from transplanting in perlite. The metal concentration causing toxicity in plants depends on the duration of exposure and the plant species, and different threshold levels for Cu or Zn have been reported in the literature. For example, in the agar-grown seedlings of *Arabidopsis thaliana* after eight days of treatment, Cu and Zn concentrations of 40.0 and 76.4 μM, respectively, reduced the root growth by 50% [6]. Concentrations up to 20 μM of Zn have been used for rocket microgreens [30], whereas rocket plants biofortified with 80 μM of Zn were used to evaluate the bioaccessibility of the element after in vitro gastrointestinal digestion [16]. In a recent review [31], data on Cu toxicity and tolerance in several species grown in different media are reported at different Cu doses and for different time periods. Both Cu and Zn are transition metals with similar atomic masses and dimensions; therefore, their effects on rocket plants mainly depend on their chemical behaviour and plant physiology.

4.1. Plant Growth

Heavy metals generally impact plant growth and development [6]; however, growth inhibition by toxic metals depends on both the time and concentration, as observed in *Lactuca sativa* exposed to mine waste [32]. Apart from the obvious effect of the plant age on biomass production, in this work, some similarities were observed in the growing parameters of the Cu- or Zn-treated plants. The root and shoot DWs were similar in the control plants and in those grown with 100 μM of Cu or Zn in the nutrient solution, suggesting that the analogous increase in the FW observed under the same conditions was likely due to dry biomass production rather than greater water absorption. Overall, the concentration of either Cu or Zn had a stronger effect on the leaves than the roots (Table 1A,B). Consistent with the values of the leaf FW, the plants exposed to excess Cu appeared slightly smaller than those exposed to the same concentration of Zn for the same time. In contrast, only at 21 DOT was the leaf DW lower in the Cu-treated plants than in the Zn-treated plants, showing that excess Cu may limit the leaf water content during the first two weeks of treatment. However, in the same time period and at concentrations above 50 μM, Zn limited the root growth more than Cu. Interestingly, while a 50 μM heavy-metal (Cu or Zn) concentration generally reduced the fresh and dry biomass production, both at the leaf and root level, no significant growth inhibition was observed with the 100 μM concentration, showing that some detoxification mechanisms against Cu or Zn toxicity

were overall more effective with a higher metal concentration. According to the correlation matrices reported in Figure 4, in both experiments, the growth parameters (DWs and FWs of leaves and roots) were negatively correlated to the N content in the tissues. This outcome may indicate that within 21 DOT, the accumulation of carbon compounds, such as sugars or secondary metabolites, could be faster than the synthesis of structural N-containing molecules, though further investigation is necessary to support this interpretation. In the experiment with excess Cu, the root content of the element was negatively correlated to the root Zn content ($R = -0.679$), suggesting an antagonistic effect in the absorption of these metal ions. Both the leaf and root Cu were negatively correlated to the leaf and root N, indicating the growth inhibition by Cu; however, the Cu concentration was positively correlated to the biomass production, suggesting a stimulation of the secondary metabolism consistent with the elicitation of the antioxidant response. In contrast, the increase in the Zn content in the plant tissue decreased the growth parameters. In the experiment with excess Zn, the concentration of Cu did not show any significant relationship with the other parameters, except for a negative correlation between the leaf Cu and N contents of both the leaves and roots. No correlation was evidenced between the root Zn content and the other parameters; only the leaf content of the metal was positively correlated to the leaf N and negatively correlated to the biomass production. This behaviour was opposite to that of Cu and suggested a lower toxicity of excess Zn, which could favour the synthesis of N compounds rather than increase the accumulation of antioxidant secondary metabolites.

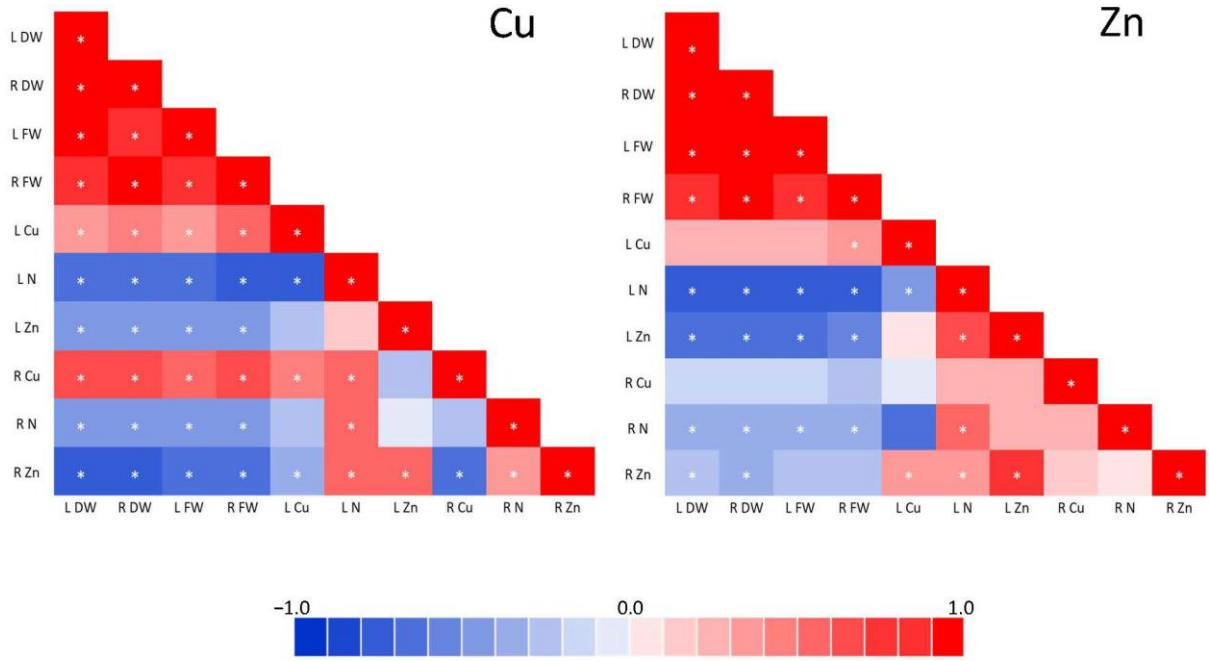

Figure 4. Correlation matrices for the dry weights and fresh weights (DWs and FW, respectively; g) of leaves (L) and roots (R), and the concentrations of nitrogen (N; g/kg DW), copper, and zinc (Cu and Zn, respectively; mg/g DW) in the tissues of rocket plants grown hydroponically in perlite, exposed to 25, 50, or 100 µM Cu (left) or Zn (right) in the nutrient solution and sampled after 7, 14, or 21 days of treatment. The concentrations of Cu and Zn in the control solution were 4.00 and 4.10 µM, respectively. Asterisks indicate significant Pearson's coefficients ($p < 0.05$).

4.2. Photosynthesis

The values of the Fv/Fm ratio (0.82–0.85) were always within the range of healthy plants, showing that the photosynthetic electron transport was not impaired by excess Cu or Zn. Higher values of stomatal resistance at 14 DOT in comparison with the other sampling dates were observed not only with excess metal, but also in control plants, and could be ascribed to the physiological effect of the plant age. Likewise, the decrease in the

chlorophyll concentration at 21 DOT (Table 2) was observed both in the treated and control plants and could not be associated with excess metal. The above results suggested that, at the concentrations used in the experiments, a 21-day exposure to either Cu or Zn does not impair photosynthesis in rocket plants. Recently, early signals of a damaged photosynthetic system were observed in *Pelargonium graveolens* plants treated with Cu concentrations up to 100 µM only after a 28-day exposure [12]. In *Colobanthus quitensis* plants grown in vitro, a 100 µM Cu concentration did not significantly alter the chlorophyll levels after 60 days of treatment [33]. After eight days of treatment, a decrease was observed in the pigment content of agar-grown *Arabidopsis thaliana*, which was not consistent with the change in the Cu or Zn concentration [6].

4.3. Plant Stress Indicators

Lipid peroxidation due to oxidative stress can occur in plants exposed to excess concentrations of heavy metals [13,34–36]. To ensure the scavenging of ROS and prevent oxidative damage, plants have developed antioxidant mechanisms involving enzymatic antioxidants as well as antioxidant molecules [31,36,37]. Under these conditions, the tissue contents of stress indicators such as H_2O_2 or MDA are modulated in terms of the dependence of the plant antioxidant response. For example, it was recently reported that toxicity from heavy metals, including Cu and Zn, activated peroxidase, which is the main H_2O_2-scavenging enzyme in plant cells [38]. According to the progression of the antioxidant response, in this study, the leaf contents of both H_2O_2 and MDA in the rocket plants were significantly affected by the sampling time, the concentration of Cu or Zn, and their interaction (Figure 1). In addition, Cu or Zn excess affected the leaf levels of both H_2O_2 and MDA in the rocket plants to a different extent. With Zn, no effect on the level of H_2O_2 was observed at concentrations up to 25 µM. However, oxidative stress was evidenced with the 50 µM treatment along with increased lipid peroxidation only in the first two weeks of exposure; with 100 µM of Zn, a slight oxidative stress was evidenced only at 7 DOT and was not associated with membrane damage. With Cu, oxidative stress occurred in all the metal treatments following a different time course in terms of the dependence of the applied dose. With a 25 µM Cu concentration, an increase in the H_2O_2 concentration was found only at 7 DOT and was associated with increased lipid peroxidation; at 50 µM Cu, oxidative stress occurred at the first two sampling dates, while a higher lipid peroxidation as compared to the control was observed during the whole growing cycle. With 100 µM of Cu, both oxidative stress and membrane damage occurred at the first two sampling dates, while no effect was observed after 21 DOT. In comparison to the Zn treatments, the above results showed a lower threshold concentration for the elicitation of Cu-induced oxidative stress and a less effective detoxification mechanism towards membrane damage in the Cu-treated plants. This outcome suggested a stronger toxic effect by Cu than Zn in the rocket.

4.4. Antioxidant Activity and Total Phenol Content

Consistent with the above findings, the effect on the synthesis and accumulation of antioxidant molecules was more pronounced for Cu than Zn (Figure 2). The behaviour of the markers linked to the antioxidant activity (that is, the leaf content of total phenols and the antioxidant capacity as determined via different assays) was related to the production of H_2O_2, as the graphs of Figure 2 resembled the pattern observed for the level of H_2O_2 across time and metal concentrations (Figure 1). Concerning the former element, at 14 DOT, the highest values for the leaf content of total phenols and the antioxidant power were observed with 25 and 50 µM Cu, whereas at 100 µM of Cu, the values of both parameters were similar to those of the control. Interestingly, at 14 DOT, the highest levels of both H_2O_2 and MDA were observed above 50 µM of Cu and indicated that, after two weeks of exposure to excess Cu, the antioxidant response was effective for concentrations up to 25 µM and became progressively less effective as the Cu concentration in the nutrient solution was increased up to 100 µM. At 21 DOT, only the highest Cu concentration was

associated with a significant decrease in all the antioxidant markers as compared to the control, along with a lower concentration of H_2O_2. The same effect at 21 DOT was caused by Zn at much lower concentrations, as lower values than those of the control were observed for the levels of antioxidant markers and H_2O_2 already with 25 µM of Zn in the nutrient solution. This outcome suggests that, after three weeks of treatment, excess Zn up to 100 µM did not have a significant toxic effect and, hence, the synthesis and accumulation of antioxidant molecules were not stimulated.

A good correlation (Pearson's coefficient ranging from 0.905 to 0.506) was found between the content of total phenols and the antioxidant capacity as determined by the FRAP, ABTS, or DPPH assay, despite a lower slope for the regression lines obtained with the latter method (Figure 5). This result evidenced that, although important components of the antioxidant response such as enzymes (superoxide dismutase, catalase, peroxidase, etc.) and glutathione were not investigated, the antioxidant capacity in rocket is largely due to phenolic compounds.

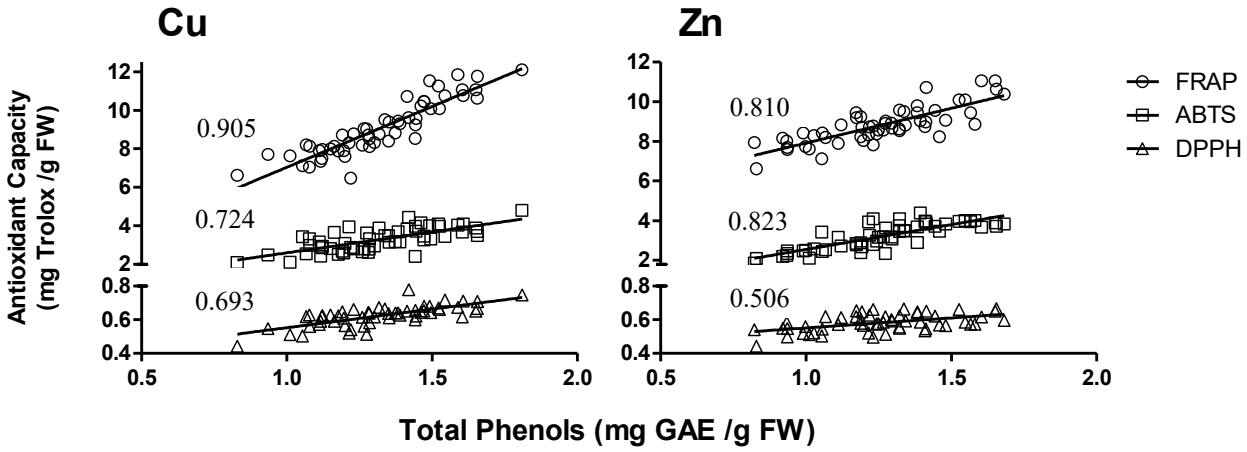

Figure 5. Linear regression between the antioxidant capacity or radical scavenging activity as determined by the FRAP, ABTS, or DPPH assay and the content of total phenols in the leaf tissues of rocket plants grown hydroponically in perlite, exposed to 25, 50, or 100 µM of copper (Cu; **left**) or zinc (Zn; **right**) in the nutrient solution and sampled after 7, 14, or 21 days of treatment. The Pearson's coefficients are reported on the graphs. The concentrations of Cu and Zn in the control solution were 4.00 and 4.10 µM, respectively. GAEs: gallic acid equivalents; FW: fresh weight.

4.5. Mineral Concentration

Plants can absorb nutrients via specific active uptake mechanisms, and the overabundance of trace elements may significantly alter the rhizosphere environment and interfere with the uptake of other nutrients. Heavy metals could compete for the same uptake transporters within the root cells or affect plant signalling for nutrient uptake regulation [39]. In this study, both Cu and Zn excesses up to 100 µM significantly affected the absorption of macronutrients and Na after 21 days of exposure, although no clear trend was found for the level of macroelements in response to Cu or Zn excess. However, higher leaf contents of N, K, and P were observed after 7 and 21 days of treatment in the Zn-treated plants than in those exposed to excess Cu (Table S5A,B of Supplementary Materials), which was consistent with the slight growth inhibition that was observed for the latter plants (Table 1A,B). Although the inhibition of root growth caused by heavy metals may indirectly affect mineral uptake, in this work, the root level of P was not directly influenced by the external concentration of either Cu or Zn. At the metal concentrations used in the experiments, the duration of the treatments with either Cu or Zn did not seem long enough to determine a significant impairment of mineral uptake. However, the experimental data evidenced some differences in the absorption of these microelements across three weeks of exposure. Due to the different tolerance mechanisms developed by plants to counteract metal toxicity, root uptake may be limited, resulting in low metal accumulation in the

root system, or translocation to the leaves could be enhanced followed by leaf storage, leading to high metal levels in the aerial parts [10]. Conversely, translocation to the leaves could be limited, resulting in higher accumulation in the root tissues than in the shoot. In both the experiments, the levels of Zn in the plant tissues were generally lower than those of Cu. In addition, in the Zn-treated plants, both the root and leaf contents of Zn in all the treatments were generally lower at the end of the experiment than at the previous samplings, indicating the limited root absorption of Zn after the first two weeks of exposure. In contrast, in the Cu-treated plants, the root content of Cu showed a marked increase as a consequence of both a higher Cu concentration in the nutrient solution and longer exposure. In contrast, the leaf Cu content at each Cu level was slightly higher after two weeks of treatment as compared to the first sampling, while no further increase was observed at 21 DOT, suggesting that, after two weeks of exposure, the plants had limited Cu translocation to the leaves rather than root absorption. However, much higher levels of Cu than Zn were found not only in the leaves of the Cu-treated plants, but also in those exposed to excess Zn, thus suggesting a considerably higher rate of translocation of the former element from roots to leaves, regardless of the treatment. The above results suggested the occurrence of different mechanisms for the absorption and translocation of Zn and Cu in rocket.

4.6. Dietary Intake

In the last years, the European Food Safety Authority (EFSA) has reported a survey of the upper levels for the dietary intake of both Cu and Zn, according to several regulatory institutions. Among the limits set by the regulations of different countries, the lowest oral dietary reference value in adults is 0.9 mg/day for Cu [31,40] and 3.0 mg/day for Zn [41]. As a serving dose of 50 g has been estimated for fresh rocket, based on several scientific studies and websites [42], the recommended Cu concentration in the leaf tissues should be not be higher than 18 mg/kg FW. In the treatments with excess Cu in the nutrient solution, this threshold was never exceeded, the highest values being 14 mg/kg FW only for two individual samples: one replicate sample collected at 14 DOT with 100 μM of Cu and one replicate sample collected at 21 DOT with 50 μM of Cu. With a similar evaluation, all the leaf samples across all the sampling times and Zn doses contained far lower levels of this element than the estimated upper limit. At the same time, the Zn levels in the plants exposed to excess Zn were significantly higher than those found in the control plants (Figure 3), showing that the enrichment of the nutrient solution with Zn concentrations up to 100 μM did not induce the metal contamination of the leaf tissues and could be considered an effective means for the Zn biofortification of rocket.

5. Conclusions

In the conditions used in the experiments, Cu showed a higher toxicity than Zn. Growth inhibition and oxidative stress were more evident in the Cu-treated plants than the Zn-treated plants due to the higher root absorption and higher translocation to the leaves of the former element. After two weeks of exposure, the plants limited the Cu translocation to the leaves rather than root absorption. In contrast, Zn showed a different behaviour, as the translocation of this element to the leaves was low and, after two weeks of exposure, the root absorption was also limited. Therefore, no relevant Zn toxicity effects were observed, and the accumulation of antioxidant molecules was not stimulated. More severe conditions, such as a longer growing period or higher metal concentration in the nutrient solution, could provide a better overview of the mechanisms of metal toxicity in rocket. The main outcome of the present study was that the short-time exposure of rocket to an excess concentration of Cu or Zn may not have toxic effects on plants and, at the same time, may not raise safety concerns linked to human consumption. Particularly, the typically short growing cycle of baby-leaf vegetables like rocket may allow cultivation with excess Cu or Zn without the occurrence of toxicity symptoms that significantly reduce the plant growth at longer exposures. Especially with zinc, which is only scarcely translocated

to the leaves, concentrations up to 100 µM could result in the biofortification rather than the metal poisoning of the aerial edible parts.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9090976/s1, Table S1: Growth parameters (mean values and standard deviations) of rocket plants grown hydroponically in perlite, exposed to different A) copper (Cu) or B) zinc (Zn) concentrations in the nutrient solution and sampled at different times. Table S2: Two-way ANOVA for height and leaf number of rocket plants grown hydroponically in perlite, exposed to different copper (Cu) or zinc (Zn) concentrations in the nutrient solution and sampled at different times. Table S3: Chlorophyll fluorescence (Fv/Fm), stomatal resistance and concentration of total chlorophylls (Total Chl) in rocket plants grown hydroponically in perlite, exposed to different copper (Cu) or zinc (Zn) concentrations in the nutrient solution and sampled at different times. Table S4: Leaf and root concentration (g/kg dry weight) of macronutrients (nitrogen, N; phosphorus, P; potassium, K) and sodium (Na) in rocket plants grown hydroponically in perlite, exposed to different A) copper (Cu) or B) zinc (Zn) concentrations in the nutrient solution and sampled at different times. Table S5: Two-way ANOVA for leaf and root concentration (g/kg dry weight) of macronutrients (nitrogen, N; phosphorus, P; potassium, K) and sodium (Na) in rocket plants grown hydroponically in perlite, exposed to different A) copper (Cu) or B) zinc (Zn) concentrations in the nutrient solution and sampled at different times.

Author Contributions: Conceptualisation, A.C. and N.T.; formal analysis, R.M.; investigation, A.C.; resources, N.T.; supervision, N.T.; writing—original draft, R.M.; writing—review and editing, A.C., L.I., A.P. and N.T. All authors have read and agreed to the published version of the manuscript.

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References

1. Ali, H.; Khan, E.; Ilahi, I. Environmental Chemistry and Ecotoxicology of Hazardous Heavy Metals: Environmental Persistence, Toxicity, and Bioaccumulation. *Hindawi J. Chem.* **2019**, *2019*, 6730305. [CrossRef]
2. Nazir, R.; Khan, M.; Masab, M.; Rehman, H.U.; Rauf, N.U.; Shahab, S.; Ameer, N.; Sajed, M.; Ullah, M.; Rafeeq, M.; et al. Accumulation of Heavy Metals (Ni, Cu, Cd, Cr, Pb, Zn, Fe) in the soil, water and plants and analysis of physico-chemical parameters of soil and water Collected from Tanda Dam kohat. *J. Pharm. Sci. Res.* **2015**, *7*, 89–97.
3. Noulas, C.; Tziouvalekas, M.; Karyotis, T. Zinc in soils, water and food crops. *J. Trace Elem. Med. Biol.* **2018**, *49*, 252–260. [CrossRef] [PubMed]
4. Rehman, M.; Liu, L.; Wang, Q.; Saleem, M.H.; Bashir, S.; Ullah, S.; Peng, D. Copper environmental toxicology, recent advances, and future outlook: A review. *Environ. Sci. Pollut. Res.* **2019**, *26*, 18003–18016. [CrossRef]
5. Manzoor, J.; Sharma, M.; Wani, K.A. Heavy metals in vegetables and their impact on the nutrient quality of vegetables: A review. *J. Plant Nutr.* **2018**, *41*, 1744–1763. [CrossRef]
6. Bae, B.; Park, H.; Kang, S. Quantitative estimation of synergistic toxicity of Cu and Zn on growth of *Arabidopsis thaliana* by isobolographic method. *Toxics* **2022**, *10*, 195. [CrossRef]
7. Briffa, J.; Sinagra, E.; Blundell, R. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* **2020**, *6*, e04691. [CrossRef]
8. Garcia, L.; Welchen, E.; Gonzalez, D.H. Mitochondria and copper homeostasis in plants. *Mitochondrion* **2014**, *19*, 269–274. [CrossRef]
9. Jeyalakshmi, S.; Radha, R. A review on diagnosis of nutrient deficiency symptoms in plant leaf image using digital image processing. *ICTACT J. Image Video Process.* **2017**, *7*, 1515–1524. [CrossRef]
10. Barrameda-Medina, Y.; Montesinos-Pereira, D.; Romero, L.; Ruiz, J.M.; Blasco, B. Comparative study of the toxic effect of Zn in *Lactuca sativa* and *Brassica oleracea* plants: I. Growth, distribution, and accumulation of Zn, and metabolism of carboxylates. *Environ. Exp. Bot.* **2014**, *107*, 98–104. [CrossRef]
11. Arif, N.; Yadav, V.; Singh, S.; Singh, S.; Ahmad, P.; Mishra, R.K.; Sharma, S.; Tripathi, D.K.; Dubey, N.K.; Chauhan, D.K. Influence of High and Low Levels of Plant-Beneficial Heavy Metal Ions on Plant Growth and Development. *Front. Environ. Sci.* **2016**, *4*, 69. [CrossRef]
12. Chrysargyris, A.; Maggini, R.; Incrocci, L.; Pardossi, A.; Tzortzakis, N. Copper Tolerance and Accumulation on *Pelargonium graveolens* L'Hér. *Grown Hydroponic Culture. Plants* **2021**, *10*, 1663. [CrossRef]
13. Balafrej, H.; Bogusz, D.; Triqui, Z.-E.A.; Guedira, A.; Bendaou, N.; Smouni, A.; Fahr, M. Zinc Hyperaccumulation in Plants: A Review. *Plants* **2020**, *9*, 562. [CrossRef] [PubMed]

14. Liščáková, P.; Nawaz, A.; Molnárová, M. Reciprocal effects of copper and zinc in plants. *Int. J. Environ. Sci. Technol.* **2022**, *19*, 9297–9312. [CrossRef]
15. Sharma, R.K.; Agrawal, M. Biological effects of heavy metals: An overview. *J. Environ. Biol.* **2005**, *26*, 301–313. [PubMed]
16. D'Imperio, M.; Montesano, F.F.; Serio, F.; Santovito, E.; Parente, A. Mineral composition and bioaccessibility in rocket and purslane after Zn biofortification process. *Foods* **2022**, *11*, 484. [CrossRef]
17. Savvas, D.; Gruda, N. Application of soilless culture technologies in the modern greenhouse industry–A review. *Eur. J. Hortic. Sci.* **2018**, *83*, 280–293. [CrossRef]
18. Pignata, G.; Ertani, A.; Casale, M.; Niñirola, D.; Egea-Gilabert, C.; Fernández, J.A.; Nicola, S. Understanding the Postharvest Phytochemical Composition Fates of Packaged Watercress (*Nasturtium officinale* R. Br.) Grown in a Floating System and Treated with *Bacillus subtilis* as PGPR. *Plants* **2022**, *11*, 589. [CrossRef]
19. Thapa, U.; Nandi, S.; Rai, R.; Upadhyay, A. Effect of nitrogen levels and harvest timing on growth, yield and quality of lettuce under floating hydroponic system. *J. Plant Nutr.* **2022**, *45*, 2563–2577. [CrossRef]
20. Karnoutsos, P.; Karagiovanidis, M.; Bantis, F.; Chatzistathis, T.; Koukounaras, A.; Ntinas, G.K. Controlled root-zone temperature effect on baby leaf vegetables yield and quality in a floating system under mild and extreme weather conditions. *J. Sci. Food Agric.* **2021**, *101*, 3933–3941. [CrossRef]
21. Chrysargyris, A.; Antoniou, O.; Tzionis, A.; Prasad, M.; Tzortzakis, N. Alternative soilless media using olive-mill and paper waste for growing ornamental plants. *Environ. Sci. Pollut. Res.* **2018**, *25*, 35915–35927. [CrossRef]
22. Tzortzakis, N.G.; Economakis, C.D. Shredded maize stems as an alternative substrate medium: Effect on growth, flowering and yield of tomato in soilless culture. *J. Veg. Sci.* **2005**, *11*, 57–70. [CrossRef]
23. Chrysargyris, A.; Antoniou, O.; Athinodorou, F.; Vassiliou, R.; Papadaki, A.; Tzortzakis, N. Deployment of olive-stone waste as a substitute growing medium component for Brassica seedling production in nurseries. *Environ. Sci. Pollut. Res.* **2019**, *26*, 35461–35472. [CrossRef] [PubMed]
24. de Azevedo Neto, A.D.; Prisco, J.T.; Enéas-Filho, J.; de Abreu, C.E.B.; Gomes-Filho, E. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ. Exp. Bot.* **2006**, *56*, 87–94. [CrossRef]
25. Loreto, F.; Velikova, V. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiol.* **2001**, *127*, 1781–1787. [CrossRef]
26. Chrysargyris, A.; Laoutari, S.; Litskas, V.D.; Stavrinides, M.C.; Tzortzakis, N. Effects of water stress on lavender and sage biomass production, essential oil composition and biocidal properties against *Tetranychus urticae* (Koch). *Sci. Hortic.* **2016**, *213*, 96–103. [CrossRef]
27. Wojdyło, A.; Oszmiański, J.; Czemerys, R. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chem.* **2007**, *105*, 940–949. [CrossRef]
28. Tzortzakis, N.G.; Tzanakaki, K.; Economakis, C.D. Effect of origanum oil and vinegar on the maintenance of postharvest quality of tomato. *Food Nutr. Sci.* **2011**, *2*, 974–982. [CrossRef]
29. Calori, A.H.; Moraes, L.A.S.; Purquerio, L.F.V.; Factor, T.L.; Júnior, S.L.; Tivelli, S.W. Electric conductivity and space between plants on baby leaf production in NFT hydroponic system inside greenhouse. *Acta Hortic.* **2015**, *1107*, 303–310. [CrossRef]
30. Di Gioia, F.; Petropoulos, S.A.; Ozores-Hampton, M.; Morgan, K.; Rosskopf, E.N. Zinc and iron agronomic biofortification of brassicaceae microgreens. *Agronomy* **2019**, *9*, 677. [CrossRef]
31. Kumar, V.; Pandita, S.; Sidhu, G.P.S.; Sharma, A.; Khanna, K.; Kaur, P.; Bali, A.S.; Setia, R. Chemosphere copper bioavailability, uptake, toxicity and tolerance in plants: A comprehensive review. *Chemosphere* **2021**, *262*, 127810. [CrossRef] [PubMed]
32. Calabrò, M.R.; Roqueiro, G.; Tapia, R.; Crespo, D.C.; Bargiela, M.F.; Young, B.J. Chronic toxicity, bioavailability and bioaccumulation of Zn, Cu and Pb in *Lactuca sativa* exposed to waste from an abandoned gold mine. *Chemosphere* **2022**, *307*, 135855. [CrossRef] [PubMed]
33. Cuba-Díaz, M.; Marín, C.; Castel, K.; Machuca, Á.; Rifo, S. Effect of copper(II) ions on morpho-physiological and biochemical variables in *Colobanthus quitensis*. *J. Soil Sci. Plant Nutr.* **2017**, *17*, 429–440. [CrossRef]
34. Shahid, M.; Pourrut, B.; Dumat, C.; Nadeem, M.; Aslam, M.; Pinelli, E. Heavy-Metal-Induced Reactive Oxygen Species: Phytotoxicity and Physicochemical Changes in Plants. In *Reviews of Environmental Contamination and Toxicology*; Whitacre, D., Ed.; Springer: Cham, Switherland, 2014; Volume 232. [CrossRef]
35. Cabot, C.; Martos, S.; Llugany, M.; Gallego, B.; Tolrà, R.; Poschenrieder, C. A Role for Zinc in Plant Defense Against Pathogens and Herbivores. *Front. Plant Sci.* **2019**, *10*, 1171. [CrossRef]
36. Shabbir, Z.; Sardar, A.; Shabbir, A.; Abbas, G.; Shamshad, S.; Khalid, S.; Natasha Murtaza, G.; Dumat, C.; Shahid, M. Copper Uptake Essentiality Toxicity Detoxification and Risk Assessment in Soil-Plant Environment. *Chemosphere* **2020**, *259*, 127436. [CrossRef]
37. Adrees, M.; Ali, S.; Rizwan, M.; Ibrahim, M.; Abbas, F.; Farid, M.; Zia-ur-Rehman, M.; Irshad, M.K.; Bharwana, S.A. The effect of excess copper on growth and physiology of important food crops: A review. *Environ. Sci. Pollut. Res.* **2015**, *22*, 8148–8162. [CrossRef] [PubMed]
38. Khalilzadeh, R.; Pirzad, A.; Sepehr, E.; Anwar, S.; Khan, S. Physiological and biochemical responses of *Phragmites australis* to wastewater for different time duration. *Acta Physiol. Plant.* **2022**, *44*, 130. [CrossRef]

39. Lešková, A.; Javot, H.; Giehl, R.F.J. Metal crossroads in plants: Modulation of nutrient acquisition and root development by essential trace metals. *J. Exp. Bot.* **2022**, *73*, 1751–1765. [CrossRef]
40. European Food Safety Authority (EFSA). Scientific Opinion on Dietary Reference Values for zinc. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *EFSA J.* **2014**, *12*, 3844. [CrossRef]
41. European Food Safety Authority (EFSA). Scientific Opinion on Dietary Reference Values for copper. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *EFSA J.* **2015**, *13*, 4253. [CrossRef]
42. Söderqvist, K.; Rosberg, A.K.; Boqvist, S.; Alsanius, B.; Mogren, L.; Vågsholm, I. Season and Species: Two Possible Hurdles for Reducing the Food Safety Risk of *Escherichia coli* O157 Contamination of Leafy Vegetables. *J. Food Prot.* **2019**, *82*, 247–255. [CrossRef] [PubMed]