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Response of Aeroponically Cultivated Baby-Leaf Lettuce (*Lactuca sativa* L.) Plants with Different Zinc, Copper, Iodine, and Selenium Concentrations

Martina Puccinelli *, Andrea De Padova, Paolo Vernieri, Giulia Carmassi and Luca Incrocci

Department of Agriculture Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy; a.depadova@studenti.unipi.it (A.D.P.); paolo.vernieri@unipi.it (P.V.); giulia.carmassi@unipi.it (G.C.); luca.incrocci@unipi.it (L.I.)

* Correspondence: martina.puccinelli@agr.unipi.it

Abstract: Over two billion people worldwide suffer from micronutrient deficiencies. Biofortifying vegetables can enhance micronutrient intake through the diet. This study assessed the biofortification of indoor-grown baby-leaf lettuce using aeroponics. Four experiments, two each, were conducted by adding different concentrations of Zn (from 10 to 450 μM) or Cu (from 3 to 250 μM) into a nutrient solution. A fifth experiment was conducted by simultaneously adding to the nutrient solution the optimal concentration of I (5 μM) and Se (13 μM), chosen on the basis of previous works, and the optimal concentration of Zn (250 μM) and Cu (150 μM), chosen on the basis of the results obtained in the first four experiments. Leaf biomass, mineral concentrations, chlorophylls, carotenoids, phenols, flavonoids, nitrates, and antioxidant capacity were measured 21 days after transplanting. Higher concentrations of Cu, Zn, I, or Se in the nutrient solution led to an increase in their concentrations in lettuce leaves, without affecting the growth or leaf quality of lettuce plants. The simultaneous application of I with the other elements induced a higher accumulation in leaves compared to when I is applied alone. One hundred grams of lettuce leaves biofortified with Se, I, Cu, and Zn would provide the 6.1%, 35.3%, and 263.0% of Adequate Intake for Cu, Se, and I, respectively, and 4.5% of Population Reference Intake for Zn. Our results suggest that simultaneously biofortifying baby-leaf lettuce with these four minerals is a practical and convenient way to integrate these micronutrients into the diet without reducing the yield or quality of lettuce.

Keywords: biofortification; soilless farming; indoor cultivation; leafy vegetables

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1. Introduction

The insufficient uptake of micronutrients affects over two billion people worldwide [1]. Copper (Cu) [2] and zinc (Zn) [3] are two of the most deficient minerals, and their deficiency can cause a number of health problems, including digestion disorders, anemia, hypertension, diabetes mellitus, hormone imbalance, exhaustion, breathing difficulties, weakened immune system, hair loss, and skin health issues [4,5]. Approximately 30% of the global population is affected by iodine (I) deficiency [6]. Iodine is an essential microelement for humans, and it is involved in the biosynthesis of thyroid hormone [7]. About 15% of the global population is affected by selenium (Se) deficiency [6]. In humans and animals, Se, is an essential element, acting as part of seleno-aminoacids and seleno-proteins, and is a cofactor of glutathione peroxidase (GSH-Px; EC 1.11.1.9), [8]. Furthermore, Se plays a role in thyroid hormone metabolism, antioxidant defense, and immune function [8].

The human intake of Cu, Zn [9], I [10], and Se [11] can be increased by the biofortification of vegetables during plant growth, which improves their nutritional quality [12].

Biofortification is the process by which the mineral content of vegetables is increased during cultivation. The application of low concentrations of minerals does not result in toxicity in plants, whereas higher doses may induce a reduction in production and a

deterioration in the quality of the plants. The toxic dose is generally dependent on both the mineral element applied and the plant species treated [13].

A food is considered a good source of a certain mineral when 100 g provides at least 15% of the recommended daily dose of the mineral in question. It can be seen that to satisfy the 15% of adequate intake (AI) of Cu [14], I [15], and Se [16], and the population reference intake (PRI) of Zn [17] proposed by the European Food Safety Authority, biofortified lettuce fresh leaves should contain 13–16 mg kg^{-1} of Cu, 6 mg kg^{-1} of I, 0.7 mg kg^{-1} of Se, and 75–163 mg kg^{-1} of Zn.

The simultaneous application of Cu, Zn, Se, and I would promote the intake by humans of four essential elements at the same time through the diet. Furthermore, the simultaneous use of Cu, Zn, Se, and I for biofortification may be toxic for plants [18]. Several works have been conducted in hydroponics on I [19–22], Se [22–25], and Zn enrichment [26–29]. Conversely, there are few studies about the biofortification of vegetables with Cu. The majority of existing studies have focused on the impact of foliar application of Cu nanoparticles [30,31]. To the best of our knowledge, and according to the review of Kathi et al. [32], there is no previous research investigating the simultaneous biofortification of plants with Cu, Zn, I, and Se. The simultaneous biofortification with Se and I has only been investigated in spinach [33] and lettuce [22,34–36]. Furthermore, the simultaneous application of Zn, I, and Se has been studied in wheat [37]. To date, no research has been conducted on the simultaneous biofortification with Cu and other minerals. The biofortification of plants with multiple mineral elements may result in an increased risk of plant toxicity, making it necessary to find the optimal biofortification dose for multiple elements simultaneously. These could be some of the reasons that determine the absence of work on contemporary biofortification with Se, I, Cu, and Zn.

The utilization of closed-loop soilless systems facilitates the production of biofortified vegetables and enables the attainment of a superior yield and quality, with less fertilizers and water. Among the various soilless cultivation systems, aeroponics, which involves suspending the roots of plants in the air and spraying them, frequently or at intervals, with the nutrient solution [38], is a nutrient solution used to grow leafy vegetables, including lettuce [21,39]. Aeroponic cultivation avoids the problem of root hypoxia. Furthermore, the radical absorption of the minerals is facilitated by the continuous mixing of the solution. It is also possible to change the solution applied during the cultivation cycle without having to drain and replace the solution, but simply by acting on the hydraulic system. The lightness of the aeroponic system allows its use in vertical cultivation systems, such as in plant factories with artificial light (PFALs) [38]. Moreover, the control of the management of the nutrient solution and the controlled growth environment, by standardizing the growth conditions, make the biofortification process more controllable and the leaf mineral concentrations obtained more predictable [40].

In numerous countries, there is a growing trend in cultivating high-value fresh vegetables, such as ready-to-eat immature leaves of leafy vegetables [41], and herbs in PFALs. In PFALs, the use of multi-tiered cultivation systems and the maintenance of optimal environmental conditions enhances crop yield [42]. Biofortified vegetables are particularly interesting for production in PFALs. Indeed, given the high production costs within the PFALs, in these facilities, it is necessary to grow vegetables that stand out in the market, for example, for a greater nutraceutical value [43].

There is a lack of knowledge on the biofortification of vegetables with Zn, Cu, and on simultaneous biofortification with Zn, Cu, I, and Se. This study was carried out to evaluate the possibility of producing lettuce biofortified simultaneously with Zn, Cu, I, and Se. The effects of the addition, to the nutrient solution, of Zn-EDDTA, Cu-EDDTA, potassium iodide (KI), and sodium selenate (Na_2SeO_4), both separately and simultaneously, on leaf Zn, Cu, I, and Se accumulation, and plant growth and leaf quality of baby-leaf lettuce, were evaluated. Lettuce plants were grown indoors in aeroponics.

2. Materials and Methods

2.1. Experimental Site and Environmental Condition

Five experiments were conducted, between November 2022 and March 2023, at the University of Pisa. The experiments involved the cultivation of baby leaf green lettuce (*Lactuca sativa* L.; cv "Nelson", ISI Sementi S.p.a, Parma, Italy) in aeroponics using a growth chamber, (Table 1). The five experiments differ only for the Cu, Zn, Se, and I concentrations in the nutrient solution.

Table 1. Description of treatments performed during the different experiments.

Experiment	Treatment		Concentration (μM)	Concentration (mg L^{-1})
Cu_1	Control	Cu	3	0.19
	Cu.25		25	1.59
	Cu.50		50	3.18
	Cu.75		75	4.76
Cu_2	Control	Cu	3	0.19
	Cu.150		150	9.53
	Cu.200		200	12.70
	Cu.250		250	15.88
Zn_1	Control	Zn	10	0.65
	Zn.50		50	3.27
	Zn.100		100	6.54
	Zn.150		150	9.51
Zn_2	Control	Zn	10	0.65
	Zn.250		250	16.35
	Zn.350		350	22.89
	Zn.450		450	29.43
Mix	Control	Cu	3	0.19
		Zn	10	0.65
		Se	0	0
		I	0	0
	Se.13	Se	13	1.03
	I.5	I	5	0.63
	Mix	Cu	150	9.53
		Zn	250	16.35
		Se	13	1.03
		I	5	0.63

Air temperature and relative humidity were kept at 24.0 °C and 65–70%, respectively, inside the growth chamber by a climate control system. Lettuce plants were illuminated by red, blue, and green (65% R, 15% B, 20% G) LED lamps (Ambralight, Vicenza, Italy) with a 16 h photoperiod at 200 μmol m^{-2} s^{-1} PPFD.

2.2. Plant Material

Lettuce seeds were sown in 240-cell trays with stone wool plugs. Seven days after sowing, the seedlings were transplanted in aeroponics. Twelve separate aeroponic systems were used. Aeroponic systems were provided by Edo Radici Felici Srl (Pontinia, LT, Italy). Each aeroponic system consisted of a 90 L growth box (67 × 41 cm, height 32 cm), closed on

the top by a plastic tray hosting 380 plants (1400 plants m^{-2}), and contained 30 L of nutrient solution at the bottom (height of the nutrient solution: 7 cm; Figure 1). The free empty space available for root growth was about 25 cm. The nutrient solution was taken from the bottom of the tank, pressurized by an external pump (electric voltage 12 volts, maximum capacity 6 L min^{-1}, max pressure 4 atm), and sprayed onto the plant roots through the nozzles (EdoMax0.5, micro sprinkler heads made of reinforced polyethylene, flow rate 0.5 L min^{-1}), for 30 s every 10 min, then collected again at the bottom of the tank. The time spraying was regulated by an Arduino IDE 2.1.0 board.

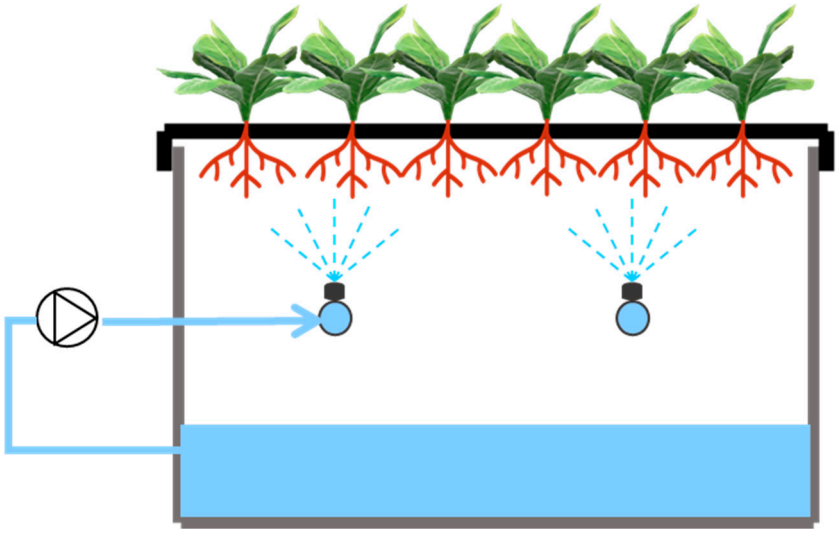

Figure 1. Operation diagram of the aeroponic systems used for the experiments.

2.3. Experimental Design and Treatments

Four days after transplant, different concentrations of Se (as Na_2SeO_4), Zn (as Zn EDTA), Cu (as Cu EDTA), and I (as KI) were added to the nutrient solutions. Each treatment had three replicates (1140 plants $treatment^{-1}$), each consisting of one aeroponic system. The plants were harvested 10 days after the start of treatments.

Two experiments were conducted on biofortification with Zn and two with Cu. To find out the best concentration for biofortification, during the first experiments on Zn (Zn_1) or Cu (Cu_1) biofortification, three concentrations of Zn or Cu, other than control, were tested, respectively. Since, during the first experiments, Zn and Cu were scarcely accumulated in lettuce leaves, in the second experiments on Zn (Zn_2) or Cu (Cu_2) biofortification, higher concentrations of Zn or Cu were used. In all the experiments, in the Control treatment, no minerals were added to the standard nutrient solution. The different elements and concentrations used in each experiment are reported in Table 1.

The Se and I concentrations used in the Mix experiment, were chosen on the base of the results obtained in experiments previously conducted on baby leaves lettuce grown in aeroponic biofortified with Se and I [22]. Whereas the concentration of Zn and Cu were chosen on the basis of the results obtained in the first 4 experiments (Zn_1, Cu_1, Zn_2, Zn_2).

2.4. Nutrient Solution Management

During the cultivation, in each aeroponic system, the nutrient solution was added to keep its level above 7 cm. The nutrient solution was never discharged during the experiment. The nutrient solution had a pH of 5.6 and an electrical conductivity (EC) of 2.37 dS m^{-1}, and contained the following concentration of nutritive elements: N-NO_3 10.0 mM, N-NH_4 0.26 mM, P 1.5 mM, K 9.0 mM, Ca 4.5 mM, Mg 2.0 mM, Fe 40.0 µM, B 40.0 µM, Cu 3.0 µM, Zn 10.0 µM, Mn 10.0 µM, and Mo 1.0 µM. During the cultivation, the pH and EC of the nutrient solution were measured every three days. The pH was adjusted

to stay in a range of ±10% of the initial value, and the EC varied by a maximum of ±20% from the initial value during the cultivation cycle.

2.5. Plant Growth

Leaf fresh (FW) and dry weight (DW) were measured in 100 plants collected in each replicate at the harvesting commercial stage, ranging between 21 days after the transplant. Dry weight was determined in plant samples that were dried in a ventilated oven at 50 °C until they reached of constant weight.

2.6. Leaf Quality Attributes

The concentration of total phenols, flavonoids, chlorophylls, and carotenoids, and the antioxidant capacity was determined at harvest in fresh leaves, after extraction in 99% *v*/*v* methanol. Using the Folin–Ciocalteu reagent technique [44], the total phenol content in the methanol extract was measured. The results were reported as mg of gallic acid equivalent (GAE) per g of fresh weight (FW). At 765 nm, the absorbance was measured, and a calibration curve comprising 0, 50, 100, 150, and 250 mg of gallic acid L^{-1} was used to determine the concentration. The method proposed by Kim et al. [45] was used to determine the flavonoid content in leaves. Briefly, 0.1 mL of the methanol extract was mixed with 0.06 mL of $NaNO_2$ (5%) and 0.04 mL of $AlCl_3$ (10%). Five minutes later, 0.4 mL of NaOH and 0.2 mL of H_2O were added. At 510 nm, the absorbance was measured. The results were represented as milligrams of catechin per gram of fresh weight. The antioxidant capacity was assessed using the ferric-reducing ability of the plasma (FRAP) method [46], and the results were expressed as μmol of Fe(II) per g of FW. Spectrophotometric measurements of total chlorophylls and carotenoids at 662.5, 652.4, and 470 nm were made on the methanol extract. The formulas provided by Lichtenthaler were used to calculate the concentrations of carotenoids and chlorophylls [47].

2.7. Mineral Concentration

The concentrations of potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) were determined in dried lettuce leaves, after mineralization with 65% $HNO_3{}^-$ and 30% H_2O_2 in a 5:2 ratio, at 220 °C for one hour, by atomic absorption spectroscopy. All determinations were made in triplicate, and the accuracy of the measurements was tested using a tomato leaf Certified Reference Material 1573a (CRM 1573a) from the National Institute of Standards and Technology (Gaithersburg, MD, USA). The same extracts were used for the determination of P-PO_4 concentration utilizing UV/Vis spectrometry [48]. The dried leaf samples were extracted with distilled water at room temperature for two hours; then, the extracts were used to determine spectrophotometrically the nitrate concentration by the salicylic sulfuric acid technique [49]. The organic nitrogen content in dried samples was assessed utilizing the Kjeldahl method [50].

The UNI EN13657:2004 [51] and UNI EN ISO 17294-2:2016 [52] methods were used for the sample digestion and selenium determination, respectively, in oven-dried ground samples. Three replicates were analyzed for each treatment by the CAIM group (Follonica, GR, Italy).

Oven-dried ground samples were used to determine the inorganic iodine concentration, as previously reported by Puccinelli et al. [21].

2.8. Contribution to Copper, Zinc, Selenium, or Iodine Dietary Intake and Maximum Daily Intake

Conventionally, a serving size of food is often indicated as 100 g. Therefore, the estimated dietary intake (EDI, μg d^{-1}) of Cu, Zn, Se, or I was calculated based on the amount of Cu, Zn, Se, or I provided by an assumed portion of 100 g of lettuce leaves and expressed as a percentage of adequate intake (AI) for Cu, Se, and I, or as the population reference intake (PRI) for Zn. The AI of Cu for adults is 1.6 and 1.3, respectively, for men and women [14]: to calculate the % of AI satisfied by the consumption of biofortified

lettuce, we used an average value of 1.45 mg d^{-1}. The AI of Se and I in adults is set at 70 µg d^{-1} [16] and 150 µg d^{-1} [15], respectively. Regarding Zn, EFSA provides PRI, which ranges between 7.5 and 16.3 mg d^{-1}, for four levels of phytate intake [17]. In this work, EDI was expressed as a percentage of the mean PRIs set for adults (11.5 mg d^{-1}). We also calculate the amount of biofortified fresh lettuce leaves that would provide 100% of AI of Cu, I, and Se or of Zn PRI.

2.9. Statistical Analysis

Cu, Zn, I, and Se treatments were used as factors in a one-way ANOVA analysis of the data, which were then presented as the mean values (±standard error) of three replicates. Levene's test was used to check for homogeneity of variances and Shapiro–Wilk test was used to determine whether the distribution of the data was normal. Tukey's post hoc test was used to differentiate the mean values ($p < 0.05$). JMP Pro 17 statistical software was used to conduct statistical analysis.

3. Results and Discussion

3.1. Biofortification with Copper (Experiments Cu_1 and Cu_2)

In the Cu_1 experiment, the concentration of Cu in lettuce leaves was slightly affected by the concentration of Cu in the nutrient solution, and a significant increment was only detected in plants treated with 50 µM of Cu, with no differences between Cu.25, Cu.50, and Cu.75 treatments (Figure 2A). In the Cu_2 experiment, the application of a higher concentration of Cu induced a higher concentration of Cu in the leaves, without differences between plants treated with 150, 200, and 250 µM of Cu (Figure 2B). There are few studies about the biofortification of vegetables with Cu, most of which investigated the effect of foliar application of Cu nanoparticles, and contrasting results are reported [30,31,53]. Our results are in agreement with a previous study conducted on lettuce plants grown with different concentrations of Cu in soil, where treatment with nanoparticles or microparticles of Cu at concentrations of 3.15 and 6.30 mmol kg^{-1} of soil slightly increased the Cu concentration in leaves (by 3.6–13.8%) [30]. On the contrary, in the same study, a higher amount of Cu was accumulated in roots [30]. Moreover, in another study, the foliar application of Cu nanoparticles at concentrations ranging from 5 to 25 mg L^{-1} did not induce an increase in Cu concentration in lettuce leaves [53]. This could explain the fact that plants accumulate Cu in roots and seeds but not in leaves [54]. On the contrary, spinach plants treated with a Cu concentration in soil ranging from 16 to 47 µmol kg^{-1} showed a Cu concentration in leaves up to 4.5-fold higher than in the control [55]. Thus, plant species and way of application could play a role in Cu accumulation by plants.

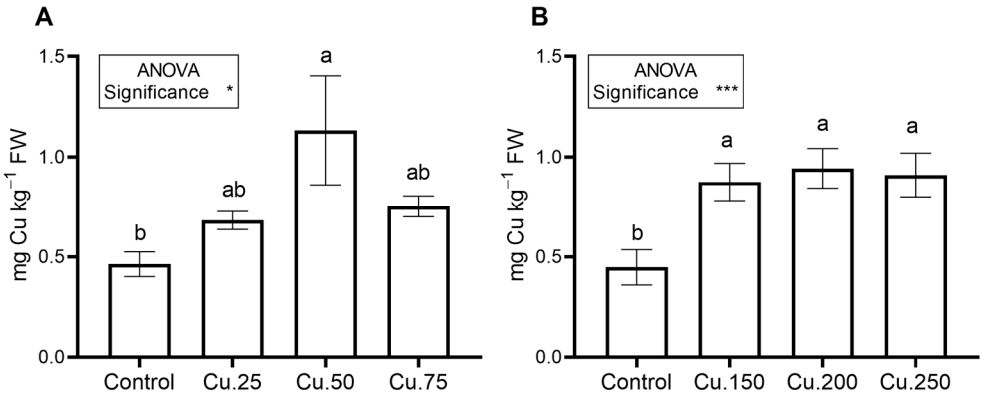

Figure 2. Experiment Cu_1 (**A**) and Cu_2 (**B**). Copper (Cu) concentration in leaves of lettuce plants grown, indoors in aeroponics, with different concentrations of Cu in the nutrient solution: 3 µM (Control), 25 µM (Cu.25), 50 µM (Cu.50), 75 µM (Cu.75), 150 µM (Cu.150), 200 µM (Cu.200), 250 µM (Cu.250). Means (n = 3) flanked by the same letter are not statistically different for $p = 0.05$ after Tukey's test. Significance level: * $p \leq 0.05$; *** $p \leq 0.001$.

The concentration of Cu in the leaf of biofortified lettuce ranged from 0.682 to 1.132 mg kg^{-1} FW. Thus, 100 g of fresh Cu-biofortified lettuce leaves would satisfy from 4.7 to 7.8 % of the adequate intake (AI) of Cu (Table 2). To satisfy the 100% AI for Cu (1.45 mg d^{-1}), it would be necessary to consume from 1319 to 2131 g of biofortified lettuce leaves.

Table 2. Percentage of adequate intake (AI), for Se, I, and Cu, or of population reference intake (PRI), for Zn, satisfied by the consumption of 100 g of fresh leaves (FW) of lettuce plant grown indoors, in aeroponics, with different concentrations of Cu, Zn, I, and Se in the nutrient solution, and the amount of FW that would provide the 100% of AI, for Se, I, and Cu, or 100% of PRI, for Zn.

Experiment	Treatment		Concentration (μM)	% AI or % PRI per 100 g FW	g FW 100% AI or PRI
Cu_1	Control	Cu	3	3.20	3154.6
	Cu.25		25	4.70	2131.2
	Cu.50		50	7.81	1319.0
	Cu.75		75	5.20	1929.3
Cu_2	Control	Cu	3	3.09	3333.4
	Cu.150		150	6.04	1672.0
	Cu.200		200	6.49	1553.6
	Cu.250		250	6.25	1616.6
Zn_1	Control	Zn	10	2.73	3677.0
	Zn.50		50	3.65	2750.4
	Zn.100		100	4.74	2109.6
	Zn.150		150	4.71	2133.1
Zn_2	Control	Zn	10	3.18	3167.6
	Zn.250		250	5.55	1805.1
	Zn.350		350	4.62	2172.3
	Zn.450		450	5.42	1863.3
Mix	Control	Cu	0	2.33	4368.5
		Zn	0	2.37	4274.6
		Se	0	0	-
		I	0	0.83	15,655.1
	Se.13	Se	13	35.79	279.9
	I.5	I	5	101.89	99.8
	Mix	Cu	150	6.12	1656.8
		Zn	250	4.48	2249.2
		Se	13	35.32	284.8
		I	5	262.9	38.2

In both our experiments, treatments with a growing concentration of Cu in the nutrient solution did not affect plant growth, since no differences in fresh (1905.7 g m^{-2} and 1965.6 g m^{-2}, on average, respectively in experiment Cu_1 and Cu_2) and dry (73.5 g m^{-2} and 72.0 g m^{-2}, on average, respectively in experiment Cu_1 and Cu_2) weight were detected (Tables S1 and S2). In our experiments, the limited leaf concentration of Cu in biofortified lettuce could explain the lack of toxic effects on plant growth. In fact, crop species can tolerate a maximum of 20–30 mg kg^{-1} DW of Cu in leaves [56].

In both experiments, qualitative parameters, such as the concentration of chlorophylls (0.956 mg g^{-1} FW and 0.960 mg g^{-1} FW, on average, respectively in experiment Cu_1 and Cu_2); carotenoids (0.115 mg g^{-1} FW and 0.122 mg g^{-1} FW, on average, respectively in experiment Cu_1 and Cu_2); flavonoids (0.558 mg g^{-1} FW and 0.881 mg g^{-1} FW, on average, respectively in experiment Cu_1 and Cu_2); phenols (1.90 mg g^{-1} FW and 2.48 mg g^{-1} FW, on average, respectively in experiment Cu_1 and Cu_2); antioxidant capacity (5.85 mmol Fe(II) kg^{-1} FW and 9.42 mmol Fe(II) kg^{-1} FW, on average, respectively in experiment Cu_1 and Cu_2); and dry matter content (3.88% FW and 3.66% FW, on average, respectively in experiment Cu_1 and Cu_2), were not affected by treatment with Cu (Tables S1 and S2).

This suggests that the treatments with Cu did not induce oxidative stress in lettuce plants. This is consistent with previous studies that have demonstrated that nanoparticles of Cu do not increase the production of reactive oxygen species (ROS) and malondialdehyde (MDA) in lettuce leaves [30]. The nitrate concentration in lettuce leaves was not affected by treatment with Cu (2328.5 mg kg^{-1} FW and 2510.7 mg kg^{-1} FW, on average, respectively in experiment Cu_1 and Cu_2), and was always below the maximum values set for lettuce (5000 mg kg^{-1} FW) grown in a greenhouse during fall–winter season [57].

The effect of copper (Cu) application on plant mineral uptake and accumulation is influenced by a number of factors, including the specific plant species, the concentration of Cu in the growing substrate, the duration of the treatment, and the growth conditions. Copper toxicity in plants typically results in a reduction in the uptake and accumulation of Ca, Mg, K, Mn, S, and Fe [58]. In our experiments, the mineral concentration of leaves was not affected by Cu treatment (Tables S1 and S2).

3.2. Biofortification with Zinc (Experiments Zn_1 and Zn_2)

In the Zn_1 experiment, a higher concentration of Zn was detected in the leaves of plants treated with 100 and 150 μM of Zn, compared to the control. No differences were observed between these two treatments (Figure 3A). In the Zn_2 experiment, all the concentrations of Zn used for biofortification induced a higher leaf concentration of Zn, with the highest value detected in plants treated with 250 μM of Zn (Figure 3B). The increase in Zn leaf concentration observed in the present study is less pronounced than that in two previous works conducted on lettuce [26] and *Brassica oleracea* [27] plants grown indoors in hydroponics. In those studies, the Zn concentration in leaves ranged from 115.7 to 265.3 mg kg^{-1} DW and from 79.7 to 464.7 mg kg^{-1} DW, respectively, in lettuce [26] and *Brassica oleracea* [27] plants treated with Zn in the nutrient solution from 20 to 100 μM. On the contrary, the highest Zn leaf concentration detected in our experiment ranged from 132.7 to 144.8 mg kg^{-1} DW, and was observed in treatments with Zn concentrations of 100 to 450 μM. Furthermore, a positive linear correlation, between the Zn concentration in leaves and in the growing medium, was detected in leafy brassica treated with Zn up to 7.6 mM [28]. Moreover, the Zn concentrations detected in lettuce leaves in the present experiment are similar to those observed by Ciriello et al. [29] in leaves of hydroponically grown basil plants treated with 50 μM of Zn in the nutrient solution. Moreover, an experiment conducted on lettuce plants by de Lima et al. [59] demonstrated that treatments with Zn concentration ranging from 15.3 to 36.7 μM linearly increase the Zn concentration in leaves, up to approximately twice the concentration in the control. On the contrary, the Zn concentration in roots increased quadratically. When Zn is applied in large amounts, Zn content is higher in root than leaf tissues due to the genetic regulation of the absorption and transport of this micronutrient, which determines the seven-to-eight-fold less Zn accumulated in the leaves than in the roots [60]. To the best of our knowledge, in the literature, there are no published papers about the biofortification of leafy vegetables in aeroponics. The cultivation system could affect the Zn uptake by plants and thus the accumulation of Zn in shoots.

The concentration of Zn in the leaves of biofortified lettuce ranged from 4.20 to 6.38 mg kg^{-1} FW. Therefore, 100 g of fresh Zn-biofortified lettuce leaves would provide between 3.7 and 5.6% of the adequate intake (AI) of Zn (Table 2). In order to satisfy the 100% PRI for Zn, it would be necessary to consume from 1805 to 2750 g of biofortified lettuce leaves per day.

In both experiments, treatments with a growing concentration of Zn in the nutrient solution did not affect plant growth, since no differences in fresh and dry weight were detected (Tables 3 and 4). The absence of toxic effects on plant growth may be attributed to the low concentration of Zn observed in plants treated with a high concentration of Zn in the nutrient solution. Indeed, the symptoms of Zn toxicity in lettuce plants are detected with a concentration of Zn in the shoot higher than those obtained in our experiments, as reviewed by Kaur and Garg [61].

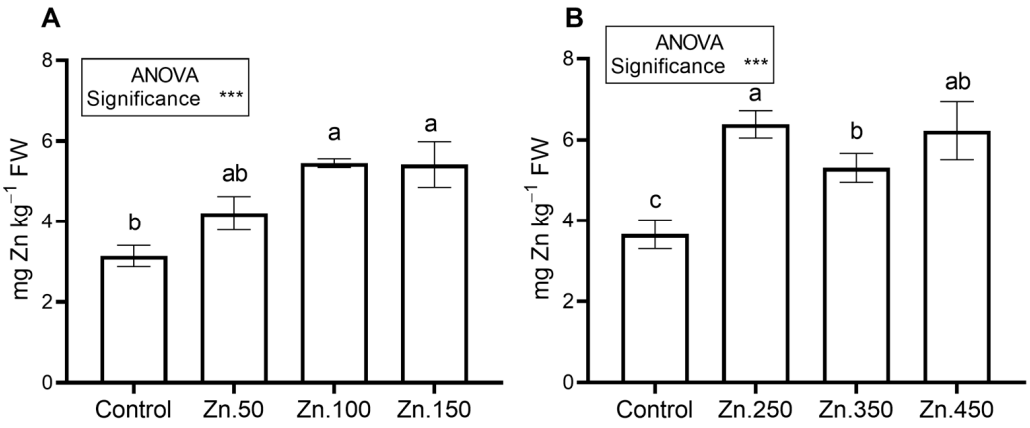

Figure 3. Experiment Zn_1 (**A**) and Zn_2 (**B**). Zinc (Zn) concentration in leaves of lettuce plants grown, in aeroponics, with different concentrations of Zn in the nutrient solution: 10 µM (Control), 50 µM (Zn.50), 100 µM (Zn.100), 150 µM (Zn.150). Means (n = 3) flanked by the same letter are not statistically different for $p = 0.05$ after Tukey's test. Significance level: *** $p \leq 0.001$.

Table 3. Experiment Zn_1. Fresh (FW) and dry (DW) biomass, dry matter content, mineral, chlorophyll, carotenoid, flavonoid, phenol, nitrate concentration, and antioxidant capacity in leaves of lettuce plants grown indoors in aeroponics, with different concentrations of Zn in the nutrient solution: 10 µM (Control), 50 µM (Zn.50), 100 µM (Zn.100), 150 µM (Zn.150). Means (n = 3) flanked by the same letter are not statistically different for $p = 0.05$ after Tukey's test. Significance level: * $p \leq 0.05$; ns = not significant.

		Treatment				
	u.m.	**Control**	**Zn.50**	**Zn.100**	**Zn.150**	**ANOVA**
Fresh weight	g m^{-2}	2062.7 ± 200.0	2060.1 ± 112.5	1982.1 ± 262.0	1915.1 ± 182.9	ns
Dry weight	g m^{-2}	82.8 ± 8.0	80.6 ± 2.9	75.5 ± 6.7	71.2 ± 1.5	ns
Dry matter content	% FW	4.01 ± 0.00	3.93 ± 0.36	3.83 ± 0.17	3.76 ± 1.44	ns
N-tot	g kg^{-1} FW	2.80 ± 0.22	3.01 ± 0.37	2.78 ± 0.13	2.80 ± 0.14	ns
K	g kg^{-1} FW	3.83 ± 0.40	3.64 ± 0.40	3.88 ± 0.13	4.05 ± 0.14	ns
P	g kg^{-1} FW	0.215 ± 0.038	0.402 ± 0.013	0.339 ± 0.057	0.364 ± 0.102	ns
Ca	g kg^{-1} FW	0.894 ± 0.142	0.861 ± 0.110	0.881 ± 0.100	0.909 ± 0.120	ns
Na	g kg^{-1} FW	0.025 ± 0.002 b	0.079 ± 0.012 a	0.061 ± 0.006 ab	0.096 ± 0.009 a	*
Mg	g kg^{-1} FW	0.207 ± 0.008	0.168 ± 0.003	0.217 ± 0.010	0.216 ± 0.015	ns
Mn	mg kg^{-1} FW	6.63 ± 0.01	5.19 ± 0.12	6.70 ± 0.11	6.23 ± 0.93	ns
Fe	mg kg^{-1} FW	14.55 ± 0.23	13.22 ± 4.27	16.63 ± 0.85	13.79 ± 6.35	ns
Cu	mg kg^{-1} FW	0.637 ± 0.012 a	0.540 ± 0.030 b	0.570 ± 0.002 b	0.541 ± 0.014 b	ns
Chlorophylls	mg g^{-1} FW	0.871 ± 0.140	1.041 ± 0.078	1.051 ± 0.068	1.081 ± 0.006	ns
Carotenoids	mg g^{-1} FW	0.145 ± 0.009	0.179 ± 0.013	0.166 ± 0.001	0.183 ± 0.004	ns
Flavonoids	mg g^{-1} FW	0.563 ± 0.107	0.772 ± 0.151	0.723 ± 0.094	0.991 ± 0.079	ns
Phenols	mg g^{-1} FW	1.50 ± 0.15	1.89 ± 0.29	1.40 ± 0.04	1.87 ± 0.07	ns
Antioxidant capacity	mmol Fe (II) kg^{-1} FW	7.68 ± 1.40	8.78 ± 1.54	7.83 ± 0.09	9.93 ± 0.08	ns
NO_3	mg kg^{-1} PF	2599.9 ± 263.8	2902.9 ± 695.7	2539.7 ± 164.8	2744.6 ± 672.5	ns

The application of elevated zinc concentrations in the nutrient solution did not result in discernible alterations in the mineral composition or quality of the leaves of lettuce plants, with the exception of the leaf concentration of sodium during the Zn_1 experiment (Table 3) and Ca during the Zn_2 experiment (Table 4). During the Zn_1 experiment, the leaf Na concentration was slightly higher than control in plants treated with 50 and 150 µM of Zn (Table 3). On the contrary, in the Zn_2 experiment, the leaf Ca concentration was decreased in the same way by treatment with 250, 350, and 450 µM of Zn, compared to control (Table 4). In both experiments, the nitrate concentration in lettuce leaves was not affected by treatment with Zn (Table 3), and was always below the maximum limit set for lettuce [57]. The limited effects on leaf quality and leaf mineral concentrations indicate that the dose of Zn used for treatments did not induce toxicity or mineral deficiencies in lettuce leaves. Ciriello et al. [29] reported a reduction in chlorophyll concentration and an increased phenol concentration and antioxidant capacity in basil leaves grown hydroponically with Zn concentrations ranging from 12.5 µM to 50 µM. An enhanced antioxidant capacity

and total phenol concentration were previously detected in lettuce leaves treated with Zn concentrations from 25 to 100 µM via foliar application [62]. Therefore, the effect of Zn treatment on leaf quality may depend on plant species, cultivation system, and way of application (as reviewed by Szerement et al. [63]).

Table 4. Experiment Zn_2. Fresh (FW) and dry (DW) biomass, dry matter content, mineral, chlorophyll, carotenoid, flavonoid, phenol, nitrate concentration and antioxidant capacity in leaves of lettuce plants grown indoor in aeroponics, with different concentrations of Zn in the nutrient solution: 10 µM (Control), 250 µM (Zn.250), 350 µM (Zn.350), 450 µM (Zn.450). Means (n = 3) flanked by the same letter are not statistically different for $p = 0.05$ after Tukey's test. Significance level: *** $p \leq 0.001$; * $p \leq 0.05$; ns = not significant.

		Treatment				
	u.m.	**Control**	**Zn.250**	**Zn.350**	**Zn.450**	**ANOVA**
Fresh weight	g m^{-2}	1892.3 ± 71.8	1637.1 ± 98.1	1726.5 ± 224.5	1762.5 ± 129.6	ns
Dry weight	g m^{-2}	86.3 ± 3.5	74.6 ± 7.8	68.5 ± 6.4	78.3 ± 6.5	ns
Dry matter content	% FW	4.59 ± 0.28	4.53 ± 0.27	4.02 ± 0.16	4.33 ± 0.19	ns
N-tot	g kg^{-1} FW	3.13 ± 0.14	3.05 ± 0.12	2.64 ± 0.10	2.99 ± 0.14	ns
K	g kg^{-1} FW	3.66 ± 0.27	3.16 ± 0.31	2.74 ± 0.07	3.36 ± 0.46	ns
P	g kg^{-1} FW	0.617 ± 0.080	0.544 ± 0.052	0.435 ± 0.022	0.453 ± 0.018	ns
Ca	g kg^{-1} FW	0.594 ± 0.027 a	0.377 ± 0.019 b	0.473 ± 0.019 b	0.440 ± 0.037 b	***
Na	g kg^{-1} FW	0.059 ± 0.003	0.056 ± 0.003	0.061 ± 0.002	0.070 ± 0.002	ns
Mg	g kg^{-1} FW	0.184 ± 0.01 a	0.146 ± 0.01 b	0.152 ± 0.00 b	0.156 ± 0.01 ab	*
Mn	mg kg^{-1} FW	7.80 ± 0.70	7.95 ± 0.35	6.86 ± 0.19	7.44 ± 0.65	ns
Fe	mg kg^{-1} FW	14.38 ± 2.03	14.70 ± 2.11	12.65 ± 1.02	13.66 ± 2.14	ns
Cu	mg kg^{-1} FW	0.573 ± 0.031	0.598 ± 0.042	0.573 ± 0.023	0.634 ± 0.014	ns
Chlorophylls	mg g^{-1} FW	1.09 ± 0.095	1.25 ± 0.030	1.29 ± 0.054	1.22 ± 0.071	ns
Carotenoids	mg g^{-1} FW	0.138 ± 0.015	0.159 ± 0.011	0.151 ± 0.014	0.122 ± 0.018	ns
Flavonoids	mg g^{-1} FW	0.585 ± 0.034	0.809 ± 0.045	0.776 ± 0.144	0.695 ± 0.117	ns
Phenols	mg g^{-1} FW	1.47 ± 0.12	1.67 ± 0.09	1.68 ± 0.14	1.55 ± 0.22	ns
Antioxidant capacity	mmol Fe (II) kg^{-1} FW	6.41 ± 0.84	8.13 ± 0.68	7.05 ± 0.74	7.95 ± 0.93	ns
NO_3	mg kg^{-1} PF	2420.6 ± 68.9	2269.3 ± 254.0	2189.8 ± 321.1	2259.6 ± 250.1	ns

3.3. Biofortification with Se, I, or Simultaneously with Zn, Cu, Se, and I (Experiment Mix)

The application of Se resulted in an increase in leaf Se concentration, irrespective of whether it was applied individually or in combination with I, Zn, and Cu (Figure 4A). In previous works, the leaf Se concentration in many leafy vegetables including chard [64], basil [65,66], lettuce [22,67], Swiss chard, sea beet [25], lamb's lettuce, wild rocket, and spinach [67] was increased by Se supplementation. The Se concentrations in lettuce leaves obtained in the present experiment are in agreement with the results obtained in a previous experiment conducted with baby leaf lettuce plants grown in aeroponics and treated with the same concentration of Se [22]. Moreover, according to the experiment conducted by Puccinelli et al. [22], aeroponic cultivation can be more effective in Se biofortification compared to a floating system. Thus, the plant species and cultivation system employed can affect Se accumulation in leaves of Se-treated plants (as reviewed by Szerement et al. [63]).

The Se concentration in leaves of lettuce biofortified only with Se was 0.251 kg^{-1} FW. Consequently, 100 g of fresh Se-biofortified leaves of lettuce would satisfy 36% of the adequate intake (AI) of Se (Table 2). In order to achieve 100% AI for Se UL, it would be necessary to consume 280 g of biofortified lettuce leaves.

The dose of Se used for treatments was found to be non-toxic for lettuce plants, thus treatment with Se did not affect plant growth and leaf quality parameters, such as chlorophyll, phenol, and carotenoid concentration and the antioxidant capacity of leaves (Table 5). In previous works, total phenol content was not affected by the biofortification of basil [68], carrot [69], lettuce [22], Swiss chard, and sea beet [25].

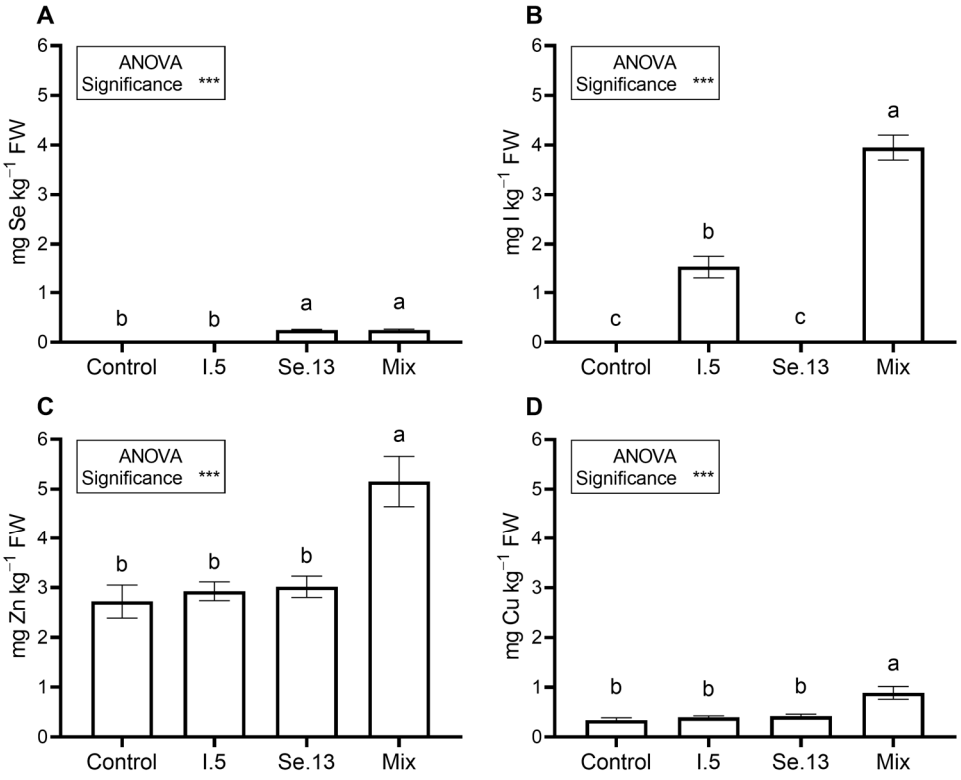

Figure 4. Experiment Mix: Selenium (Se; (**A**)), iodine (I; (**B**)), zinc (Zn; (**C**)), and copper (Cu; (**D**)) in leaves of lettuce plants grown indoors in aeroponics, with different concentrations of Se and I, applied alone or simultaneously with Cu and Zn, in the nutrient solution: 3 µM Cu, 10 µM Zn, 0 µM Se, 0 µM I (Control); 5 µM I (I.5); 13 µM Se (Se.13); 150 µM Cu, 250 µM Zn, 13 µM Se, 5 µM I (Mix). Means (n = 3) flanked by the same letter are not statistically different for $p = 0.05$ after Tukey's test. Significance level: *** $p \leq 0.001$.

Table 5. Experiment Mix: Fresh (FW) and dry (DW) biomass, dry matter content, mineral, chlorophyll, carotenoid, flavonoid, phenol, nitrate concentration, and antioxidant capacity in leaves of lettuce plants grown indoors in aeroponics, with different concentrations of Se and I, applied alone or simultaneously with Cu and Zn, in the nutrient solution: 3 µM Cu, 10 µM Zn, 0 µM Se, 0 µM I (Control); 5 µM I (I.5); 13 µM Se (Se.13); 150 µM Cu, 250 µM Zn, 13 µM Se, 5 µM I (Mix). Means (n = 3) flanked by the same letter are not statistically different for $p = 0.05$ after Tukey's test. Significance level: *** $p \leq 0.001$; * $p \leq 0.05$; ns = not significant.

		Treatment				
	u.m.	**Control**	**I.5**	**Se.13**	**Mix**	**ANOVA**
Fresh weight	g m^{-2}	2064.5 ± 185.3	2201.2 ± 186.0	2198.9 ± 95.8	2404.2 ± 267.1	ns
Dry weight	g m^{-2}	73.34 ± 9.30	81.49 ± 8.53	83.39 ± 5.13	82.05 ± 7.74	ns
Dry matter content	% FW	3.52 ± 0.16	3.69 ± 0.15	3.78 ± 0.08	3.43 ± 0.09	ns
N-tot	g kg^{-1} FW	2.39 ± 0.06	2.49 ± 0.08	2.61 ± 0.04	2.36 ± 0.04	ns
K	g kg^{-1} FW	4.18 ± 0.40	3.93 ± 0.38	3.85 ± 0.47	4.11 ± 0.34	ns
P	g kg^{-1} FW	0.313 ± 0.008 ab	0.332 ± 0.013 ab	0.359 ± 0.008 a	0.306 ± 0.014 b	*
Ca	g kg^{-1} FW	1.181 ± 0.051 ab	1.014 ± 0.116 ab	1.299 ± 0.073 a	0.891 ± 0.022 b	*
Na	g kg^{-1} FW	0.062 ± 0.002 b	0.080 ± 0.007 b	0.063 ± 0.003 b	0.102 ± 0.004 a	***
Mg	g kg^{-1} FW	0.152 ± 0.006 ab	0.167 ± 0.006 ab	0.174 ± 0.004 a	0.148 ± 0.005 b	*
Mn	mg kg^{-1} FW	3.33 ± 0.07	3.45 ± 0.21	3.87 ± 0.27	3.40 ± 0.17	ns
Fe	mg kg^{-1} FW	6.20 ± 0.79 ab	8.11 ± 0.85 a	6.21 ± 0.53 ab	4.65 ± 0.40 b	*
Chlorophylls	mg g^{-1} FW	1.28 ± 0.065	1.23 ± 0.068	1.244 ± 0.021	1.36 ± 0.053	ns
Carotenoids	mg g^{-1} FW	0.117 ± 0.008	0.120 ± 0.003	0.129 ± 0.005	0.118 ± 0.003	ns
Flavonoids	mg g^{-1} FW	0.67 ± 0.05	0.44 ± 0.09	0.51 ± 0.07	0.57 ± 0.07	ns
Phenols	mg g^{-1} FW	1.40 ± 0.03	1.25 ± 0.08	1.29 ± 0.10	1.26 ± 0.08	ns
Antioxidant capacity	mmol Fe (II) kg^{-1} FW	9.08 ± 0.17	8.00 ± 0.73	8.48 ± 0.74	8.33 ± 0.60	ns
NO_3	mg kg^{-1} PF	1922.6 ± 58.7	2128.1 ± 225.3	2152.3 ± 109.9	1896.5 ± 69.4	ns

When I was individually added to the nutrient solution, the I concentration in leaves increased compared to the control (Figure 4B). The addition of I, as potassium iodide (KI) or potassium iodate (KIO_3), in the nutrient solution in hydroponics, was shown to be effective for the biofortification of several leafy vegetables such as water spinach [70], basil [21,71], and lettuce [22,72]. The I concentrations detected in leaves of lettuce plants treated with iodine in the present work are comparable to those reported by Puccinelli et al. [22] for lettuce plants grown in aeroponics and treated with the same I concentrations in the nutrient solution. There are contrasting results about the effect of the cultivation system on biofortification with I of lettuce. Indeed, Puccinelli et al. [22] detected higher I concentrations in the leaves of lettuce plants grown in aeroponics than in a floating system, but the same authors obtained the opposite result in another experiment conducted with basil plants [21]. Thus, the plant response to iodine enrichment may depend on plant species, the applied concentration, and the cultivation system.

In I-enriched plants, the leaf iodine concentration was 1.53 mg kg^{-1} FW. The consumption of 100 g of lettuce biofortified with I could thus satisfy 102% of the adequate intake (AI) of I (Table 2).

Plant growth and leaf quality parameters were not affected by treatment with I (Table 5). This could be explained by the non-toxic dose of I used for treatments, and it is in agreement with previous works: total phenol content was not affected by treatments with I in carrot [69], tomato [73], lettuce [22], Swiss chard, and sea beet [74].

The addition of I to the nutrient solution in combination with Zn, Cu, and Se induced a leaf concentration of I higher than in plants treated only with I (Figure 4B). Our results are in contrast with a previous work where no differences were detected in leaf I concentration between biofortification with only I or in combination with Se [22]. Moreover, in a previous study, the foliar application of I in combination with Zn and Se did not induce a higher leaf concentration of I, compared to the application of only I [37]. Plant species, cultivation system, and the way of application may affect the interaction of I uptake with other micronutrients.

The Zn (Figure 4C) and Cu (Figure 4D) leaf concentrations were higher in the Mix treatment than in control plants. Combined treatment with Zn, Cu, Se, and I did not affect the growth of lettuce plants. Qualitative parameters of leaves were not affected by individual treatment with Se or I, nor by the combined treatment with Zn, Cu, Se, and I (Table 5). This can mean that the concentration of these minerals accumulated in lettuce leaves did not induce toxicity to lettuce plants (as described above).

Combined treatment with Zn, Cu, Se, and I scarcely affected the mineral concentration of lettuce leaves (Table 5), which showed a lower concentration of P, Ca, and Mg compared to plants treated only with Se; and a lower concentration of Fe compared to plants treated only with I. Leaves of lettuce plants biofortified simultaneously with Zn, Cu, Se, and I showed a higher Na concentration than other treatments (Table 5). In a previous study conducted with soilless-grown lettuce, the simultaneous application of Se, I, and Zn did not affect the leaf concentration of P, K, Ca, Mg, Na, and Cu, whereas it decreased the leaf concentration of Fe [75].

The consumption of 100 g of fresh lettuce leaves biofortified simultaneously with Se, I, Cu, and Zn would allow the 6.1, 35.3 and 263.0% of the AI of Cu, Se, and I to be satisfied, respectively, and the 4.5% of the PRI of Zn (Table 2). Even if the amount of I provided by the consumption of 100 g of fresh lettuce leaves (394 µg) is over the AI, it is still below the tolerable upper limit fixed for I (600 µg d^{-1}; [76]).

4. Conclusions

The concentrations of Cu, Zn, I, and Se tested in the present study allow for the simultaneous biofortification of lettuce leaves with these minerals without a concomitant reduction in yield and quality. Moreover, the simultaneous application of I with Cu, Zn, and Se has a positive effect on I accumulation in lettuce leaves, compared to when I is applied alone. The contribution to meeting human needs for these minerals also depends on the

Adequate Intake (AI) and Population Reference Intake (PRI) values set for each mineral. Indeed, the AI for Cu and the PRI for Zn are much higher than the AI values set for I and Se. Consequently, even though the foliar concentrations of Zn are higher than those of I and Se, and the concentration of Cu is higher than that of Se, the satisfaction rates of AI/PRI for Cu and Zn are lower compared to those for I and Se. Despite this, the consumption of lettuce leaves simultaneously biofortified with Cu, Zn, I, and Se would represent a practical and convenient way to integrate these four micronutrients into the diet.

Further research is required to elucidate the mechanisms by which Cu, Zn, or Se enhance the uptake of I by lettuce plants. Furthermore, it would be beneficial to test the effects of lower I concentrations when applied together with the other minerals.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10070726/s1, Table S1. Experiment Cu_1. Fresh (FW) and dry (DW) biomass, dry matter content, mineral, chlorophyll, carotenoid, flavonoid, phenol, nitrate concentration, and antioxidant capacity in leaves of lettuce plants grown indoors in aeroponics, with different concentrations of Cu in the nutrient solution: 3 µM (Control), 25 µM (Cu.25), 50 µM (Cu.50), 75 µM (Cu.75). Means (n = 3) flanked by the same letter are not statistically different for P = 0.05 after Tukey's test. Significance level: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns = not significant. Table S2. Experiment Cu_2. Fresh (FW) and dry (DW) biomass, dry matter content, mineral, chlorophyll, carotenoid, flavonoid, phenol, nitrate concentration, and antioxidant capacity in leaves of lettuce plants grown indoors in aeroponics, with different concentrations of Cu in the nutrient solution: 3 µM (Control), 150 µM (Cu.150), 200 µM (Cu.200), 250 µM (Cu.250). Means (n = 3) flanked by the same letter are not statistically different for P = 0.05 after Tukey's test. Significance level: *** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns = not significant.

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Data Availability Statement: Data are contained within the article. Additional data can be obtained by contacting the corresponding author of the article.

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