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Iron Biofortification of Greenhouse Soilless Lettuce: An Effective Agronomic Tool to Improve the Dietary Mineral Intake

Camila Vanessa Buturi [1,†], Leo Sabatino [2,†], Rosario Paolo Mauro [1,*], Eloy Navarro-León [3], Begoña Blasco [3], Cherubino Leonardi [1] and Francesco Giuffrida [1]

[1] Dipartimento di Agricoltura, Alimentazione e Ambiente (Di3A), University of Catania, Via Valdisavoia 5, 95123 Catania, Italy; camila.buturi@phd.unict.it (C.V.B.); cherubino.leonardi@unict.it (C.L.); francesco.giuffrida@unict.it (F.G.)
[2] Dipartimento Scienze Agrarie, Alimentari e Forestali (SAAF), University of Palermo, Viale delle Scienze, ed. 5, 90128 Palermo, Italy; leo.sabatino@unipa.it
[3] Department of Plant Physiology, Faculty of Sciences, University of Granada, 18071 Granada, Spain; enleon@ugr.es (E.N.-L.); bblasco@ugr.es (B.B.)
* Correspondence: rosario.mauro@unict.it
† These authors contributed equally to this work.

Abstract: The present experiment addressed the effects of different iron (Fe) concentrations in the nutrient solution supplied as Fe-HBED, i.e., 0.02 (Fe0, control), 1.02 (Fe1), and 2.02 mmol L^{-1} (Fe2) on lettuce ('Nauplus' and 'Romana') yield and compositional traits. This experiment was carried out in a greenhouse using an open soilless cultivation system, at the experimental farm of the University of Catania (Sicily, Italy: 37°24′31.5″ N, 15°03′32.8″ E, 6 m a.s.l.). The addition of Fe-HBED reduced the plants' aboveground biomass (−18%, averaged over Fe1 and Fe2), but promoted their dry matter content (+16% in Fe2). The concentration of chlorophylls, carotenoids, anthocyanins, and antioxidants peaked at Fe2, along with the antioxidant capacity and concentration of stress indicators in leaves. The Fe content in leaves was promoted in the Fe-treated plants (+187% averaged over Fe1 and Fe2). 'Romana' showed the highest Fe accumulation (reaching 29.8 mg kg^{-1} FW in Fe1), but 'Nauplus' proved a higher tolerance to the Fe-derived oxidative stress. The Fe2 treatment maximized leaf N, P, K, S, and Zn contents, while those of Ca, Mg, Mn, and B peaked at Fe1. Overall, our study revealed the effectiveness of Fe-HBED in increasing the Fe content and improving the nutritional quality of lettuce grown in soilless cultivation systems.

Keywords: mineral biofortification; *Lactuca sativa* L.; antioxidants; oxidative stress; hidden hunger

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1. Introduction

It is well known that iron (Fe) is an essential mineral element for humans, being involved in the synthesis of hemoglobin and myoglobin [1]. However, the importance of Fe goes beyond the oxygen transport, as it plays a key role in neural systems, immune cell functioning, and homeostasis, it is required for energy metabolism and exercise, being fundamental in the maintenance of human health [2]. Moreover, Fe deficiency symptoms usually include weakness, fatigue, difficulty in concentrating, motor and mental impairment, and anemia [3].

The amount of Fe required daily by the human body ranges between 8 and 18 mg, which represents the recommended daily allowance (RDA); in contrast, the tolerable upper intake level (UL) for adults is 40 mg day^{-1} [4]. However, in some cases, the minimum intake requirement is not fulfilled with the diet, resulting in cases of micronutrient deficiencies, also called hidden hunger [5,6]. This kind of malnutrition is not always easy to detect, and it does not affect only developing countries, but it is also present in the developed world [7]. The causes of the insufficient intake of micronutrients, such as mineral elements, can be attributed to poverty, but also to the rise of new diets (e.g., veganism) and bad eating

habits in developed countries, which include daily intake of high-calorie, low-nutrient-dense foods [8].

Moreover, in the specific case of minerals, not all the elements present in the food matrix are available for the absorption. In fact, only around 14–18% of the Fe present in the diet is bioavailable [9]. This happens because Fe absorption can be limited by many factors such as the presence of inhibiting substances (calcium, phytates, and tannins), age, pregnancy, surgical procedures, and medical conditions [9,10].

An alternative to increase the intake of micronutrients is to include, in the diet, foods containing higher concentrations of those elements. Given that, strategies aiming to increase the Fe content in food can be good tools to improve human dietary patterns [11]. At the same time, vegetables contain a variety of natural health-promoting benefits, such as vitamins, minerals, and antioxidants, being excellent functional food options [12,13].

When mineral micronutrients are concerned, an efficient approach to improve their concentrations in vegetables may be agronomic biofortification, i.e., by growing them with targeted applications of fertilizers [14–16]. In addition, this strategy, when well-managed, can provide more than simply an increase in the target element. Indeed, by using specific elements as eustressors, biofortification can also increase the concentration of many antioxidant compounds, establishing a link between plant nutrition and human nutrition [17].

Soilless cultivation systems offer benefits such as the possibility to control water availability, pH, and nutrient concentrations in the root zone [18]. In fact, currently about 3.5% of the total area cultivated under tunnels and greenhouses for vegetable production adopts soilless cultivation systems. This method can increase not only yield but also the quality and the shelf life of fresh vegetables, meeting the highest demands of modern consumers [19].

Biofortification of vegetables can be carried out in soilless systems by adding higher concentrations of target fertilizers in the nutrient solution [20]. Moreover, in the specific case of Fe, which presents a low solubility in the soil [21], a soilless cultivation system can be a good option to increase micronutrient availability, since it facilitates the pH management in the nutrient solution [22].

Another factor that can affect the biofortification effectiveness, is the chemical form of the added micronutrients in the nutrient solution. Considering Fe, chelate forms are highly recommended since they are more easily available for plants and can optimize mineral absorption when compared to inorganic salts [23].

In addition, it should be taken into consideration that the introduction of higher amounts of fertilizers in the nutrient solution can also affect vegetable yield and quality [24]. Since Fe excess can be toxic to the plant, causing damages to the membrane, DNA, and proteins, it is important to understand the activation of the antioxidant enzymes involved in the Fe biofortification [25]. So far, few biofortification studies have been conducted aiming to improve the Fe and antioxidant content of vegetables and at the same time assess the stress conditions of plants submitted to high Fe levels in the nutrient solution.

Besides being a model plant, lettuce (*Lactuca sativa* L.) is one of the most popular and consumed leafy vegetables in the world [26]. In this study, we chose two different genotypes of lettuce to compare their tolerance to high doses of Fe introduced in the nutrient solution, i.e., *L. sativa* L. var. *capitata* (Looseleaf) and *L. sativa* L. var. *longifolia* (Romaine) as they are among the most commonly consumed [27–29].

Given the scarcity of biofortification studies, our investigation aimed to address the effects of different iron (Fe) concentrations in the nutrient solution supplied as Fe-HBED on yield and compositional traits of two cultivars of greenhouse soilless lettuce and compare the tolerance of these genotypes to the exposure of high levels of this element in the nutrient solution.

Therefore, the hypothesis of this study is that the application of Fe-HBED to lettuce plants will modify the compositional traits of the plants in a genotype-specific manner.

2. Materials and Methods

2.1. Experimental Site and Plant Material

A greenhouse experiment was carried out from December 2020 to January 2021, at the experimental farm of the University of Catania (Sicily, Italy: 37°24′31.5″ N, 15°03′32.8″ E, 6 m a.s.l.). The climate of the area is semi-arid Mediterranean, with mild winters and hot, dry summers. An 810 m^2, multi-aisle cold greenhouse was used, having a steel tubular structure with adjustable windows on the roof and along the sides, and covered with polycarbonate slabs. Two lettuce cultivars were selected for the study, i.e., 'Nauplus' (var. *capitata*; Blumen vegetable seeds, Piacenza, Italy) and 'Romana' (var. *longifolia*; Topseed, Sarna, Italy). Plantlets were transplanted on 10 December 2020 in the greenhouse at the stage of four true leaves, in an open soilless cultivation system using 5 L plastic pots (20 cm height, 19 cm width) and perlite as growing medium (particle size 2–6 mm). Before transplanting, plantlets were selected for uniform size and healthy appearance. Pots were arranged in simple rows, adopting a 0.25m × 0.50 m rectangular format (center to center) and 1 plant per pot (8 plants m^{-2}). Plants were harvested on 25 January 2021. Each net experimental unit contained 12 plants.

2.2. Treatments

The treatments consisted of three concentrations of Fe chelate added to the nutrient solution: Fe0: 0 mmol L^{-1} Fe (just the standard nutrient solution, equal to 0.022 mmol L^{-1} Fe); Fe1: 1 mmol L^{-1} Fe; Fe2: 2 mmol L^{-1} Fe in the chelate form HBED. Thus, the final concentrations were 0.02, 1.02 and 2.02 mmol L^{-1} Fe. During the cycle, the crop was fertigated with a standard nutrient solution [30], having the following composition: 8.0 mM N-NO_3^-, 1.5 mM S, 1.0 mM P, 3.0 mM K, 3.0 mM Ca, 1.0 mM Mg, 1.0 mM N-NH_4^+, 22 µM Fe, 9 µM Mn, 2 µM Cu, 4 µM Zn, 9 µM B, and 1 µM Mo, with an electrical conductivity (EC) of 1400 µS cm^{-1} and a pH of 5.8 ± 0.2. Control plants received only the standard nutrient solution whereas treated plants received the same solution enriched with Fe-HBED. A leaching fraction of ~25% was adopted, to reduce root zone salinization [31].

Lettuce harvest was manually carried out on 25 January 2021, avoiding any damage to the leaves. Soon after harvest, plants were transported to the laboratory, characterized for physical variables, flash frozen with liquid nitrogen, and stored at −80 °C for further analysis. Overall, 72 lettuce heads were collected and analyzed (2 cultivars × 3 Fe concentrations × 3 replicates × 4 lettuces).

2.3. Lettuce Measurements

In the laboratory, variables such as average fresh weight (FW) and dry matter content (DM) were measured. Average fresh weight was determined using an electronic gage (0.01 g accuracy). For the dry matter content, samples of lettuce leaves were dried at 70 °C in a laboratory oven (Thermo scientific-Herathermoven) with a forced air circulation until constant weight. For biochemical analyses, frozen material was grounded in an IKA A11 analytical mill (Staufen, Germany) using liquid nitrogen. For the mineral content, frozen samples were lyophilized in a Telstar Cryodos-80 freeze dryer (Terrassa, Barcelona, Spain) and grounded in a Taurus aromatic grinder (Oliana, Barcelona, Spain). All biochemical analyses were performed using fresh frozen material, all mineral analyses were performed using lyophilized plant material. All biochemical analyses as well as the forms of nitrogen were measured through using a spectrophotometer Infinite 200 Nanoquant (Tecan, Switzerland).

2.4. Biochemical Analyses

2.4.1. Leaf Chlorophylls and Carotenoids Concentration

The determination of photosynthetic pigments was performed according to Lichtenthaler and Wellburn [32], with slight modifications. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of methanol, vortexed, and centrifuged for 5 min at 5000 rpm. After that, the absorbance of the supernatant was measured at 3 different

wavelengths: 666 nm, 653 nm, and 470 nm. The values obtained were applied in the following equations: -Chlorophyll *a* (Chl *a*) = 15.65 × A_{666} − 7.34 × A_{653} -Chlorophyll *b* (Chl *b*) = 27.05 × A653 − 11.21 × A_{666} -Carotenes = (1000 × A_{470} − 2.86 × Chl *a* − 129.2 × Chl *b*)/221. The results are expressed in μg g^{-1} FW.

2.4.2. Total Phenol and Flavonoid Concentration

Total phenol and flavonoid concentration were determined according to Rivero et al. [33], with minor modifications. For the extraction, 100 mg of macerated plant material was mixed with 500 μL of methanol, 500 μL of chloroform, and 250 μL of NaCl (1%), the material was vortexed and centrifuged for 10 min at 5000 rpm. For the total phenols, 90 μL of supernatant was mixed with 240 μL of Na_2CO_3 (5%) and 90 μL of Folin–Ciocâlteu reagent (50%). Samples were agitated and incubated at room temperature for 40 min. The absorption was measured at 725 nm. The results are expressed in μg caffeic acid (CA) g^{-1} FW. For total flavonoid concentration, 85 μL of supernatant was mixed with 180 μL of distilled water and 26 μL $NaNO_2$ (5%). Samples were agitated and incubated at room temperature for 5 min. Finally, 26 μL of $AlCl_3$ (10%) and 170 μL of NaOH (1 M) were added to the mixture, and samples were incubated as previously. The absorption was measured at 415 nm. The results are expressed in μg rutin g^{-1} FW.

2.4.3. Anthocyanin Concentration

The concentration of anthocyanins was measured according to Giusti and Wrolstad [34], with minor modifications. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of methanol acidified with 1% HCl, agitated in a vortex, and centrifuged for 5 min at 5000 rpm. Then, 250 μL of supernatant was added to react with 1 mL of buffers potassium chloride, pH 1.0 (0.025 M) and sodium acetate, pH 4.5 (0.4 M). The absorption of both solutions was measured at 640 and 710 nm. The values obtained were applied in the following equation: [(A640–A710)–(A640–A710)] × 449.2/26900. The results are expressed as mg cyanidine-3-glucoside per g^{-1} FW.

2.4.4. Ascorbic Acid Concentration

Total ascorbic acid (AsA), reduced AsA, and dehydroascorbate (DHA) concentration were determined according to Law et al. [35], with slight modifications. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of meta-phosphoric acid, agitated in a vortex, and centrifuged for 15 min at 13,000 rpm. Then, 200 μL of supernatant was mixed with 500 μL of buffer sodium phosphate (150 mM; pH 7.5) and, only, for total ascorbic acid reaction 60 μL of dithiothreitol (DTT) (10 mM) were added. Samples were agitated and incubated at room temperature for 10 min. After, 60 μL of N-ethylmaleimide (0.5%), 240 μL of trifluoroacetic acid, 240 μL of orthophosphoric acid (44%), 240 μL of bipyridyl (4%, in ethanol 70%), and 120 μL of $FeCl_3$ (3%) were added. Finally, samples were incubated at 40 °C for 40 min. The absorption of both solutions was measured at 525 nm. The results are expressed in μg g^{-1} FW.

2.4.5. Antioxidant Capacity: FRAP and TEAC Assays

The FRAP (ferric reducing antioxidant power) assay was determined according to Benzie and Strain [36], with minor adaptations. The TEAC (Trolox equivalent antioxidant activity) assay was performed following Cai et al. [37], with modifications. For both extractions, 100 mg of macerated plant material was mixed with 1 mL of methanol (100%), agitated in a vortex, and centrifuged for 2 min at 10,200 rpm. Then, for the FRAP reaction, 10 μL of supernatant was mixed with 190 μL of FRAP reagent (acetate sodium, 0.25 M, pH 3.6; TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), 1 mM and $FeCl_3$, 20 mM). The absorption was measured at 593 nm. The results are expressed in μM $FeSO_4$ g^{-1} fresh weight (FW). For the TEAC reaction, 10 μL of supernatant was mixed with 190 μL of TEAC reagent (ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (7 mM) and potassium persulfate

(2.45 mM)). The absorption was measured at 734 nm. The results are expressed in mg Trolox g^{-1} FW.

2.4.6. Superoxide Anion

The superoxide anion (O_2^-) detection was performed according to Kubiś [38], based on the reduction of NBT, with slight modifications. For the extraction, 100 mg of macerated plant material was mixed with 300 µL of buffer potassium phosphate (50 mM, pH 7.8). Then, the material was gently agitated and centrifuged for 15 min at 10,000 rpm. Subsequently, 250 µL of supernatant was mixed with 225 µL of buffer and 250 µL of hydroxylamine (10 mM). Samples were agitated and incubated for 20 min at room temperature. Subsequently, 180 µL of the extract was mixed with 460 µL of sulfuric acid (17 mM) and 460 µL of 1-Naphthylamine (7 mM). The absorption was measured at 580 nm. The results are expressed in µg g^{-1} FW.

2.4.7. Proline

The proline concentration was conducted following Bieleski and Turner [39], with some adaptations. For the extraction, 100 mg of macerated plant material was mixed with 1.2 mL of ethanol (83%), agitated in a vortex, and centrifuged for 10 min a 5500 rpm. Then, 1 mL of supernatant was added to 4 mL of Milli-Q water, 2.5 mL of ninhydrin (140 mM), and 2.5 mL of glacial acetic acid (100%). Samples were agitated and incubated for 45 min in a water bath at 100 °C. Subsequently, samples were cooled in ice and 5 mL of benzene (100%) were added and samples were incubated for 10 min at room temperature. The absorption of the organic phase was measured at 515 nm. The results are expressed in µg g^{-1} FW.

2.4.8. MDA

The MDA (malondialdehyde) concentration was carried out according to Fu and Huang [40], with minor modifications. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of trichloroethanoic acid (TCA; 10%) and thiobarbituric acid (TBA; 0.25%). Samples were agitated and incubated for 30 min in a water bath at 95 °C. Subsequently, samples were cooled in ice and centrifuged at 9500 rpm for 10 min. The absorption of the organic phase was measured at 532 and 600 nm. The values obtained were applied in the following equation: [(A532–A600)]/155. The results are expressed in µM g^{-1} FW.

2.4.9. APX

The ascorbate peroxidase (APX) activity was determined according to Rao et al. [41], with slight modifications. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of buffer potassium phosphate (100 mM, pH 7.5). Samples were gently agitated and centrifuged for 20 min at 12,000 rpm. Subsequently, 40 µL of extract was mixed with 80 µL of buffer potassium phosphate, 40 µL of sodium ascorbate (0.5 mM), and 40 µL of H_2O_2 (0.2 mM). The absorption was measured at 290 nm every 30 s for 5 min. The results are expressed in Δ Abs mg $protein^{-1}$ min^{-1} FW.

2.4.10. GPX

The glutathione peroxidase (GPX) activity was measured following Elia et al. [42], with minor modifications. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of buffer tris hydrochloride (100 mM), and added with EDTA (1 mM), and DTT (2 mM). Samples were gently agitated and centrifuged for 20 min at 15,000 rpm. Subsequently, 30 µL of extract was mixed with 170 µL of buffer potassium phosphate (100 mM). The absorption was measured at 340 nm every 30 s for 5 min. The results are expressed in Δ Abs mg $protein^{-1}$ min^{-1} FW.

2.4.11. CAT

The catalase (CAT) activity was performed according to Nakano and Asada [43], measuring the consumption of H_2O_2, with some adaptations. For the extraction, 100 mg of macerated plant material was mixed with 1 mL of buffer sodium phosphate (25 mM, pH 7). Samples were gently agitated and centrifuged for 20 min at 11,500 rpm. Subsequently, 40 µL of extract was mixed with 40 µL of buffer HEPES (25 mM), 40 µL of EDTA (0.8 mM), and 80 µL of H_2O_2 (40 mM). The absorption was measured at 240 nm every 30 s for 5 min. The results are expressed in Δ Abs mg protein^{-1} min^{-1} FW.

2.5. Mineral Analyses

Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), manganese (Mn), zinc (Zn), boron (B), and copper (Cu) mineralization were conducted through wet digestion. For the digestion, 150 mg of lyophilized material was mixed with 5 mL of nitric acid (HNO_3) and placed in a sand bath at 100 °C for one week, and drops of H_2O_2 at 33% were added daily. Subsequently, the extract was filtered with filter paper and a working solution of 20 mL was prepared with the addition of Milli-Q water. Mineral element concentrations were measured by ICP-OES (Perkin Elmer, Waltham, MA, USA), according to Martín Peinado et al. [44]. Each measurement was made with three replicates. For calibration, two sets of multi-element standards containing all the analytes of interest at five different levels of concentration were prepared using rhodium as the internal standard. All standards were prepared from ICP single-element standard solutions (Merck, Darmstadt, Germany), after dilution with 10% HNO_3. The analytical precision of the analyses was better than ±5% in all cases. The average recoveries ranged between 91% and 105% of the certified reference values. Macronutrients were calculated and expressed as mg 100 g^{-1} FW, while micronutrients as µg 100 g^{-1} FW.

Forms of Nitrogen

The contents of organic nitrogen (N) and ammonium (NH_4^+) were determined according to Krom [45]. For the organic N digestion, 150 mg of lyophilized material was mixed with 5 mL of sulfuric acid (H_2SO_4) and placed in a sand bath at 100 °C for three days; drops of H_2O_2 at 33% were added daily. Subsequently, the extract was filtered with filter paper and a working solution of 20 mL was prepared with the addition of Milli-Q water.

For the NH_4 extraction, 10 mg of dry plant material was mixed with 1 mL of Milli-Q water. Then, 30 µL of supernatant of both extracts was added to 285 µL of reactive 1 (sodium salicylate, 0.5 M; sodium nitroprusside, 2 mM) and 285 µL of reactive 2 (NaOH, 1 M; sodium dichloroisocyanurate, 28 mM). After, samples were agitated and incubated at 37 °C for 45 min. The absorption was measured at 630 nm. Results are expressed in mg g^{-1} dry weight (DW).

The content of NO_3^- (nitrate) was measured according to Cataldo et al. [46]. For the extraction, 10 mg of dry plant material was mixed with 1 mL of Milli-Q water. Samples were agitated in an agitator for 120 min. Then, 12 µL of supernatant was added to 24 µL of salicylic acid diluted in H_2SO_4 (10%) plus 565 µL sodium hydroxide (NaOH; 2 N). Samples were agitated and the absorption of the solution was measured at 410 nm. The results are expressed in mg NO_3^- g^{-1} DW).

N total was estimated as the sum of organic N and nitrate. Mineral N was estimated as the sum of NH_4^+ and NO_3^-. Assimilated N was assumed as organic N subtracted of NH_4. Results are expressed in mg g^{-1} DW.

2.6. Statistical Procedures

Collected and calculated data were firstly subjected to a two-way analysis of variance (ANOVA), based on a factorial combination (cultivar × Fe concentration in the nutrient solution). Means comparisons were carried out using Fisher's protected least significant difference (LSD) test ($p \leq 0.05$). All statistical analyses were carried out using the Statgraphics Centurion XVI software (The Plains, VA, USA).

3. Results

3.1. Lettuce Main Traits and Bioactive Compounds Concentration

When compared to the untreated control, the Fe application reduced the total plant dry biomass (−18%, on the average of Fe1 and Fe2), but promoted lettuce DM content, total Chls content, and the Chl *a*/*b* ratio (by up to 16, 40 and 24%, respectively) (Table 1). Excepting the Chl *a*/*b* ratio, 'Romana' proved to have the highest values for all these variables. When compositional traits were concerned, total phenol, anthocyanin, flavonoid, and carotenoid contents peaked at Fe2, with 'Romana' showing the highest carotenoids content, together with the highest rise in anthocyanin and flavonoid content passing from Fe0 to Fe2 (+97 and +210%, respectively) (Table 1). Similarly, the Fe application progressively increased both AsA and DHA contents, with 'Romana' proving the sharpest rises passing from Fe0 to Fe2 (+60 and +62% for AsA and DHA, respectively) (Table 1). Regarding the antioxidant activity, the highest FRAP values were recorded in Fe2 and 'Romana', while for TEAC, a higher increase was recorded in 'Romana' compared to 'Nauplus' passing from Fe0 to Fe2 (+111%) (Table 1).

3.2. Oxidative Stress Indicators

The Fe supply gradually increased O_2^- concentration, with 'Romana' showing a higher increase passing from Fe0 to Fe2 (+40%) compared to 'Nauplus' (+26.5%) (Table 2). When compared to the untreated control, the Fe supplementation promoted proline concentration (by +24% and +61%, at Fe1 and Fe2, respectively) and increased MDA content and APX activity at Fe1 and Fe2, (by up to +47 and +53%, respectively). Meanwhile, when compared to the control, Fe1 and Fe2 plants showed a reduction in the activity of GPX (by −9 and −13%, respectively), and a gradual reduction in the activity of CAT (by +9 and −18%, respectively). Among the tested genotypes, 'Nauplus' proved to have the highest values of APX and CAT activity, whereas the highest proline content was recorded in 'Romana' (Table 2).

3.3. Macronutrients and Micronutrients Content

Compared to the control, the Fe supply generated a progressive increase in N, P, K, and S concentrations of lettuce (by up to 13, 30, 29, and 45% in Fe2), while Mg concentration peaked at Fe1 (+62%) (Table 3). Regarding Ca, the response to Fe supply proved to be genotype-dependent, as in 'Nauplus' its concentration increased passing from Fe1 to Fe2 (by 44%), whereas in 'Romana', it raised within the Fe0–Fe1 range (+44%) and declined thereafter (−33%) (Table 3). When the genotype per sé was concerned, 'Romana' showed higher concentrations of P, K, Mg, and S than 'Nauplus' (Table 3).

Regarding the micronutrient content, the supplemental Fe fertilization boosted the accumulation of Fe, Mn, Zn, and B, though in a genotype-dependent way (Table 3). Indeed, when compared to the untreated control, 'Romana' showed the highest Fe increase within the Fe1–Fe2 range (+209%, on average), but the Mn, Zn, and B differences were higher at Fe1 (+124, +117 and +96%, respectively), while in 'Nauplus', all these micronutrients were maximized under the Fe2 supply (ranging from +173 to +69% in Fe and B, respectively) (Table 3). No differences were found in Cu concentrations.

The amount of Fe accumulated in the dry leaves of 'Romana' ranged from 522 to 520 mg kg^{-1}, at Fe1 and Fe2, respectively. Meanwhile, lower values were observed in 'Nauplus' plants, which varied from 315 to 335 mg kg^{-1} DW, at Fe1 and Fe2, respectively (Figure 1).

Table 1. Lettuce main traits and bioactive compound concentrations as affected by the studied factors.

	Plant Biomass (g DW plant^{-1})	Dry Matter (%)	Total Chls (mg g^{-1} FW)	Chl *a*/*b* Ratio	Total Phenols (μg g^{-1} FW)	Anthocyanins (mg g^{-1} FW)	Flavonoids (μg g^{-1} FW)	Carotenoids (μg g^{-1} FW)	AsA (μg g^{-1} FW)	DHA (μg g^{-1} FW)	FRAP (μM $FeSO_4$ g^{-1} FW)	TEAC (mg trolox g^{-1} FW)
Fe concentration												
Fe0	20.0 a	4.47 b	2.33 c	1.53 c	535 c	1.50 c	542 c	153 c	100 c	61.9 c	6.19 c	0.637 c
Fe1	16.5 b	5.03 a	2.64 b	1.70 b	781 b	2.05 b	901 b	220 b	126 b	79.4 b	9.17 b	0.881 b
Fe2	16.2 b	5.17 a	3.26 a	1.89 a	926 a	2.42 a	1134 a	304 a	143 a	95.9 a	12.3 a	1.173 a
Cultivar												
'Nauplus'	14.6 b	4.44 b	2.67 b	1.69	727	2.11 a	881	203 b	116	70.0 b	8.56 b	0.840 b
'Romana'	20.5 a	5.34 a	2.82 a	1.72	767	1.87 b	838	249 a	130	88.2 a	9.86 a	0.954 a
Fe × Cv												
Fe0 'Nauplus'	15.1	4.09	2.21	1.52	559	1.77 c	686 d	134	97 d	51.2 c	6.07	0.65 d
Fe1 'Nauplus'	15	4.36	2.85	1.76	766	2.11 b	924 bc	234	131 b	83.2 b	9.35	0.84 c
Fe2 'Nauplus'	13.8	4.89	2.96	1.80	856	2.44 a	1032 b	242	121 bc	75.5 b	10.28	1.03 b
Fe0 'Romana'	24.8	4.85	2.46	1.54	511	1.22 d	399 e	173	104 cd	72.7 b	9.00	0.62 d
Fe1 'Romana'	18.0	5.71	2.43	1.65	796	2.00 bc	879 c	207	120 bc	75.7 b	10.28	0.92 bc
Fe2 'Romana'	18.7	5.45	3.56	1.97	995	2.40 a	1236 a	367	166 a	116.4 a	14.28	1.32 a
Significance												
Fe concentration	*	*	***	***	***	**	***	***	**	**	***	***
Cultivar	**	**	**	NS	NS	**	NS	***	NS	**	**	*
Fe × Cv	NS	NS	NS	NS	NS	*	***	NS	*	**	NS	*

Different letters within each column's factor indicate significance at Fisher's protected LSD test (p = 0.05). NS: not significant; *, ** and ***: significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

Table 2. Oxidative stress indicators and enzyme activity in lettuce, as affected by the studied factors.

	O_2^- (μg g^{-1} FW)	Proline (μg g^{-1} FW)	MDA (μM g^{-1} FW)	APX (Δ Abs mg $protein^{-1}$ min^{-1} FW)	GPX (Δ Abs mg $protein^{-1}$ min^{-1} FW)	CAT (Δ Abs mg $protein^{-1}$ min^{-1} FW)
Fe concentration						
Fe0	6.91 c	15.3 b	2.92 b	0.055 b	0.171 a	0.011 a
Fe1	8.09 b	18.9 ab	4.16 a	0.084 a	0.149 b	0.010 b
Fe2	9.21 a	24.7 a	4.28 a	0.086 a	0.155 b	0.009 c
Cultivar						
'Nauplus'	7.86	15.6 b	3.61	0.084 a	0.153	0.011 a
'Romana'	8.28	23.7 a	3.97	0.066 b	0.163	0.008 b
Fe × Cv						
Fe0 'Nauplus'	6.61 d	15.7	2.81	0.064	0.163	0.013
Fe1 'Nauplus'	8.61 b	15.2	4.27	0.097	0.146	0.011
Fe2 'Nauplus'	8.36 bc	16.0	3.74	0.090	0.150	0.010
Fe0 'Romana'	7.21 cd	14.8	3.03	0.046	0.179	0.009
Fe1 'Romana'	7.58 bcd	22.7	4.05	0.071	0.151	0.008
Fe2 'Romana'	10.06 a	33.5	4.82	0.082	0.160	0.007
Significance						
Fe concentration	**	*	**	*	*	**
Cultivar	NS	*	NS	*	NS	***
Fe × Cv	*	NS	NS	NS	NS	NS

Different letters within each column's factor indicate significance at Fisher's protected LSD test ($p = 0.05$). NS: not significant; *, ** and ***: significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

Table 3. Macronutrient (mg g^{-1} FW) and micronutrient (μg g^{-1} FW) composition of lettuce affected by the studied factors.

	Macronutrients						Micronutrients				
	N	P	K	Ca	Mg	S	Fe	Mn	Zn	B	Cu
Fe concentration											
Fe0	4.41 b	3.88 c	2.90 c	0.330 c	0.143 c	0.107 c	7.7 b	3.49 c	3.16 c	1.21 c	0.689
Fe1	4.68 ab	4.60 b	3.07 b	0.385 a	0.231 a	0.135 b	21.8 a	6.28 a	5.74 b	1.93 a	0.607
Fe2	4.99 a	5.06 a	3.75 a	0.371 b	0.215 b	0.155 a	22.4 a	5.64 b	6.04 a	1.80 b	0.695
Cultivar											
'Nauplus'	4.84 a	3.96 b	3.07 b	0.347 b	0.166 b	0.123 b	12.0 b	4.33 b	4.32 b	1.51 b	0.603
'Romana'	4.54 b	5.06 a	3.40 a	0.377 a	0.227 a	0.141 a	22.5 a	5.94 a	5.64 a	1.78 a	0.725
Fe × Cv											
Fe0 'Nauplus'	4.59	3.75	3.00	0.327 c	0.132	0.109	6.0 d	3.20 d	3.00 d	1.16 d	0.602
Fe1 'Nauplus'	4.23	3.64	2.45	0.292 c	0.162	0.117	13.7 b	4.09 c	4.25 c	1.41 cd	0.775
Fe2 'Nauplus'	5.70	4.51	3.77	0.421 b	0.204	0.144	16.4 b	5.69 b	5.71 b	1.96 b	0.555
Fe0 'Romana'	4.22	4.02	2.80	0.332 c	0.154	0.104	9.4 c	3.78 cd	3.33 cd	1.25 d	0.660
Fe1 'Romana'	5.12	5.56	3.69	0.478 a	0.300	0.153	29.8 a	8.46 a	7.23 a	2.45 a	0.651
Fe2 'Romana'	4.28	5.62	3.72	0.321 c	0.226	0.166	28.3 a	5.59 b	6.36 ab	1.64 c	0.740
Significance											
Fe concentration	*	***	***	***	***	**	***	***	***	***	NS
Cultivar	*	***	**	*	***	**	***	***	**	**	NS
Fe × Cv	NS	NS	NS	***	NS	NS	***	***	**	***	NS

Different letters within each column's factor indicate significance at Fisher's protected LSD test ($p = 0.05$). NS: not significant; *, ** and ***: significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

3.4. Nitrogen Forms in Lettuce Leaves

The analysis of variance revealed that the supplemental Fe application promoted the concentration of NH_4^+ (+40 and +21%, in Fe1 and Fe2, respectively), whereas it decreased the concentration of NO_3^- (−20 and −14%, in Fe1 and Fe2, respectively). When compared to the control plants, the mineral N content was reduced in Fe1 plants (−15%), while the variable assimilated N was reduced in both Fe1 and Fe2 (−24 and −22%, respec-

tively) (Table 4). Regarding the genotype effect, the cultivar Nauplus revealed the highest concentrations of organic N, NO_3^-, total N, mineral N, and assimilated N (Table 4).

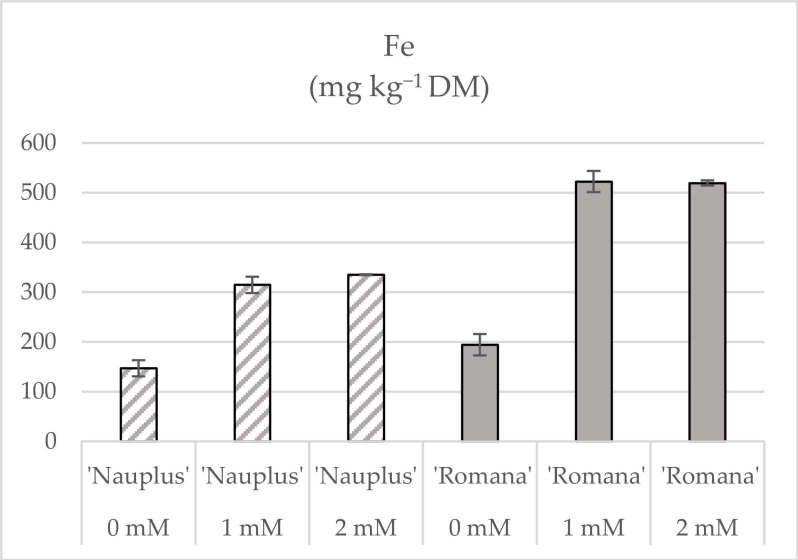

Figure 1. Fe content in the leaves of lettuce affected by the studied factors.

Table 4. Forms of N of lettuce as affected by the studied factors.

	Organic N (mg g^{-1} DW)	NH_4^+ (mg g^{-1} DW)	NO_3^- (mg g^{-1} DW)	Total N (mg g^{-1} DW)	Mineral N (mg g^{-1} DW)	Assimilated N (mg g^{-1} DW)
Fe concentration						
Fe0	33.4	6.28 c	66.2 a	99.6	72.5 a	27.1 b
Fe1	40.6	8.79 a	52.8 b	93.4	61.6 b	31.8 a
Fe2	40.7	7.62 b	56.9 b	97.6	64.5 ab	33.1 a
Cultivar						
'Nauplus'	42.2 a	7.48	66.4 a	108.7 a	73.9 a	34.7 a
'Romana'	34.3 b	7.65	50.8 b	85.1 b	58.5 b	26.7 b
Fe × Cv						
Fe0 'Nauplus'	37.1	7.00	75.2	112.3	82.2	30.1
Fe1 'Nauplus'	44.5	9.13	52.6	97.1	61.8	35.4
Fe2 'Nauplus'	45.2	6.31	71.4	116.6	77.7	38.9
Fe0 'Romana'	29.8	5.55	57.2	87.0	62.7	24.3
Fe1 'Romana'	36.8	8.46	52.9	89.7	61.4	28.3
Fe2 'Romana'	36.2	8.94	42.4	78.6	51.3	27.3
Significance						
Fe concentration	NS	**	*	NS	*	*
Cultivar	**	NS	***	***	***	**
Fe × Cv	NS	NS	NS	NS	NS	NS

Different letters within each column's factor indicate significance at Fisher's protected LSD test ($p = 0.05$). NS: not significant; *, ** and ***: significant at $p \leq 0.05$, 0.01 and 0.001, respectively.

4. Discussion

The plant biomass reduction and the DM content increase observed in the plants for our study were also reported by Giordano et al. (2019) [27], when submitting green and red Salanova cultivars (*Lactuca sativa* L. var. *capitata*) to 1 and 2 mM of Fe-EDDHA in the nutrient solution. The limitation in the growth parameters observed in this and other studies [47,48], dealing with Fe supplementation, supports the fact that, despite being essential to the plant, Fe excess produces phytotoxic effects [20]. Moreover, 'Romana' showed a higher plant biomass and a higher DM content, when compared to 'Nauplus'; this can be attributed to the plant's genetic diversity [49,50], in fact, the difference in the dry matter between the typologies used in this study is confirmed by Serio and Elia (2001) [51].

Flavonoids are a group of healthy phenolic compounds found in lettuce plants [52]. In our work, the increased concentration of flavonoids in Fe1 and Fe2 plants can be attributed to the plant's defense mechanism, since this antioxidant plays a key role in protecting plants against ROS-related damage and in alleviating oxidative stress caused by Fe excess [53,54]. This protection ability is a result of the strong chelating properties of flavonoids, capable of forming high-affinity complexes with transition metals, such as Fe [54]. In addition, this antioxidant compound has received considerable attention for its wide spectrum of pharmacological properties. The use of flavonoids has been linked to the prevention of cancer, cardiovascular diseases, gastric and intestinal problems, vascular fragility, and infections [55]. The fact that our lettuce contains such high concentrations of flavonoids contributes to its healthy characteristics.

An important subgroup of flavonoids are anthocyanins, a pigment family responsible for the red color found in some lettuce types [56]. In our study, we observed a gradual increase in the anthocyanin content compatible to the increment described by Giordano et al. [27], when submitting lettuce plants to 2 mM of Fe. The increased concentration of anthocyanins in the presence of Fe could be, also, due its metal chelating properties, as demonstrated by Sigurdson et al. (2017) [57]. In addition, Giordano et al. [27] described differences in anthocyanin concentration among cultivars, being the higher values observed in the red-pigmented cultivar. Similarly, in our study, this parameter was higher in the cultivar Nauplus, as expected, since this is also a red-pigmented cultivar. The same authors highlighted a progressive increase in the profile of other important antioxidants, such as carotenoids. Similarly, we observed a progressive increase in the carotenoid content in Fe1 and Fe2 plants, probably linked to the high ROS scavenging ability of this antioxidant [58].

Fe-biofortification studies can also benefit from the presence of Fe absorption promotors. It is well known that ascorbic acid is the most efficient enhancer of Fe absorption, overcoming the effects of all possible dietary Fe absorption inhibitors [59,60]. Lettuce plants from our study showed a progressive increase in the ascorbic acid content. Ascorbic acid is also a key antioxidant, which have probably been promoted as a protection against Fe excess [61]. Comparing the cultivars, 'Romana' AsA concentration peaked at 2 mM, indicating a more intense stress response. Moreover, the content of the oxidized form of ascorbate (DHA) in our study, followed the same path as AsA (Table 1). This oxidized form of vitamin C can be effectively reconverted to AsA in the human body and it is the most common vitamin C form in supplements and cosmetics [62].

Based on our assays, lettuce plants supplied with Fe1 and Fe2 showed a significantly higher antioxidant capacity when compared to the control plants (Table 1). This increase can be the result of the metal stress caused by the high accumulation of Fe within the plant organs. Similar increases in the antioxidant power of lettuce were observed by Jibril et al. [63] when plants were subjected to Cd stress.

The increase in the content of all above bioactive compounds suggests, that by enhancing Fe concentration in the nutrient solution, at 1 and 2 mM, we create a condition of metal stress in the lettuce, which produce reactive oxygen species (ROS) [25]. In turn, plants increase the production of non-enzymatic antioxidants such as AsA, phenols, flavonoids, carotenoids, whose main role is to scavenge or control ROS generation [64].

From a human nutrition perspective, this mechanism favors the production of health-promoting substances, making Fe biofortification a simple strategy to produce a healthier lettuce and attend an important consumer's demand.

As suggested by the biomass reduction, the application of Fe produced a stress response in lettuce plants. This fact is confirmed by the increase in stress indicator parameters such as ROS (O_2^-), lipid peroxidation indicators (MDA), or osmoprotector compounds (proline). Several studies support our results with an increase in these variables in plants subjected to Fe toxicity [25,65]. The values obtained for these indicators were higher in 'Romana', highlighting higher proline values, which is consistent with a higher stress response and a greater biomass loss.

Furthermore, plants possess mechanisms to cope with stresses such as those caused by Fe excess. For instance, enzymatic activities such as APX and CAT and antioxidant compounds such as AsA that are key for ROS detoxification are enhanced [65,66]. Thus, several studies observed that adequate Fe fertilization promotes these antioxidant systems because Fe is an enzyme cofactor acting as a catalyst for electron transfer reactions necessary for proper antioxidant functioning [66,67]. Likewise, in our study, a clear increase in antioxidant capacity (antioxidant tests), APX activity, and AsA was observed in biofortified lettuce plants, although no clear response of CAT and GPX enzyme activities was observed. Comparing between the two varieties, the higher activity of APX and CAT enzymes of the 'Nauplus' cultivar could favor ROS detoxification and would support its higher tolerance to Fe and lower biomass loss.

The biofortification treatments progressively stimulated the accumulation of other minerals such as total N, P, K, and S. A similar increase was described by Giordano et al. [27]. The authors noticed that lettuce plants submitted to 2 mM of Fe showed a higher N (in the form of nitrate) and P content. In contrast, the same authors noticed a progressive decrease in Ca and Mg contents, when the Fe concentration in the nutritive solution was enhanced. In our study, as for the Ca and Mg content, the two cultivars showed different responses when submitted to the different Fe doses. 'Nauplus' presented the higher Ca and Mg concentrations at 1 mM Fe while 'Romana' showed the higher increase at 2 mM Fe. Since this is a Fe-biofortification study, optimizing Fe absorption is a priority, in view that the Fe doses that do not cause an increase in the Ca content are preferable, because Ca is an inhibitor of Fe absorption [68]. When comparing both cultivars, the macronutrient contents (P, K, Ca, Mg, and S) were significantly higher in the cultivar Romana, when compared to 'Nauplus'. These results could be explained by the higher DM content accumulated in the former genotype.

The increase in micronutrient content (Mn, Zn, and B) observed in this study is consistent with the promotion of Mn and Zn in the leaves of African marigolds (*Tagetes erecta*) and zonal geraniums (*Pelargonium* × *hortorum*) subjected to high levels of Fe in the nutrient solution (1, 2, 4, and 6 mM) [48]. The genotype responses in our study suggest that for 'Romana', the optimal concentration of Fe in the nutrient solution should not exceed 1 mM, since this concentration allowed for the maximization of the mineral composition of leaves, mostly in terms of Ca, Mn, and B.

Regarding Fe accumulation, both additional doses of the mineral were able to produce Fe-biofortified lettuce. 'Romana' showed the highest Fe accumulation capacity, when compared to 'Nauplus'. This variability in Fe accumulation among cultivars of lettuce is common [69,70]. In fact, our results are in accordance with Giordano et al. [27], as they also highlight, in their Fe-biofortification study, a significant difference in the ability to accumulate Fe among the studied cultivars, being 'Red-Salanova', the one with the highest Fe content.

The highest amount of Fe accumulated in the leaves of 'Romana' could also explain its higher decrease in plant biomass and the higher antioxidant accumulation, when compared to 'Nauplus'. This is supported by the fact that concentrations above 500 mg kg^{-1} DW are reported as phytotoxic to the plant [71]. In fact, 'Romana' exceeded the Fe phytotoxicity limits in the tissues, in Fe1 and Fe2, meanwhile 'Nauplus' did not reach a phytotoxic range, in either of the treatments. This hypothesis is also supported by the highest proline increase observed in 'Romana', confirming the extreme stress condition of this genotype.

From a nutritional point of view, 100 g of fresh biofortified lettuce (under 1 mM of Fe) can provide 0.94 mg and 2.98 mg of Fe, 'Nauplus' and 'Romana', respectively. These values are comparable to the amount of Fe present in 100 g of prime beef (2.11 mg) and superior to pork loin and chicken breasts (0.68 and 0.62, respectively) [72]. Leaving aside considerations about the bioaccessibility of the element, these data support the hypothesis that Fe-biofortified lettuce can significantly contribute to increase the Fe concentration in the diet, facilitating Fe intake by humans and helping to fight the hidden hunger crisis.

N is a key element in plant growth and plays an important role in plant metabolism. The increase in the organic and assimilated N showed by the plants treated with 1 and 2 mM of Fe, is consistent with the increase in DM, as also observed by Giordano et al. [27].

In the context of human health, NO_3^- excess is a threat and its consumption should be minimized, because when it encounters the saliva and bacteria in the gastrointestinal tract, NO_3^- is partially converted to nitrite. Nitrite is associated with diseases such as infantile methemoglobinemia and carcinogenesis [73]. Efforts to reduce NO_3^- can involve different fertilization practices, as the use of organic fertilizers [74]. The European Commission [75] has set the maximum nitrate content allowed for the commercialization of fresh lettuce (grown in winter, under cover) as 5000 mg kg^{-1} FW. Both cultivars in this study presented NO_3^- levels within the limit, showing the 'Romana' cultivar to have the lowest NO_3^- content. In addition, the treated plants (Fe1 and Fe2) showed a reduction in NO_3^- concentration, suggesting that Fe supplementation increases the quality of lettuce, by improving an important food safety parameter. A similar effect was verified when the concentration of another metal mineral (Zn) was increased in the nutrient solution of lettuce, as Barrameda-Medina et al. [76] found a decrease in the NO_3^- presence.

5. Conclusions

Our findings indicate that Fe-biofortification of greenhouse-grown soilless lettuce is an effective tool to promote the dietary intake of Fe. We demonstrated that adding 1 mM of Fe (as Fe-HBED) in the nutrient solution not only increased the Fe content in leaves, but also stimulated the plant to produce and accumulate higher concentrations of health-promoting compounds, thus adding a possible market value to the product. Regarding the studied genotypes, 'Romana' showed higher concentrations of dry matter, Fe, minerals (N, P, K, Mn, and Zn), and a higher antioxidant power. However, high doses of Fe induced plants to stress, and from an agronomic perspective, the Nauplus genotype proved a higher tolerance to Fe exposure, showing the lowest biomass loss. Moreover, biofortification in soilless systems, through the management of the nutrient solution, proved to be simple and effective and should be further investigated. In this sense, studies aiming to mitigate the effects of metal stress on plants and the use of different molecules and concentrations are recommended to optimize the efficiency of lettuce biofortification.

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