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ORIGINAL PAPER

The multifaceted response of lettuce (*Lactuca sativa* L.) to biofortification with iron

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Abstract

The biofortification of plants seems to be a beneficial dietary strategy for alleviating iron deficiency around the world. The main aim of this study was to determine the content and bioaccessibility of minerals in biofortified lettuce in relation to plant morphological parameters. The experiment was conducted in a climate chamber under controlled conditions. Plants were grown in hydroponics with the application of nutrient solutions with varied iron levels (mg dm^{-3}): 1.5, 3.0, and 4.5. The contents of Fe, Zn, Cu, Mn, Ni, Mg, Ca, Na, and K in the lettuce were assayed after the dry ashing of the samples. The enzymatic digestion *in vitro* was performed using fresh lettuce plants, and then the contents of released elements were assayed. The concentrations of elements in lettuce before and after digestion were determined by atomic absorption spectrometry. In the lettuce, the contents of total, soluble, and insoluble fiber were also measured. It was found that the highest Fe level significantly increased the yield of dry matter. The studied trends of chlorophyll fluorescence change indicated a positive effect of Fe biofortification on plants but, at the same time, an increase in the carotenoid content was observed along with an increase in Fe, which may indicate the plants' initial defense response to increasing oxidative stress. Increasing Fe biofortification also caused an increase in the chlorophyll content in leaves. The higher content of Fe and of most other elements in the plant was determined in biofortified lettuces. Generally, biofortification of iron decreased Fe bioaccessibility and increased the bioaccessibility of other elements. In conclusion, the content of iron increases, but its bioaccessibility decreases in biofortified lettuce, and plant responses can be affected by biochemical reactions connected among others to carotenoids and the chlorophyll content.

Keywords: biofortification, lettuce, bioaccessibility, iron, nutrients

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INTRODUCTION

Iron deficiency and related negative health consequences are a serious global problem. Iron deficiency mainly affects women of childbearing age and pregnant women. The World Health Organization (WHO) reports that around 30% of the population suffers from iron deficiency anemia (Kumar et al. 2022). Iron deficiency in the body leads to anemia, fatigue, and heart problems, and in pregnant women is associated with premature birth and low birth weight newborns and growth problems in children (Pasricha et al. 2021). Epidemiological and clinical studies confirm that iron deficiency affects the development and health of entire societies worldwide (Kumar et al. 2022).

Providing the body with an adequate amount of iron is ensured by eating a diet with good sources of this element. In addition to an adequate supply of iron, its bioavailability is also important. In general, the bioavailability of iron is quite low, relatively high for heme iron found in meat and low for non-heme iron found mainly in plant foods (Man et al. 2022). In *in vitro* and *in vivo* studies, the average percentage of bioavailable iron from food products was mainly from 2% to 30%, and depended on the food matrix (Suliburska, Krejpcio 2014). The iron from a vegetarian diet is likely to be less bioavailable than from a non-vegetarian diet even if its content in both diets is similar. This is because of differences in the chemical form of iron (non-hem iron) and the nutritional factors that inhibit iron absorption, e.g. phytic acid and polyphenols (Hunt 2003). Various endogenous and exogenous factors affect the bioavailability of iron: for example, phytates significantly reduce its absorption of iron and ascorbic acid or natural antioxidants improve the bioavailability of this element (Piskin et al. 2022). The fiber content in the food affects iron absorption in the intestine. Insoluble dietary fiber is known to inhibit iron absorption, and inulin enhances the absorption of iron (Piskin et al. 2022). It is considered that poor bioaccessibility and bioavailability are the important contributors to mineral deficiency (Duijsens et al. 2023).

There are various dietary strategies to increase iron intake, such as food fortification with iron or using supplements. Products fortified with Fe had usually higher content and potential bioavailability of Fe in comparison with the non-fortified analogs (Suliburska et al. 2011, Bryszewska 2019). Iron availability from supplements depends on the iron form, salts, chelates, and also other components. The bioaccessibility of iron from supplements is usually high; however they can have many side effects on the organism (Piskin et al. 2022). The fortification strategy is regarded as the most effective, especially in the long-term approach to reducing the prevalence of iron deficiency, with fewer side effects than supplementation (Bryszewska 2019).

The beneficial dietary strategy in reducing iron deficiency may be the inclusion of biofortified edible plants because iron is supplied to the plant at

the stage of its growth and is built into the plant matrix, creating a natural source of iron. Some studies confirmed that biofortification improved iron bioaccessibility in lentils, rice, or beans (Huey et al. 2022, Ofori et al. 2022). The achieved results indicate a possible solution to the global problem of iron deficiency through the use of biofortified plants. In our work, we developed lettuce biofortified with iron. The main aim of this study was to determine the contents and bioaccessibility of minerals and multifaceted response of lettuce biofortified with iron.

MATERIALS AND METHODS

Vegetation experiment

The aim of the first part of this study was to make an evaluation of the effect of increasing Fe nutrition of lettuce (*Lactuca sativa* L. cv. 'Zeralda') on biofortification of plants with that mineral and other selected nutrients. The experiment was conducted in a climate chamber with LED lighting (16 h day/8 h night; PPFD 220 μmol m^{-2} s^{-1}), temp. 18/17°C (day/night), and RH 60-75%. 45 plants were grown on a cultivation table, of which 3 typical plants for each combination were selected for detailed chemical analysis (a replication was one single plant). A view of the experimental setup is shown in Photo 1.

Photo. 1. A view of the experiment in a climate chamber (February 2022)

Seedlings were prepared 3 weeks before the vegetation experiment. Lettuce seeds were sown on 12 January, into multipot trays filled with standard peat substrate recommended for seedlings production. Seedlings at the stage of 4-5 leaves were placed in drip-free pots (V 500 cm^3) filled with perlite. Plants were grown for 40 days, during which they were supplied different levels of Fe nutrition and watered to the stable weight. Plants were

fertigated with a nutrient solution of the following chemical compositions (mg dm^{-3}): $N\text{-}NH_4$ < 15, $N\text{-}NO_3$ – 135, $P\text{-}PO_4$ – 40, K – 250, Ca – 150, Mg – 50, Mn – 0.33, Zn – 0.21, Cu – 0.08, B – 0.2; pH – 5.50; EC – 1.9 mS cm^{-1}. The following fertilisers for hydroponic cultivation were used to prepare the nutrient solutions: potassium nitrate (13% $N\text{-}NO_3$, 38.2% K), calcium nitrate (14.7% $N\text{-}NO_3$, 18.5% Ca), mono potassium phosphate (22.3% P, 28.2% K), potassium sulphate (44.8% K, 17% S), magnesium sulphate (9.9% Mg, 13% S), manganese sulphate (32.3% Mn), copper sulphate (25.6% Cu), borax (11.3% B) and sodium molybdate (39.6% Mo). Three increasing levels of iron nutrition were applied (mg dm^{-3}): 1.5, 3.0, and 4.5 (denoted as Fe-I, Fe-II, and Fe-III, respectively). The source of iron was Librel Fe-DP 7 (7% Fe; DTPA form, Royal Brinkman, Poznan, Poland).

Chlorophyll fluorescence and leaf spectral parameter measurements

On the day of harvest (40th day of cultivation in pots; 7 April), chlorophyll a fluorescence was measured on all the plants in the experiment using a PAR-FluorPen FP 110D fluorimeter (PSI Company, the Czech Republic). Leaf fragments were shaded with a special leaf-clip for 30 minutes. Then, the OJIP test was conducted to measure the following chlorophyll fluorescence parameters: F_0 – initial fluorescence, F_M – maximum fluorescence intensity, F_V – maximum variable fluorescence, F_V/F_M – maximum photochemical quantum PSII after dark adaptation, Fv/F_0 – primary photochemical reaction yield rate, ABS/RC – light energy absorbed by the PSII antenna photon flux per active reaction centre, TR_0/RC – total energy used to reduce QA by the unit reaction centre of PSII per energy captured by a single active RC, ET_0/RC – rate of electron transport through a single RC, DI_0/RC – non-photochemical quenching per reaction centre of PSII, total dissipation of energy not captured by the RC in the form of heat, fluorescence and transfer to other systems, and PI_{Abs} – performance index (potential) for energy conservation from excitation to the reduction of the intersystem electron acceptors.

On the last day of the experiment, a handheld spectro-radiometer system was used for measurement of spectral reflectance of an internal light source (Xenon incandescent lamp 380 - 1050 nm) from leaves, and for measurements of transmittance and absorbance of any external light source (The PolyPen, PSI, the Czech Republic). The PolyPen incorporates formulas of commonly used reflectance indexes (e.g. NDVI, G, and PRI) into its software and displays values for the selected indexes.

Enzymatic digestion

The whole lettuce crop (edible part of the plants) was collected for the analysis. The lettuce was shredded and mixed, then divided into two batches – one used to determine the dry mass, and the other frozen at -80°C. Prior to the digestion process *in vitro*, the lettuce was thawed and homogenized,

then 2 g of sample were taken for each analysis. Samples of lettuce were dried at 105°C and the dry mass was measured. All analyses were made in 3 replications.

Enzymatic digestion *in vitro* was performed according to the method of Suliburska and Krejpcio (2014). To a 2 g sample, deionized water (20 ml) was added and the sample was shaken for 10 min. The pH was brought to 2 using 0.1 M HCl aqueous solution (Suprapure, Merck), then pepsin solution (0.5 ml 100 ml^{-1}) was added to the homogenate. Subsequently, samples were placed in a thermostatic shaker (37°C) for 2 h. During the incubation process, pH was checked or corrected when necessary by an addition of 6 M HCl aqueous solution. After 2 h, digested samples were treated with 6% $NaHCO_3$ aqueous solution (Extrapure, Merck) to bring pH to 6.8-7.0, the pancreatin solution (10 ml 40 ml^{-1} of homogenate) was added, and the samples were placed in a thermostatic shaker (37°C) for 4 h. Afterwards, the digested samples were centrifuged at 3.800 rpm min^{-1} for 10 min. The supernatant was taken and 65% nitric acid (Merck) was added, after which the samples were mineralized in a Speedwave XPERT Microwave Digestion System (Berghof, Eningen, Germany). Control (blank) samples were prepared by adding no product but all reagents.

In order to determine the total content of minerals in native products, lettuce samples (2 g) were ashed in a muffle furnace at 450°C until complete mineralization, and then dissolved in 1 N nitric acid. All analyses were made in 3 replications.

Mineral contents

The contents of minerals in native and *in vitro* digested products were determined by atomic absorption spectrometry (Atomic Absorption Spectrophotometer ZA3000, Hitachi) using an air acetylene flame, after obtaining an appropriate dilution with deionized water (for Fe, Zn, Cu, Mn, Ni, Na, and K) or with LaCl3 (0.3% solution, for Ca and Mg). The methods were validated by a simultaneous analysis of the reference material (LGC7162, LGC standards), with an average recovery of 92-98% accuracy for elements. The content of minerals in lettuce was expressed in mg kg^{-1} dry mass, while the degree of a mineral released (bioaccessibility) was expressed as the percent of mineral (mg) liberated during the enzymatic digestion *in vitro* from 100 g of product. Deionized water and acid-washed glassware were used in this study. Each lettuce sample from the *in vitro* digestion was analyzed in triplicate.

Total dietary fiber (TDF) and its soluble (SDF) and insoluble (IDF) fractions were estimated by the AOAC Method 991.43 (Ferjancic et al. 2022). A sample (about 0.2 g of dry matter) was placed and incubated with MES/TRIS buffer containing 2(N-morpholino) ethane sulfonic acid (MES, Sigma, M 8250), and tris(hydroxymethyl) aminomethane (TRIS, Sigma, T1503). Samples were digested by using thermostable α-amylase (Megazyme

E-BLAAM-40ML), protease (Megazyme E-BSPRT-40ML), and amyloglucosidase (Megazyme, E-AMGDF-100ML). After incubation, the non-digested residue was weighed (IDF), and SDF was precipitated from the soluble fraction. TDF was calculated as the sum of IDF and SDF.

Uptake of nutrients

The uptake of nutrients by the aerial parts of the plants was calculated by taking into account the dry matter yield and the determined contents of nutrients in the dry matter.

Statistical analysis

The results of chemical analysis were given as mean ± SD of three parallel measurements. The statistical analysis was carried out using the Statistica 13.0 software (StatSoft Inc., Tulsa, OK, USA), and the ANOVA with the Duncan test were performed at the significance level α=0.05. The Euclidean distance was used in the cluster analyses. The principal component analysis (PCA) was used to identify relationships between the parameters.

RESULTS AND DISCUSSION

Plant yield was determined by DM (dry matter). An increase in yield was found at the highest iron nutrition level (Fe, 4.5 mg L^{-1}), compared with lower-level Fe treatments. Simultaneously, a positive effect was found for total fiber and soluble fiber contents (Table 1).

Table 1

Basic characteristics of the Fe biofortified lettuce

Fe treatment	Yield of dry matter (g $plant^{-1}$)	Total fiber (g kg^{-1} d.m.)	Soluble fiber (g kg^{-1} d.m.)
Fe I	0.793±0.10*a**	367.3±14.1	89.1±9.5
Fe II	0.760±0.01*a*	376.6±17.6	90.2±16.9
Fe III	1.383±0.11*b*	394.8±21.2	102.7±12.2

insoluble fiber – total fiber - soluble fiber; d.m. – dry mass

* Key for Tables 1-4: data followed by the same letter do not differ significantly at α=0.05 for each parameter.

The spectrum analyses indicated that two phenomena were occurring in parallel: (1) improvement in chlorophyll content, and (2) simultaneous increase in carotenoid content (Table 2*A*). For example, an increasing tendency was determined in the case of NDVI, which may indicate improvement

Table 2

The influence of Fe biofortification on spectral reflectance of leaves

a

Fe treatment	G*	GM1	GM2	NDVI
Equal	R554/R677	R750/R550	R750/R700	(RNIR-RRED) / (RNIR+RRED)
Fe I	2.77±0.18*a*	2.57±0.12*a*	2.60±0.12*a*	0.67±0.02*a*
Fe II	2.85±0.14*a*	2.83±0.10*b*	3.01±0.13*b*	0.72±0.01*b*
Fe III	3.26±0.18*b*	3.08±0.17*c*	3.11±0.10*b*	0.73±0.01*b*

* G – Greenness Index, GM – Gitelson and Merzlyak Indexes, NDVI – Normalized Difference Vegetation Index

b

Fe treatment	OSAVI*	SR	PRI	CRI2	SIPI*
Equal	(1+0.16) * (R790-R670) / (R790-R670+0.16)	RNIR / RRED	(R531-R570) / (R531+R570)	(1/P550)-(1/P700)	(R790-R450)/ (R790+R650)
Fe I	0.72±0.03	4.81±0.38*a*	-0.009±0.001*a*	5.94±0.50*a*	0.75±0.02
Fe II	0.73±0.03	5.58±0.23*b*	0.008±0.002*b*	6.65±0.43*b*	0.76±0.04
Fe III	0.75±0.03	5.95±0.45*b*	0.013±0.003*c*	7.52±0.50*c*	0.79±0.03

* OSAVI – Optimized Soil-Adjusted Vegetation Index, SR – Simple Ratio Index, PRI – Photochemical Reflectance Index, CRI2 – Carotenoid Reflectance, SIPI – Structure Intensive Pigment Index

in leaf colour, towards green, and this positively correlates with the value of the G parameter (greenness). An increase in GM1 indicates an increase in the chlorophyll content. At the same time, the SR index indicates improvement in the plant's overall wellbeing. Similar tendencies were found in the OSAVI index, which indicates improvement in plant vitality, positively correlating with the level of Fe nutrition. Also, PRI increased under the studied Fe biofortification levels.

Protective function of carotenoids against ROS, which determines the plant's growth, can be associated with a gradual increase in Fe-induced oxidative stress (Maoka 2020). Changes in the carotenoid content relative to chlorophyll are indicated by the CRI1 index (Table 2*b*). The value of the Structure Intensive Pigment Index (SIPI) indicates a tendency of a growing content of carotenoids in relation to chlorophyll (Penuelas et al. 1995), which may indicate the emergence of early oxidative stress symptoms in plants that are not yet morphologically visible.

Equations were determined to describe the trend of changes in the values of the indexes under study (Figure 1). The determined R^2 values for the indexes with significant changes in values under the influence of iron biofortification ranged from 0.45 (for G index) to 0.81 (for PRI index).

The studied trends indicate a positive effect of iron biofortification on plants' response: a significant decrease in F_0 was found, which may positi-

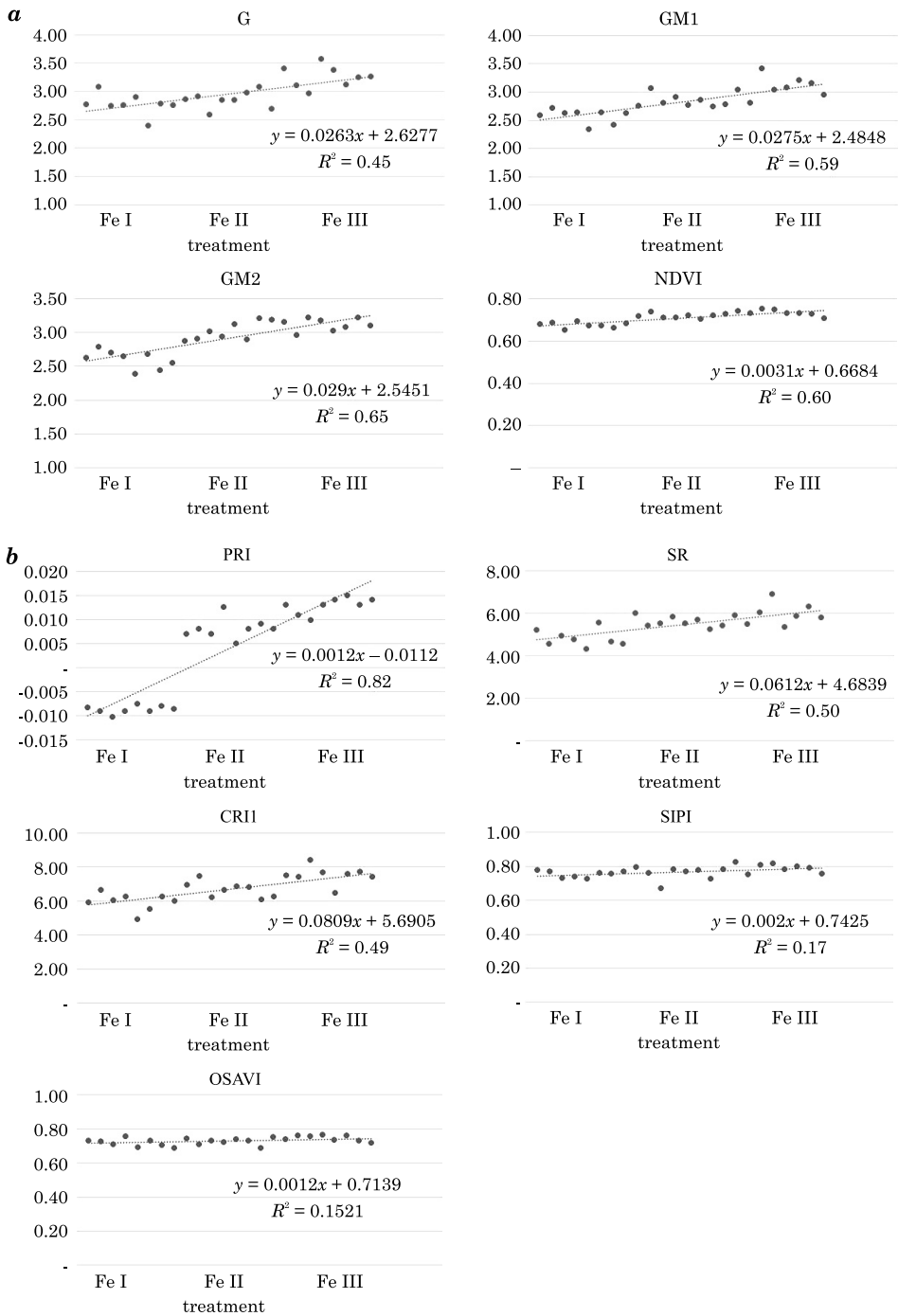

Fig. 1. The influence of Fe biofortification on the trend of values of the indexes demonstrating spectral reflectance of leaves (mean values from determinations)

vely indicate improvement in the transfer of excitation energy between chlorophyll molecules (Table 3*a*). The efficiency index of the primary photochemical reaction, characterizing the efficiency of oxygen release in PSII (F_V/F_0), was improved. No negative changes in F_M and F_V were shown, which may indicate that the plants were not affected by strong oxidative stress associated with the levels of Fe nutrition. At the same time, plants' wellbeing is further evidenced by a significant increase in the F_M/F_0 ratio: its significant decrease to < 4 is characteristic of severe stresses.

Table 3

The influence of Fe biofortification on selected chlorophyll fluorescence parameters

a

Fe treatment	F_0	F_M	F_V	F_M/F_0	F_V/F_0	F_V/F_M
Fe I	4 947±227*b*	30 991±1448	26 045±1440	6.29±0.38*a*	5.29±0.48*a*	0.84± 0.01
Fe II	4 468±178*a*	29 920±2096	25 452±1863	6.70±0.24*ab*	5.70±0.24*ab*	0.85±0.01
Fe III	4 450±200*a*	31 046±4568	26 596±4452	6.99±0.34*b*	5.99±0.94*b*	0.86±0.03

b

Fe treatment	Pi_Abs	ABS/RC	TRo/RC	ETo/RC	DIo/RC
Fe I	10.46±1.60*a*	1.21±0.07	1.03±0.06	0.71±0.03*a*	0.18±0.01
Fe II	13.42±1.70*b*	1.19±0.03	1.02±0.02	0.74±0.03*a*	0.17±0.01
Fe III	14.61±1.53*b*	1.23±0.05	1.05±0.04	0.79±0.04*b*	0.18±0.01

As the Fe level increased, the PSII function index calculated from energy absorption (Pi_Abs) increased significantly, which positively correlates with the dry matter yield achieved. Electron flow rate behind QA per RC (ETo/RC) was also significantly improved. Changes in other fluorescence parameters were not significant.

Iron biofortification had relatively different effects on the studied parameters of spectral reflectance of leaves and of chlorophyll fluorescence (Figures 2, 3).

The mineral contents of lettuce depending on the Fe biofortification levels tested are shown in Table 4. The concentration of iron in the dry mass of lettuce was significantly higher under 3.0 and 4.5 mg dm^{-3} of Fe biofortification, and was over three times higher than that in the control group Fe I (1.5 mg dm^{-3}). In the dry mass of lettuce, the higher level of biofortified Fe (Fe III) significantly increased the concentration of Zn, Mn, and Mg in the plants. The Ni content in the dry mass of lettuce was the lowest in the Fe III group and the highest in the Fe II group. The concentrations of sodium and potassium in lettuce dry matter demonstrated the opposite trend; the sodium concentration significantly decreased in Fe II and Fe III groups, and potassium concentration increased in these groups.

The Pearson's correlation analysis showed varying relationships between the parameters studied and increasing levels of iron biofortification. The cor-

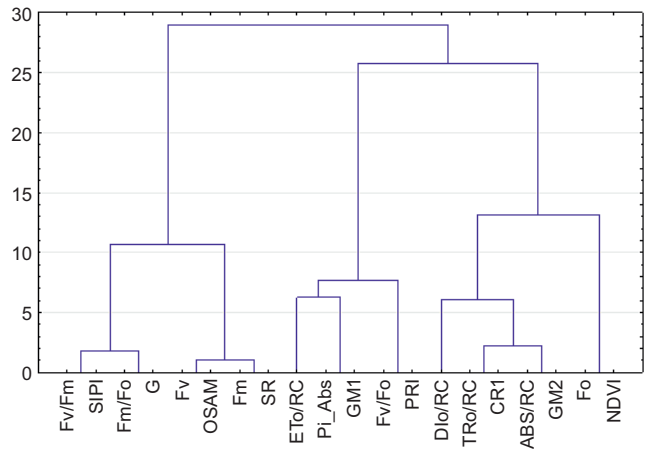

Fig. 2. The cluster analysis of the studied indexes of spectral reflectance of leaves and of chlorophyll fluorescence

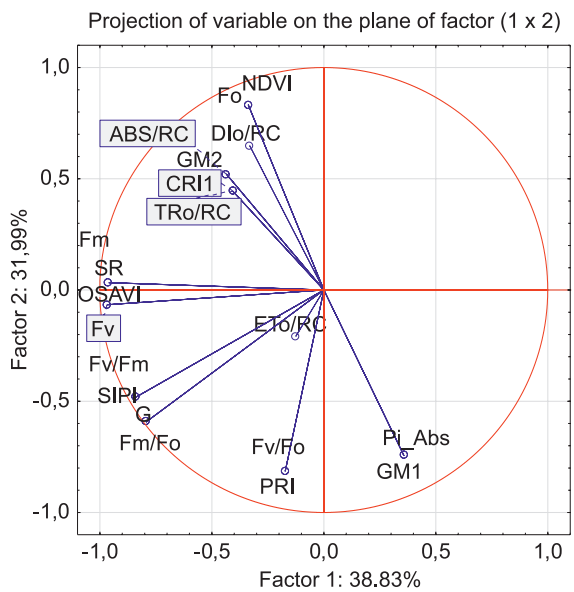

Fig. 3. Principal component analysis (PCA) of indexes of spectral reflectance of leaves and of chlorophyll fluorescence

relation between Fe nutrition level and uptake of this nutrient by aerial parts of lettuce plants was positive (0.99), similarly to dry matter yield (0.83). However, there was a negative relationship between the iron uptake and its bioavailability (-0.43), which is also confirmed by the previously conducted analysis of variance (Table S1). These relationships are displayed in Figures 4 and 5.

Results of the determinations of bioaccessibility indicated that iron bioaccessibility significantly decreased in any biofortified group compared to the

Table 4

Mineral contents and their bioaccessibility in Fe biofortified lettuce

Parameters	Content in dry mass	Bioaccessibility
Fe	(mg kg^{-1} d.m.)	(%)
Fe I	53.2±4.0*a*	27.27±1.86*c*
Fe II	177.4±23.7*b*	3.53±0.15*a*
Fe III	167.5±6.2*b*	16.66±1.05*b*
Zn	(mg kg^{-1} d.m.)	(%)
Fe I	21.5±2.9*a*	46.46±5.48*b*
Fe II	21.1±2.9*a*	28.04±2.83*a*
Fe III	24.0±0.7*b*	39.22±5.49*ab*
Cu	(mg kg^{-1} d.m.)	(%)
Fe I	3.245±0.464	47.83±8.38
Fe II	3.236±0.303	36.14±8.22
Fe III	3.410±0.076	58.03±3.05
Mn	(mg kg^{-1} d.m.)	(%)
Fe I	62.3±4.6*a*	20.25±0.96*a*
Fe II	64.3±2.9*a*	18.93±0.60*a*
Fe III	126.6±20.3*b*	23.72±2.14*b*
Ni	(mg kg^{-1} d.m.)	(%)
Fe I	2.065±0.462*ab*	28.22±5.42*a*
Fe II	2.250±0.594*b*	25.54±5.75*a*
Fe III	1.740±0.146*a*	71.55±4.97*b*
Mg	(% in d.m.)	(%)
Fe I	0.221±0.05*a*	28.63±4.08*a*
Fe II	0.217±0.06*a*	27.26±6.20*a*
Fe III	0.317±0.02*b*	44.08±3.87*b*
Ca	(% in d.m.)	(%)
Fe I	1.096±0.08	21.00±4.03*a*
Fe II	1.073±0.05	18.33±1.36*a*
Fe III	1.082±0.04	30.24±1.70*b*
Na	(% in d.m.)	(%)
Fe I	0.096±0.0014*b*	37.74±1.18*a*
Fe II	0.079±0.008*a*	35.12±3.37*a*
Fe III	0.083±0.007*a*	76.52±3.47*b*
K	(% in d.m.)	(%)
Fe I	1.581±0.021*a*	33.79±1.21*ab*
Fe II	1.783±0.073*b*	23.43±4.50*a*
Fe III	1.630±0.059*ab*	48.41±3.14*b*

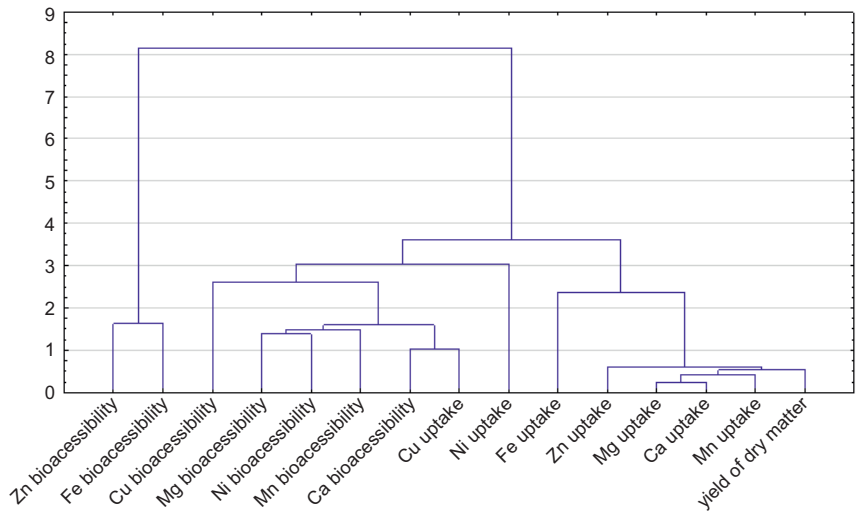

Fig. 4. The cluster analysis of the selected chemical parameters

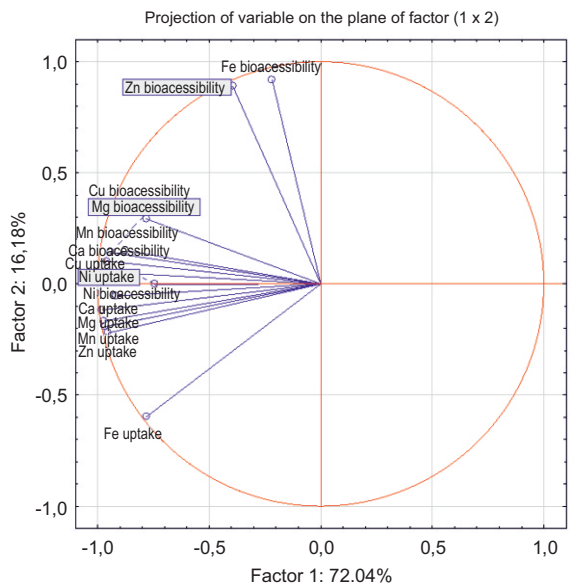

Fig. 5. Principal component analysis (PCA) of indexes of mineral uptake and bioacessibility

control group, and was the lowest in the Fe II group. Similar changes in bioaccessibility between groups were observed for zinc, with the lowest bioaccessibility of Zn in Fe II. Bioaccessibility of Mn, Ni, Mg, Ca, Na, and K was significantly higher in Fe III than in other groups (for K, marked differences were found between Fe III and Fe II groups).

The amounts of minerals released (potentially bioavailable) from 100 g of wet lettuce are shown in Figure 6. The amounts of released Fe were com-

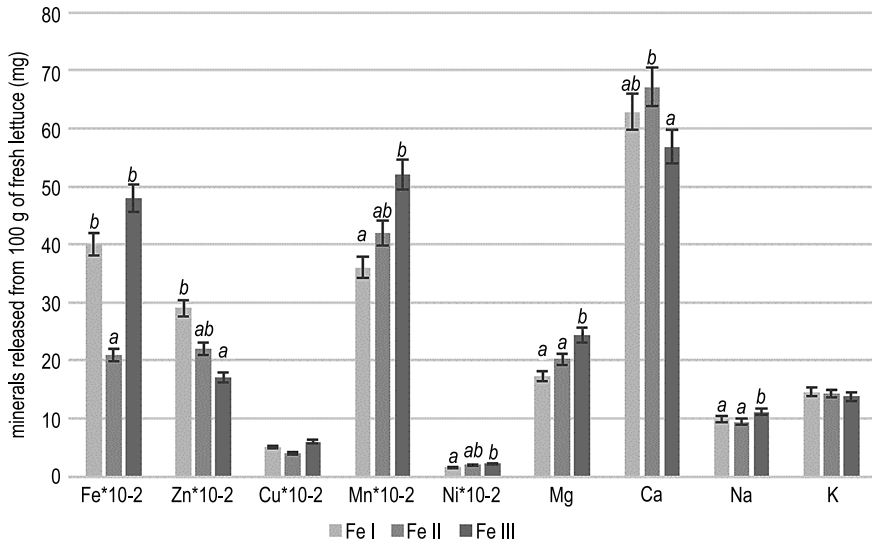

Fig. 6. The amounts of minerals (mg) released from fresh lettuce (100 g): *a*, *b* – significant differences (p<0.05)

parable in Fe I and Fe III groups, but drastically lower in the Fe II group. Increased Fe levels in the nutrient solution markedly decreased released Zn, but significantly increased released Mn and Ni. The significantly higher potentially bioavailable Mg and Na were in the Fe III group compared to Fe I and Fe II groups. The amounts of released Ca were significantly lower in Fe III than in Fe II (Figure 6).

The most important finding in this study is that iron biofortification of lettuce increases the yield of dry matter and iron content, but decreases iron bioaccessibility. Iron biofortification of lettuce at the levels of 3.0 mg dm^{-3} and 4.5 mg dm^{-3} seems to be ineffective when it comes to iron absorption in the body. However, lettuce biofortified with iron may be a better source of Mn, Mg, and Na for the organism, and it may increase nickel exposure of the consumer compared with common lettuce. The determined trends need to be confirmed for other plant species, with the inclusion of other iron compounds (7% Fe-DTPA was used in our study).

The increasing dry matter yield of lettuce at the Fe level of 4.5 mg dm^{-3} in the nutrient solution is desirable for food production. This effect is beneficial for lettuce growers. Based on the results obtained in this study, the applied dose of 4.5 mg dm^{-3} positively affected the growth dynamics of lettuce, unlike the dose of 3 mg L^{-1}, which did not affect the growth of plants. The previous study demonstrated that 3.0 mg dm^{-3} of Fe in the nutrient solution did not affect the yield of fresh matter of kohlrabi, peas, radish, and spinach (Frąszczak, Kleiber 2022), with a simultaneous decrease in dry matter production in kohlrabi and radish at a Fe level of 3.0 mg dm^{-3}.

Iron is an essential element for the physiological and biochemical processes in plants, and is required for chlorophyll biosynthesis and photosynthesis (Roosta et al. 2018, Kobayashi et al. 2019), but the response to this metal could vary among plant species (Frąszczak, Kleiber 2022). The important role of iron in plant growth explains the significant increase in yield of lettuce grown on the nutrient solution with the highest iron content. In an experiment conducted with a sufficient amount of water, there was an increase in biomass production. Biomass yield actually reflects the course of physiological processes occurring in the plants, especially photosynthesis and respiration. In our study, we determined an increase in yield under the increasing Fe nutrition level. The reasons for this can be seen in the increased chlorophyll content in leaf tissues (indexes G, GM1, and GM2, NDVI) and overall plant vigour (indexes OSAVI and SR). This is confirmed by changes in the PRI index value, with an increasing trend parallel to the increasing Fe levels ($y = 0.0087x - 0.0165$; $R^2 = 0.93$). The PRI index is significantly correlated with both net CO_2 uptake and radiation use efficiency as measured by gas exchange (Kohzuma et al. 2021), explaining the upward trend in dry matter yield. Inordinately intensive Fe nutrition could inhibit chlorophyll synthesis. Pigment disruption of the photosynthetic complex promotes changes in electron transport, reduces the net assimilation rate of CO_2, and deprives plants of essential sugars (Lanquar et al. 2005). Nutrition of plants with metal micronutrients (including Fe) has influence on various parts of the photosynthetic apparatus and biomass production (Roosta et al. 2018). In the conducted study, we showed the impact of iron nutrition on photosynthetic activity by tracing distinctive chlorophyll fluorescence changes. For example, F_V/F_M decreasing at lower Fe levels (however, not proven statistically) could have occurred as a result of some damage to the PSII reaction centre.

In this study, despite an increase in the iron content in biofortified lettuce tissues (Fe II and Fe III), a decrease in iron bioaccessibility was observed, which was an unexpected result. Significantly reduced bioavailability of Fe in group Fe II could have been caused by a significant increase in dry matter. The relationship between the bioavailability of Fe and the amount of dry matter was confirmed by a significant inverse correlation between the content of Fe in dry matter and its bioavailability (Supplementary, Table 1S). Accumulation of Fe and other elements, as well as other components in the matrix that were not analyzed, may induce interaction between components (also other minerals) and decrease Fe release from the matrix (Kobayashi et al. 2019, Zhao et al. 2021, Frąszczak, Kleiber 2022). In our experiment, we used DTPA, which contributes to the optimal nutrition of the plant with Fe. However, the results obtained indicate that the presence of iron chelate in lettuce tissue may have contributed to reducing the bioaccessibility of iron from this plant to the organism. From a cognitive point of view, it would be interesting to determine the DTPA content in plant tissues. The bioavailability of iron was negatively correlated with

its content in both fresh and dry mass. Interaction with calcium, magnesium or zinc could have influence on bioavailability.

Chelate DTPA (iron complex with diethylenetriaminepentaacetic acid) contains valuable Fe(III) iron. Plants use two different strategies for Fe uptake ("strategy I" – dicotyledonous plants. and "strategy II" – graminea plants) – Römheld, Marschner (1986). Strategy I is a reduction-based mechanism that involves acidification of the rhizosphere by root-released protons (Robe et al. 2020*a*,*b*). Increased H+-ATPase activity causes the roots to release coumarin and riboflavin, which increases Fe3 mobility. A membrane-localized reductase then reduces Fe^{3+} to Fe^{2+}, and a membrane-localized high-affinity Fe transporter then absorbs Fe^{2+} from the roots. The details of this process are described in a number of articles (Santi et al. 2003, Fan 2022, Pei et al. 2022). Fe^{3+} reduction system, comprising reductive coenzyme II (NADPH) dehydrogenase and trivalent iron chelating reductase (FRO). After generating chelates with chelators in the plant, Fe^{3+} released by the H+-ATPase protease system enters the cell. The reduction system then transforms Fe^{3+} chelates into Fe^{2+} chelates that are released for plant uptake and utilization. The Fe^{2+} transporter protein (IRT) system, which brings Fe^{2+} chelates reduced by Fe^{3+} chelating reductase into the cell by transmembrane transport and then transported by other transporter proteins to various organelles and organs of the plant to supply the plant for its growth and development. The above process requires energy. However, in our research, it was determined that the use of increasing Fe-EDTA concentrations significantly increased the yield of dry mass, which indicates the efficiency of photosynthesis. This was also confirmed by the tendencies of changes in the fluorescence of chlorophyll. However, excessively intensive Fe-EDTA nutrition can cause a toxic effect, which may be due to the effect of chelate itself (or possible admixtures) or the sensitivity of the plant species (Siebach et al. 2015). Contrary to our results, peas and beans biofortified with Fe were good sources of Fe and also improved the iron status in organisms, as evidenced in *in vivo* studies (Sant' Ana et al. 2019, Vaiknoras, Larochelle 2020, Coelho et al. 2021). However, another study indicated that biofortified beans did not provide more iron to the organism than a non-biofortified plant did, which complies with our findings (Glahn et al. 2020).

Iron biofortification in lettuce affects the concentration and bioaccessibility of other elements. In this study, we observed a positive relationship between zinc and iron in lettuce, as zinc in biofortified lettuce changed similarly to iron. The level of fortification of 4.5 mg dm^{-3} significantly increased the content and bioavailability of elements such as Zn, Mn, Mg, and K. Therefore, the real content of Mn, Mg, Ca, and Na released from fresh lettuce was higher in the group with the higher level of biofortification.

Effects of biofortification of Fe on mineral contents in lettuce may indicate the competition between Fe and other cations, as some minerals (Cu and Zn) share the same cell membrane transporters (De Dorlodot et al.

2005). In kohlrabi, pea, radish, and spinach biofortified with 3 mg dm^{-3} of Fe, decreased contents of zinc and copper, and an increased concentration of Mn in dry mass were determined, an effect which was partly (in the case of Mn) confirmed in this study in lettuce (De Dorlodot et al. 2005). The higher bioaccessibility of the analyzed minerals in lettuce Fe III could be related to the change in the ratios between minerals and lesser competition between the components during release, and to the change in other parameters not analysed in our experiment. The percent bioaccessibility of Cu, Mn, and Zn obtained in this study was lower than that from lettuce biofortified with Se (Do Nascimento de Silva, Cadore 2019), which may confirm strong competition between Fe and other minerals, but also may be due to different plant growth conditions in both studies.

The significant increase in the amount of Ni released from lettuce Fe III can be alarming, although the content of Ni decreased in the dry matter of the plant. However, any increase in Ni potential bioavailability indicates that we can absorb more of this element from this lettuce than from unfortified one. The ratio of iron to nickel in the substrate affects the uptake of these elements by the plant, and it can explain why the excess of iron in Fe III reduced the nickel content in lettuce (Merlot 2020). In the organism, nickel interacts with iron at the absorption level because these elements can compete for the same transport mechanisms in the intestine (Tallkvist, Tjalve 1997). In a human study, the reverse interaction between iron and nickel status was observed (Pieczyńska et al. 2021). The functions of nickel in the body are not fully understood, but exposure to this element can be dangerous due to the elevated oxidative stress and damage to mitochondria. Common symptoms of nickel exposure include allergies, cardiovascular diseases, kidney dysfunctions, and cancer (Genchi et al. 2020).

Fe and Mn compete for the same protein in blood serum (transferrin) and the protein divalent metal transporter system DMT 1 (Roth, Garrick 2003). As the level of iron nutrition increased, the Mn:Fe ratios changed; however, the indicated trends were not linear and also suggest a possible interaction between other nutrients. On the other hand, as the intensity of lettuce nutrition with manganese increases, its relationship with iron increases, and the trend of change (up to 19.2 mg dm^{-3}) is linear (R^2=0.95) – Kleiber (2014).

A limiting factor in plant biofortification may be the oxidative stress it induces. Chemically, iron is a metal, and when used in excess, it can cause stress. However, the intensity of stress can vary depending on the plant species and developmental stage (Roosta et al. 2018). This has been confirmed in a study on broccoli microgreens (Vastakaite-Kairiene et al. 2022), where different doses of Fe (ranging from 2 to 15 ppm) in the medium did not significantly affect the biometric parameters. This assertion has also positively correlated to the other results (Przybysz et al. 2016, Di Gioia 2019). In the case of microgreens such as kohlrabi and radish, a significant reduction

in dry matter yield was found at Fe levels of 3 mg dm^{-3} (Frąszczak, Kleiber 2022). Peas, on the other hand, appeared to be more tolerant, and at 3 mg dm^{-3}, the pea yield showed an increasing trend compared to the plants growing at 1.5 mg Fe dm^{-3} (Frąszczak, Kleiber 2022). In our study, we showed changes in the carotenoid content. The protective function of carotenoids against ROS (reactive oxygen species) can be associated with a gradual increase in Fe-induced oxidative stress (Maoka 2020). Changes in the carotenoid content relative to chlorophyll are indicated by the CRI1 index. The value of the Structure Intensive Pigment Index (SIPI) indicates a tendency to increase the content of carotenoids in relation to chlorophyll (Penuelas et al. 1995), which may indicate the emergence of stress symptoms in plants, not yet morphologically visible. The PRI is also sensitive to changes in carotenoid pigments in leaves, such as xanthophyll pigments (Wong, Gamon 2015). However, deficient iron nutrition of lettuce has a negative impact leaves by reducing their carotenoid content (Roosta et al. 2018). At the same time, it is also important to mention the beneficial effects of carotenoids, including antioxidants, in the human diet (Eggersdorfer, Wyss 2018).

Although iron is a micronutrient for plants, excess iron in plant tissues can cause a number of metabolic disorders, primarily due to increased ROS levels caused by participation in the Fenton reaction (Thongbai, Goodman 2000). The reason for the toxic effect of iron is its reaction with hydrogen peroxide (H_2O_2) and hydroxyl radical (OH–), which is the most powerful ROS oxidant (Dixon, Stockwell 2014). Therefore, it is important to select species capable of accumulating high-affinity iron, and to conduct research on the optimisation of plant biofortification with that micronutrient.

This is the first study to analyse the bioaccessibility of minerals from lettuce biofortified (via nutrient solution directly to rootzone) with iron on two levels. In our research, we used our proven *in vitro* digestion method, although other *in vitro* digestion models are currently being used (Duijsens et al. 2023). Using the same method, however, allows us to compare the results we have obtained in different studies, and these results are comparable to ones from other widely cited models (Suliburska, Krejpcio 2014).

CONCLUSIONS

Biofortification of Fe at 4.5 mg dm^{-3}significantly increases the DM yield of the lettuce. The content of iron in biofortified lettuce increases, but the bioaccessibility of iron decreases. From the nutritional point of view, the biofortification of lettuce with levels of 3.0 mg dm^{-3} and 4.5 mg dm^{-3} (if in the form of DPTA) is ineffective. However, the biofortification with 4.5 mg dm^{-3} may improve the amount of Mn, Mg, Ni, and Na ions released

from lettuce. At the same time, as confirmed by trends in changes in the carotenoid content (non-destructive measurement of pigments), increasing the level of Fe nutrition can increase oxidative stress and reduce crop yields.

Author contributions

J.S. – conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization, writing – original draft preparation; writing – review & editing; T.K. – conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization, resources, writing – original draft preparation, writing – review & editing; R.G. – conceptualization, funding acquisition, investigation, project administration, resources, writing – review & editing; K.D.- methodology, writing – review & editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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