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Short Term Elevated CO₂ Interacts with Iron Deficiency, Further Repressing Growth, Photosynthesis and Mineral Accumulation in Soybean (*Glycine max* L.) and Common Bean (*Phaseolus vulgaris* L.)

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Abstract: Elevated CO₂ (eCO₂) has been reported to cause mineral losses in several important food crops such as soybean (*Glycine max* L.) and common bean (*Phaseolus vulgaris* L.). In addition, more than 30% of the world's arable land is calcareous, leading to iron (Fe) deficiency chlorosis and lower Fe levels in plant tissues. We hypothesize that there will be combinatorial effects of eCO₂ and Fe deficiency on the mineral dynamics of these crops at a morphological, biochemical and physiological level. To test this hypothesis, plants were grown hydroponically under Fe sufficiency (20 μM Fe-EDDHA) or deficiency (0 μM Fe-EDDHA) at ambient CO₂ (aCO₂, 400 ppm) or eCO₂ (800 ppm). Plants of both species exposed to eCO₂ and Fe deficiency showed the lowest biomass accumulation and the lowest root: shoot ratio. Soybean at eCO₂ had significantly higher chlorophyll levels (81%, $p < 0.0001$) and common bean had significantly higher photosynthetic rates (60%, $p < 0.05$) but only under Fe sufficiency. In addition, eCO₂ increased ferric chelate reductase activity (FCR) in Fe-sufficient soybean by 4-fold ($p < 0.1$) and in Fe-deficient common bean plants by 10-fold ($p < 0.0001$). In common bean, an interactive effect of both environmental factors was observed, resulting in the lowest root Fe levels. The lowering of Fe accumulation in both crops under eCO₂ may be linked to the low root citrate accumulation in these plants when grown with unrestricted Fe supply. No changes were observed for malate in soybean, but in common bean, shoot levels were significantly lower under Fe deficiency (77%, $p < 0.05$) and Fe sufficiency (98%, $p < 0.001$). These results suggest that the mechanisms involved in reduced Fe accumulation caused by eCO₂ and Fe deficiency may not be independent, and an interaction of these factors may lead to further reduced Fe levels.

Keywords: elevated CO₂; iron deficiency (Fe); plant nutrition; organic acids; photosynthesis

1. Introduction

Atmospheric CO₂ is one of the main drivers of climate change. In June of 2021 [1] global CO₂ concentrations were 418.7 ppm, but these are predicted to double by the end of this century [2] Elevated CO₂ (eCO₂) is known to affect plant growth, crop yield and nutritional status of agricultural products. Although it seems to induce higher growth and yield [3–9] a number of studies making use of meta-analysis methodologies showed reduced mineral or protein concentrations in several crop species such as wheat, barley, rice, common bean, soybean, among others [10–14]. In previous studies, eCO₂ has been reported to induce photosynthesis [15], stomatal closure [2,16,17] and increase the levels of leaf organic acids concentrations [18–20]. Organic acid release is well known to be associated with iron (Fe) uptake and transport within the xylem [21–23], and reduced stomatal conductance and transpiration rates, may reduce mineral uptake through the xylem sap since transpiration-driven mass flow from root to other organs may be reduced [24,25].

Fe deficiency is another factor highly affecting the nutritional status of food crops. The insoluble nature of Fe in calcareous soils, which represent about 30% of the world's arable land, imposes restricted Fe supply for plant growth leading to reduced yields and lower nutritional quality [26]. Under Fe deficiency, plants develop typical symptoms of Fe deficiency chlorosis (IDC), such as yellowing of the upper leaves and interveinal chlorosis. These symptoms, depending on the crop, are particularly visible in the first two to three weeks after seed emergence. Legumes are particularly prone to IDC, their nutritional value is further jeopardized by eCO₂ and they provide a large share of the global population's diet. Common bean (*Phaseolus vulgaris*) comprises 50% of grain legumes consumed worldwide being the most important for direct human consumption [27] especially in Latin America and Africa [28] and in 2017 the production surpassed 31 million metric tons (FAO statistics). Soybean (*Glycine max.*) is the most produced legume crop worldwide, more than 230 million metric tons being produced per year [29], and cultivated on an estimated 6% of the world's arable land.

In this scenario of global legume dependency, reductions on the mineral levels of these important crops, due to increasing CO₂ and Fe deficiency, will aggravate current dietary deficiencies, particularly in countries whose primary dietary sources are grain legumes. Dietary deficiencies are already a major global public health problem. More than two billion people suffer from Fe deficiency anemia, leading to reduced growth in childhood, reduced ability to fight off infections and higher rates of maternal and child deaths [13], causing the loss of 63 million life-years annually [30].

Although there is evidence that there will be nutrient losses from eCO₂ and Fe deficiency, there is little knowledge on the mechanisms involved in these losses, and no studies have been done on the combined effect of these two environmental changes in legumes. Previous studies mainly focused on eCO₂ combined with changes in nitrogen (N) [15,31–36], phosphorous (P) [37–40], magnesium (Mg) [41,42] and potassium (K) availability [7,43], with only one on Fe [44].

Strategy I plants such as legumes rely on an Fe reduction mechanism in their roots to acquire Fe (Blair et al., 2010). This mechanism involves (i) the activity of a membrane bound Fe chelate reductase, and the extrusion of organic acids (particularly malate and citrate, which are associated with Fe uptake and transport within the xylem), and protons, which solubilizes Fe prior to uptake [45].

The objective of this study is to understand the individual and combined effects of Fe deficiency and eCO₂ on plant growth (biomass accumulation and partitioning), physiology (chlorophyll and photosynthetic activity), mineral nutrition (Fe, Zn, Mg, Mn, K and P) and biochemistry, by looking at rate limiting steps associated with Fe uptake including Fe reductase activity and malate and citrate accumulation. This study also aims to understand if the influence of eCO₂ and Fe deficiency on Fe-related processes in legumes is species dependent.

2. Materials and methods

2.1. Plant Material and Growth Conditions

Seeds of *G. max* cv. "Thorne" and *P. vulgaris* cv. "Papo de Rola" were rolled in filter paper humidified daily with water and placed vertically for seven days in the dark, at 25 °C. Germinated seedlings were transferred to 5 L hydroponic vessels (three vessels with five seedlings per vessel). The vessels were placed in a climate chamber (Aralab Fitoclima 10000EHF) with 16 h day photoperiod providing 325 μmol s⁻¹ m⁻² of photosynthetic photon flux density at plant level supplied by a mixture of incandescent bulbs and fluorescent lights. Temperatures were set to 25 °C during the light period and to 20 °C during the dark period, whereas relative humidity (RH) was maintained at 75% throughout day and night.

Plants grew in hydroponics under ambient CO₂ (aCO₂, 400 ppm) and eCO₂ (800 ppm) for three weeks. The standard solution for hydroponic growth of both cultivars included: 1.2 mM KNO₃; 0.8 mM Ca(NO₃)₂; 0.2 mM MgSO₄·H₂O; 0.3 mM; NH₄H₂PO₄; 25 mM CaCl₂; 25 mM H₃BO₃; 0.5 mM MnSO₄; 2 mM; ZnSO₄·H₂O; 0.5 mM CuSO₄·H₂O; 0.5 mM MoO₃;

0.1 mM NiSO₄. The hydroponic solution was buffered with 1 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5. Plants grew for six days under complete solution with 20 µM Fe(III)-EDDHA (ethylenediamine-N,N-bis(o-hydroxyphenyl) acetic acid) followed by two weeks treatment with 0 and 20 µM Fe(III)-EDDHA. After two weeks under the settled conditions, a set of measurements was performed in these plants including chlorophyll levels, photosynthetic rates, Fe reductase activity and dry weight of leaves, stems and roots. One set of leaf and root samples from five individual plants of each treatment was dried for mineral analysis whereas another set of samples from the same tissues of other five individual plants of each treatment was frozen under N₂ for the analysis of organic acids.

2.2. Morphometric Parameters

The chlorophyll levels were assessed with Soil and Plant Analyzer Development (SPAD) readings, measured with a portable chlorophyll meter (Konica Minolta SPAD-502 Plus; Minolta, Osaka, Japan), using the youngest trifoliate leaf of five independent biological replicates. The roots, shoots and leaves were separated, and the dry weights of these organs were determined.

2.3. Photosynthesis

The photosynthetic rate was measured using an infrared gas analyzer (IRGA) LI-6400XT Portable Photosynthesis System (LI-COR Inc., Lincoln, CA, USA) by attaching the IRGA chamber to the most expanded leaf of the youngest trifoliate. The measurements were taken in the following conditions: leaf temperature at 25 °C, photosynthetically active photon flux density at 500 µmol m⁻² s⁻¹, CO₂ concentration at 400 µmol CO₂ mol⁻¹ for plant grown under aCO₂ and 800 µmol CO₂ mol⁻¹ for plants grown under eCO₂, and flow rate at 500 µmol s⁻¹. The measurements were conducted in five individual plants from each growth condition.

2.4. Ferric Chelate Reductase (FCR) Assay

Fe reductase activity was measured as described by Grusak et al. (1990) except that ferrozine was used instead of BPDS (bathophenanthroline disulfonic acid). Measurements were performed in whole intact roots of five individual plants where Fe reduction was followed by the spectrophotometric measurement of Fe²⁺ chelated to ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt). Roots of each intact plant were submerged in assay solution containing 1.2 mM KNO₃, 0.8 mM Ca(NO₃)₂, 0.3 mM NH₄H₂PO₄, 0.2 mM MgSO₄, 25 µM CaCl₂, 25 µM H₃BO₃, 0.5 µM MnSO₄, 2 µM ZnSO₄, 0.5 µM CuSO₄, 0.5 µM MoO₃, 0.1 µM NiSO₄, 100 µM Fe(III)-EDTA (ethylenediaminetetraacetic acid) and 300 µM ferrozine; all buffered with 1 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 5.5. The assays were conducted under dim light conditions at 20 °C. After 45 min the roots were removed from the assay solution. Ferrozine-Fe(II) was measured by absorbance at 562 nm (subtracting blanks of assay solution with no plants) and reduced Fe was calculated using the extinction coefficient of 28.16 mM cm⁻¹.

2.5. Organic Acids

The extraction and analysis of organic acids was performed as described by Vasconcelos et al. (2014) with slight modifications. Leaf and root samples from five individual plants of each treatment, previously frozen in liquid nitrogen, were grinded in a ceramic mortar and pestle with 2 mL of 2.5 mM sulfuric acid. Homogenates were boiled for 30 min, filtered with a 0.2 µm PTFE filter, and kept at -80 °C until HPLC analysis. Organic anions were analysed with an HPLC system (Lachrom, Merck Hitachi, Darmstadt, Germany) composed of an ion exchange Aminex HPX 87EC column (300 × 7.8 mm) maintained at 65 °C and two detectors in series: refractive index and absorbance (220 nm). The mobile phase was 2.5 mM sulfuric acid, flown at a rate of 0.8 mL min⁻¹. The injection volume was 50 µL and the running time, 30 min. Peaks corresponding to citric and malic acids were identified by

comparison of their retention times with those of known standards from Sigma (St. Louis, MO, USA). Quantification was made with known amounts of each organic anion using peak areas.

2.6. Minerals

Dried leaf and root powder (200 mg) was mixed with 5 mL of 65% HNO₃ plus 1 mL 30% H₂O₂ in a Teflon reaction vessel and digested in a microwave system (Speedwave MWS-3+, Berghof, Eningen, Germany). Digestion was conducted in five steps: 130 °C for 5 min; 160 °C for 10 min; 170 °C for 10 min; 100 °C for 2 min and 100 °C for 2 min. The resulting solutions were filtered and brought up to 50 mL with ultrapure water for analysis. Mineral concentrations were analysed by inductively coupled plasma argon spectrometry (ICP; ICP-OES Optima 7000 DV, PerkinElmer, Waltham, MA, USA). Five biological replicates of each treatment were analysed in triplicate. Mineral concentrations were expressed in mg kg⁻¹ dry weight.

2.7. Statistical Analysis

Data were analyzed with GraphPad Prism version 6. Differences among treatments were tested with two-way ANOVA, and the separation of means was performed using the corrected multiple comparisons Tukey test ($p < 0.05$).

3. Results

3.1. Biomass Accumulation

Plants grown under different Fe supplies and atmospheric CO₂ concentrations showed distinct biomass accumulation patterns. The trifoliolate biomass of soybean plants was not affected by Fe deficiency or eCO₂ individually. However, Fe deficiency combined with eCO₂ (−Fe/eCO₂) decreased leaf biomass accumulation by 52%. ($p < 0.05$) (Figure 1a). The stem and root biomass of soybean plants were not significantly affected by restricted Fe supply (Figure 1b,c) but under Fe sufficiency, plants exposure to eCO₂ stimulated root biomass accumulation by 52% ($p < 0.05$) whereas under Fe deficiency, eCO₂ decreased root biomass accumulation by 38% ($p < 0.05$) (Figure 1c). In addition, the root to shoot ratio was significantly higher (52%, $p < 0.05$) in the plants growing under Fe sufficiency at eCO₂, compared with plants from the other conditions (Figure 1d). Similarly to the observed for soybean, in common bean plants Fe restriction and eCO₂ individually had no effect on trifoliolate biomass (Figure 1e). However, when Fe deficiency was combined with eCO₂, the trifoliolate biomass sharply decreased by about 88% ($p < 0.0001$) compared with control plants (+Fe/aCO₂). Fe restriction slightly induced stem and root biomass accumulation but eCO₂ seemed not to have a significant effect on stem biomass (Figure 1f,g). In contrast, eCO₂ highly reduced root biomass of Fe deficient plants (Figure 1g). Still, the root: shoot ratio of common bean plants of all treatments was not significantly different (Figure 1h) indicating that the metabolic changes caused by both factors may not have disturbed significantly common bean growth.

3.2. Photosynthesis and Photosynthetic Pigments

In contrast to plants growing under Fe sufficiency, plants of both species grown under Fe deficiency showed IDC symptoms, presenting 40–65% lower chlorophyll levels (Figure 2a,d). Under Fe sufficiency, soybean plants growing at eCO₂ showed significantly higher chlorophyll levels (81%, $p < 0.0001$) and higher photosynthetic rates (65%, not significant) than under aCO₂ (Figure 2a,b). These CO₂ effects were only observed under Fe sufficiency. Similarly, in common bean, the photosynthetic rate of Fe sufficient plants growing under eCO₂ was significantly higher (6%, $p < 0.05$) (Figure 2e) but not the chlorophyll levels, for which differences were not significant (Figure 2d). Chlorophyll levels were not significantly affected by CO₂ (Figure 2a,d) under Fe deficiency. However, in common bean, eCO₂ seemed to have acted synergistically with Fe deficiency in decreasing photosynthesis (Figure 2e).

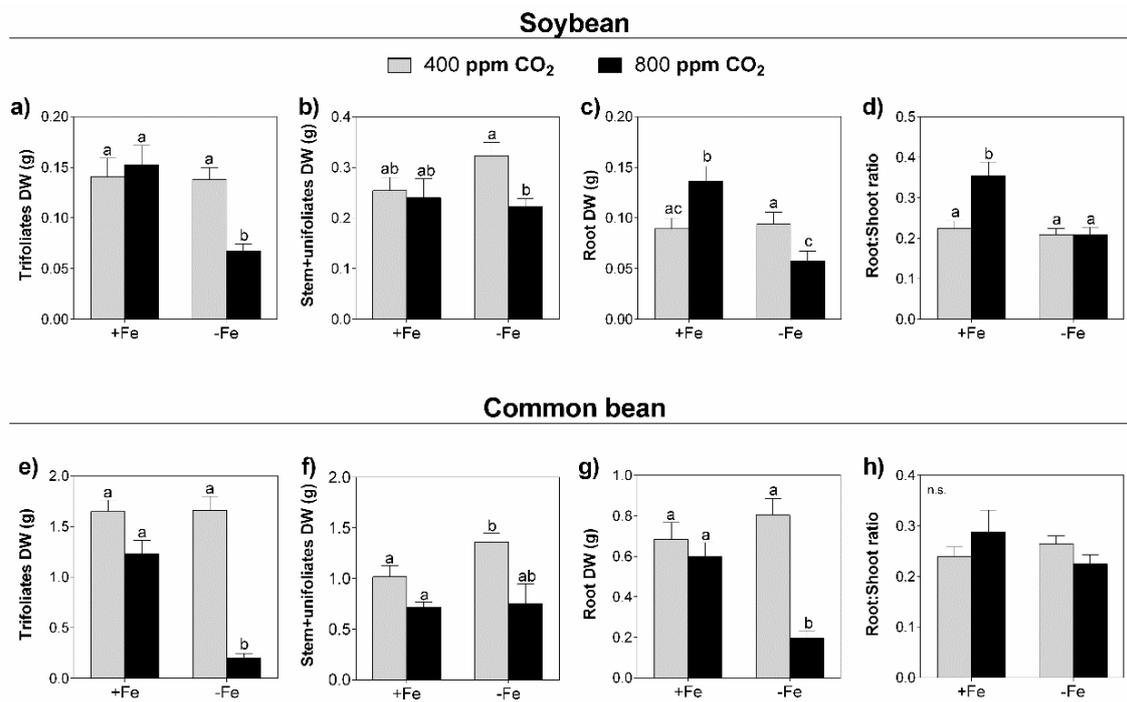


Figure 1. Trifoliolate (a,e), stem+unifoliolate (b,f), root (c,g) and root:shoot ratio (d,h) of soybean and common bean plants grown at 400 ppm and 800 ppm CO₂ and with 0 (–Fe) and 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biological replicates ± SE. Different letters above bars indicate significant differences $p < 0.05$ using the Tukey test.

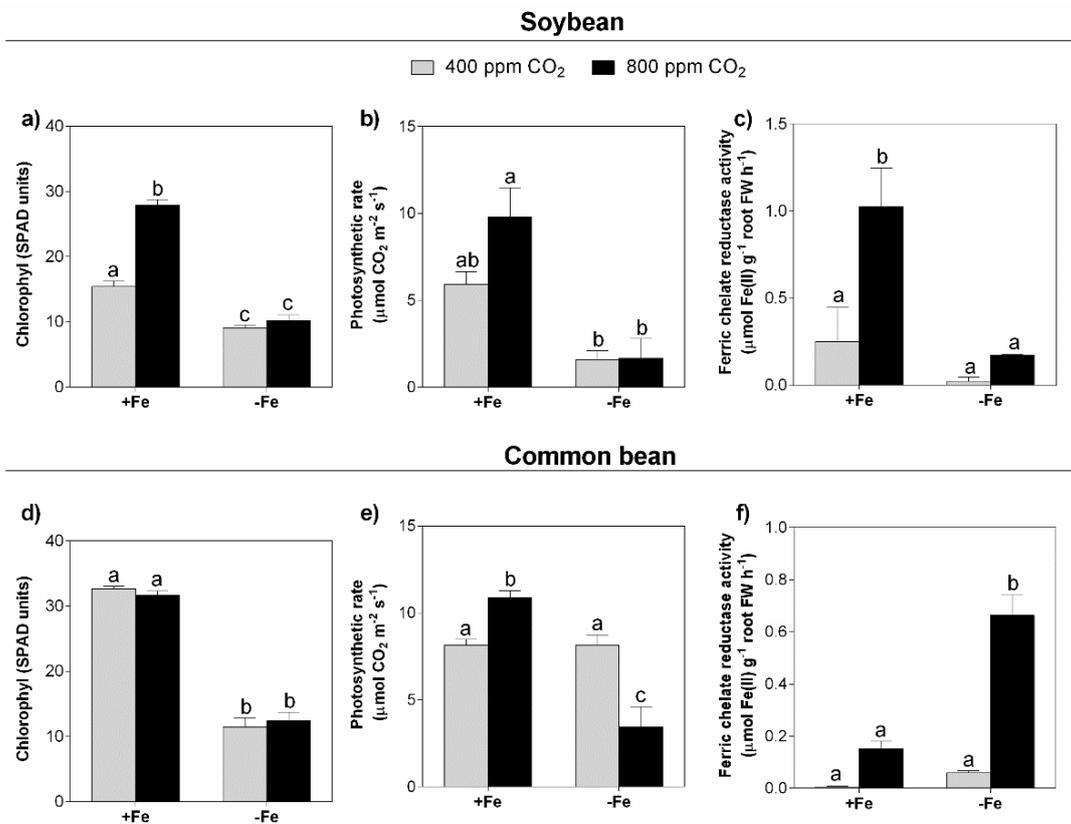


Figure 2. SPAD values (a,d), photosynthetic rate (b,e) and ferric chelate reductase activity (c,f), of soybean and common bean plants grown at 400 ppm and 800 ppm CO₂ and with 0 (–Fe) and 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biological replicates ± SE. Different letters above bars indicate significant differences $p < 0.05$ using the Tukey test.

3.3. Ferric Chelate Reductase (FCR) Activity

Fe deficiency did not significantly affect FCR activity under aCO₂, but under eCO₂, FCR activity was increased in both Fe treatments (Figure 2c,f). In soybean the increase was more pronounced in the presence of Fe (4-fold higher) whereas in bean it was most significant in the absence of Fe (10-fold higher) (Figure 2c,f).

3.4. Organic Acids

In soybean, citric and malic acid concentrations decreased by 98% ($p < 0.05$) in the leaves and roots of Fe sufficient plants at eCO₂ compared with control plants (Figure 3a); and the levels of malic acid were reduced by 95% ($p < 0.01$) in the leaves of Fe deficient plants exposed to eCO₂ compared with control plants (Figure 3b). However, under Fe deficiency, despite that the levels of these organic acids tended to decrease slightly, the difference was not significant when compared to control plants (Figure 3a). A similar pattern was observed for common bean. Both citric and malic acid concentrations tended to be lower in the plants grown under eCO₂ in each Fe condition. At +Fe/aCO₂ they decreased 82% ($p < 0.05$) in the roots, and at -Fe/eCO₂ the levels of these organic acids were totally depleted (Figure 3c,d).

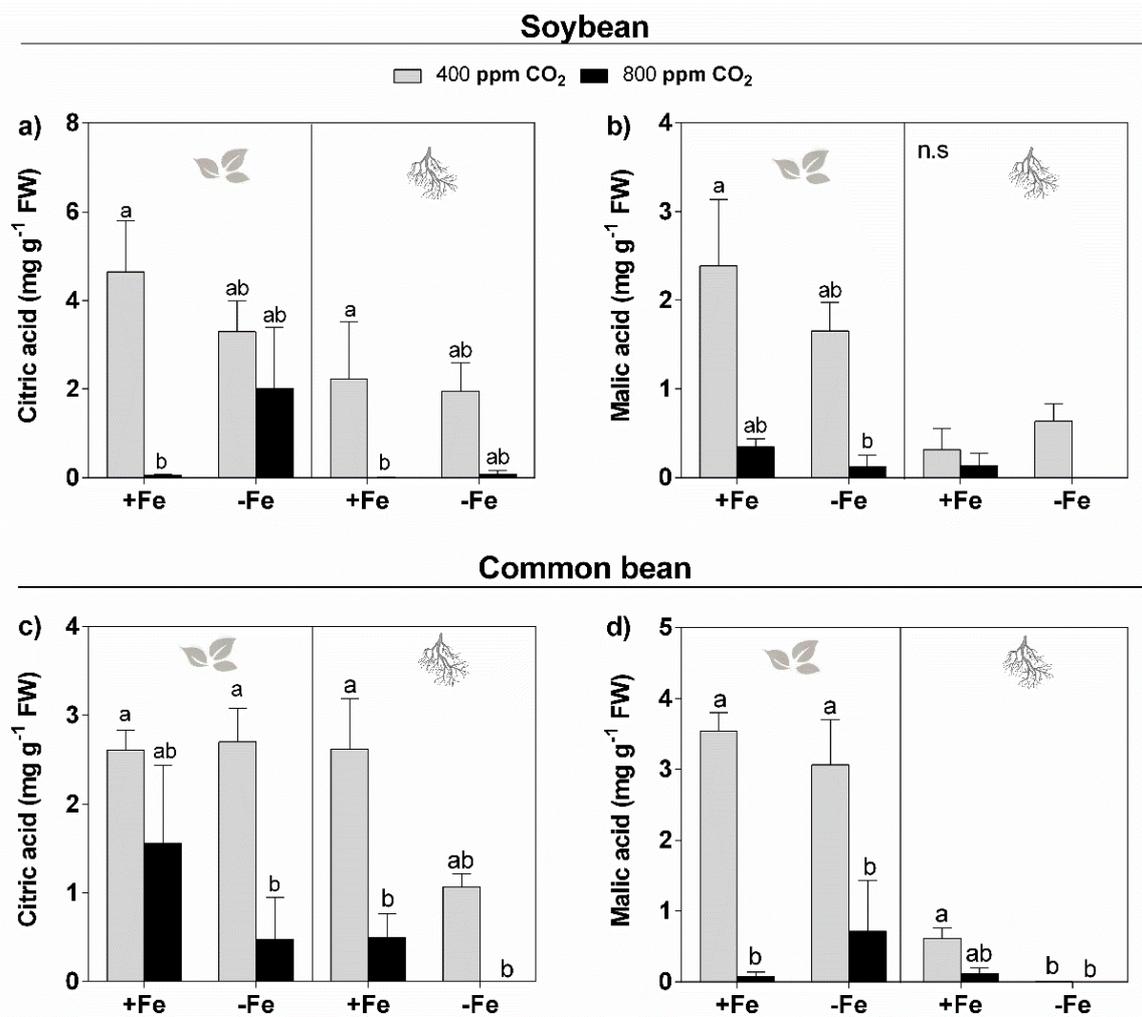


Figure 3. Concentrations of citric (a,c) and malic (b,d) acids in the leaves and roots of soybean and common bean plants grown at 400 ppm and 800 ppm CO₂ and with 0 (−Fe) and 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biological replicates ± SE. Different letters above bars indicate significant differences $p < 0.05$ using the Tukey test.

In common bean, under −Fe/aCO₂, citric acid was reduced 59% ($p < 0.1$) and malic acid by 99% ($p < 0.05$) in the roots but not in the leaves (Figure 3c,d). In addition, under

–Fe/eCO₂ the levels of malic acid were totally depleted in the roots of soybean and common bean and the levels of citric acid were undetected in the roots of common bean.

3.5. Mineral Accumulation

3.5.1. Effect of eCO₂

In the leaves of both soybean and common bean plants grown under eCO₂, the levels of the analysed minerals were not significantly different, independently of the Fe supply, indicating that under eCO₂, Fe restriction has no effect on leaf mineral concentrations (Figure 4). In contrast, when looking only at the effect of eCO₂, the results clearly showed a direct effect of eCO₂ in decreasing leaf mineral levels. In soybean grown under Fe sufficiency, plant exposure to eCO₂ decreased the levels of Fe by 82% ($p < 0.01$), Zn by 78% ($p < 0.01$), Mg, 57% ($p < 0.05$) and Mn by 62% ($p < 0.01$), whereas in common bean leaves the levels of Zn and Mn decreased by 76% ($p < 0.01$) and 81% ($p < 0.01$) under eCO₂, respectively (Figure 4).

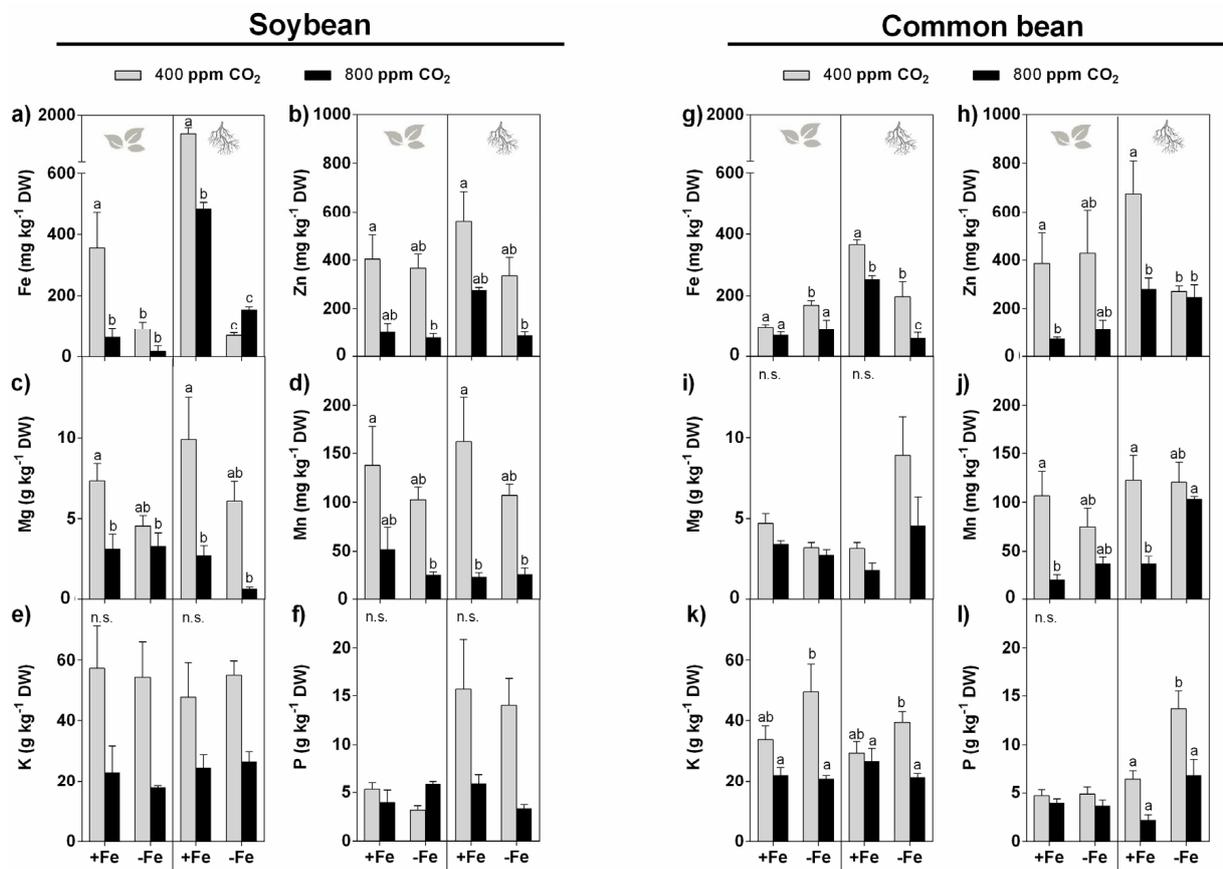


Figure 4. Concentrations of iron (Fe), zinc (Zn), magnesium (Mg), manganese (Mn), potassium (K) and phosphorous (P) in the leaves and roots of soybean (left panel) and common bean plants (right panel) grown at 400 ppm or 800 ppm CO₂, under iron sufficiency, with iron at 20 μM Fe-EDDHA (+Fe), and iron deficiency without Fe (–Fe). The results represent the mean of five biological replicates ± SE. Different letters above bars indicate significant differences $p < 0.05$ using the Tukey test.

In the roots of plants of both species, the same pattern was observed. Under each Fe condition, the levels of all analysed minerals decreased in the plant roots of both species under eCO₂ compared to aCO₂ (Figure 4).

3.5.2. Effect of Fe Restriction

The mineral levels of both soybean and common bean plants were highly impacted by restricted Fe supply. In the leaves of soybean plants grown under –Fe/aCO₂, except for phosphorous (P), the levels of all analysed minerals were lower than the observed in

plants grown under +Fe/aCO₂ (Figure 4a–e). Among the analysed minerals, Fe was the only one that decreased significantly, by 75% ($p < 0.01$), under –Fe/aCO₂ compared to +Fe/aCO₂. In contrast, in common bean leaves the levels of Fe, Zn and K tended to be higher in –Fe/aCO₂ compared with +Fe/aCO₂. For instance, the levels of Fe increased significantly by 73% ($p < 0.01$) whereas the levels of Zn increased by 11% and K levels augmented 48% but not significantly (Figure 4g,h,k).

In the roots of soybean plants grown under –Fe/aCO₂, the levels of Fe, Zn, Mg and Mn tended to be lower than under +Fe/aCO₂ but the differences were significant only for Fe which levels decreased by 95% ($p < 0.0001$) under Fe limitation (Figure 4a–d,f). The same tendency was observed in common bean roots in regard to Fe and Zn. However, whereas under –Fe/aCO₂ the levels of Fe decreased significantly by 46% ($p < 0.0001$) compared with plants grown under +Fe/aCO₂, the levels of P doubled under Fe restriction.

3.5.3. Interactive Effect of eCO₂ and Restricted Fe Supply

An interactive effect of both factors leading to reduced mineral concentration was evident on Fe accumulation in common bean roots and to some extent soybean leaves (Figure 4a,g).

The results clearly showed an effect of eCO₂ in decreasing the Fe levels in the leaves of both species. In soybean, eCO₂ had a similar effect of Fe restriction, and the Fe levels were the lowest when both factors were combined. Under eCO₂ at each Fe supply regime the Fe levels were reduced by 80–82% ($p < 0.05$) (compared to aCO₂ plants, and under Fe deficiency the levels of Fe were reduced by about 71–72% in each CO₂ concentration ($p < 0.0$). The lowest Fe levels were measured in the leaves of Fe deficient soybean plants under eCO₂ (–Fe/eCO₂) (Figure 4g),

In common bean leaves the levels of Fe increased under Fe restriction at aCO₂ (–Fe/aCO₂) but at eCO₂ (–Fe/eCO₂) the levels were similar to those of Fe sufficient plants regardless the CO₂ concentration (Figure 4g). However, a cumulative effect of these factors leading to further reduced Fe levels was observed in common bean roots. In soybean roots the effect of Fe deficiency alone was not significantly different from the effect of Fe deficiency combined with eCO₂ (–Fe/eCO₂) (Figure 4a). However, Fe deficient common bean plants grown under eCO₂ (–Fe/eCO₂) showed the lowest Fe levels in the roots and the difference was significant compared with the levels found in the plants of all the other growing conditions (Figure 4g). In addition, under aCO₂ Fe deficient common bean plants (–Fe/aCO₂) had root–Fe levels significantly higher than Fe sufficient plants (+Fe/aCO₂); and Fe deficient plants grown under eCO₂ (–Fe/eCO₂) showed lower root Fe levels than plants grown under aCO₂ (–Fe/aCO₂) (Figure 4g).

Regarding the other analysed minerals, no significant differences were found when looking at the interaction of the effects of both factors.

4. Discussion

4.1. Elevated CO₂ and Fe Restriction Led to General Biomass Decrease in Both Legume Species

Plants growing under different Fe supply and atmospheric CO₂ concentrations showed different biomasses. Elevated CO₂ on its own tended to stimulate soybean but not common bean growth, suggesting a species dependent effect (Figure 1). The largest effect of eCO₂ occurred in soybean roots, with Fe sufficient plants showing higher biomass. Increased leaf and root biomass accumulation under eCO₂ have been previously reported for soybean [46,47] and common bean plants [37,48], and the root is the organ more often reported to be highly affected by increasing atmospheric CO₂ concentrations. Despite the results of several studies conducted in soybean consistently showing that eCO₂ increases leaf and root biomass [15], for common bean, the effect of eCO₂ on increased biomass allocation seems to be dependent on the bean cultivar [48]. For instance, Salsman et al. [49] showed that eCO₂ had no impact on root and shoot growth and this result is in accordance with our findings where no significant differences on leaf and root biomass were found in common bean plants.

An interactive effect between eCO₂ and Fe restriction led to general biomass decrease in both crop species (Figure 1a,c,e,g). Under eCO₂ it is known that photosynthesis and biomass accumulation are stimulated [46,50–52] and the requirement for nutrients increases [37,38,41,53]. Therefore, stimulation of biomass build-up may be a drawback when plants are growing under Fe limitation. Fe plays an important role in plant development since it is involved in several metabolic processes such as photosynthesis, respiration, nitrogen fixation, DNA synthesis, hormone production, and chlorophyll formation being also an important co-factor of several enzymes [54]. Under eCO₂, as plant growth is stimulated, the requirements for Fe increase and the plant may invest its resources on increasing Fe uptake and transportation. Since these processes require increasing energy supply, the plant utilizes its resources in these processes and biomass accumulation is reduced. In contrast, at eCO₂, plants under optimal nutritional conditions may invest in root growth in order to cope with the increasing plant demand for nutrients.

Here the results clearly show for both species that there is an interactive effect between Fe deficiency and eCO₂ on decreasing trifoliolate and root biomass accumulation. To the best of our knowledge there is only one study addressing the combined effect of Fe deficiency and eCO₂ in plant growth and nutrition [44]. This previous study was conducted in tomato and showed that plant exposure to eCO₂ significantly increased root and shoot biomass regardless of Fe supply, but the root:shoot ratio was significantly higher only in plants growing at eCO₂ under Fe limitation and not under Fe sufficiency. These results indicate that in tomato there is also an interactive effect of Fe deficiency and eCO₂ impacting the whole plant growth but, in contrast to our study, in tomato the effect was positive, increasing biomass accumulation. Despite the contrasting results, in both studies an interactive effect of Fe deficiency and eCO₂ interfering in biomass accumulation was observed. The differences in biomass accumulation patterns suggest that the interaction of both factors is species dependent or there may be differences in trial conditions, namely in the method used for restricting Fe supply to the plant and time-point of analysis.

4.2. Depending on the Legume Species, eCO₂ Stimulates Photosynthesis, but in Combination with Fe Deficiency, This Effect Is Lost

In contrast with plants growing under Fe sufficiency, plants of both species grown under Fe deficiency showed IDC symptoms with 40–65% ($p < 0.0001$) lower chlorophyll levels (Figure 2a,d). It is well known that Fe deficiency leads to a decrease in the concentration of several foliar pigments, such as chlorophylls and carotenoids, and results in a sharp growth reduction [55,56]. The main symptom of this deficiency is the leaf yellowing and development of chlorosis in the youngest plant leaves.

Under Fe sufficiency, soybean and common bean plants growing at eCO₂ showed higher photosynthetic rates than under aCO₂ (Figure 2a,b). As C₃ plants, soybean and bean are highly dependent on atmospheric CO₂ levels for photosynthesis catalyzed by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This enzyme has higher affinity for CO₂ than O₂, but when CO₂ concentrations are limited, which is the case for example during stomatal closure, Rubisco binds oxygen, and photorespiration is induced. Thus, carboxylation becomes the rate-limiting step in C₃ photosynthesis [50] and carbon fixation. Accordingly, in an environment with enriched CO₂ levels, photosynthesis is expected to increase, as observed in this study, and this effect has been previously reported for several species [16,39,50,57–60].

In contrast with soybean, in common bean plants, eCO₂ seems to have acted synergistically with Fe deficiency in decreasing photosynthesis (Figure 2e). This result suggests that under Fe deficiency, eCO₂ may induce a downward acclimation, i.e., a downregulation of photosynthesis. The acclimation process is believed to result from plants' inability to fully utilize the extra photosynthate produced as a result of plant exposure to eCO₂ [41,61,62]. Since, under Fe deficiency the chlorophyll levels of common bean plants grown under both CO₂ conditions was almost the same, the absence of Fe may possibly have determined this inability. The observed sharp induction of ferric chelate reductase (FCR) activity in these

plants (Figure 2f) indicates that they were highly stressed and requiring increased Fe uptake, probably because Fe is involved in many metabolic processes including plant respiration.

In general, the results suggest that under Fe sufficiency, eCO₂ may induce photosynthesis but under Fe restriction, it may contribute to increase the incidence, severity, and earlier occurrence of IDC depending on the legume species.

4.3. Ferric Chelate Reductase Activity Is Stimulated under eCO₂

Both soybean and common bean are Strategy I plants, i.e., they first reduce Fe from the ferric to ferrous form in order to transport it via xylem or phloem [63]. There are a number of studies reporting the induction of FCR activity under Fe deficiency in several species including common bean plants [64–67] among others [68–70]. For both soybean and common bean growing under aCO₂, Fe restriction did not significantly affect FCR activity (Figure 2a,d). FCR activity in plants growing under Fe deficiency is generally induced [71], but some studies report a decreasing activity of this enzyme in soybean plants growing under restricted Fe supply [72,73]. In fact, there are studies evidencing that FCR activity is species and cultivar dependent [67,74], which may explain our contrasting results.

When looking at the effect of CO₂, for both species, FCR activity was higher under eCO₂ regardless of the Fe treatment (Figure 2c,f); however, the effect was species' specific. In soybean FCR was 4-fold higher in the presence of Fe, whereas in common bean it was 10-fold higher in the absence of Fe (Figure 2c,f). In tomato, eCO₂ has been shown to induce FCR activity when plants were grown under Fe deficiency but not under Fe sufficiency [44]. These are contrasting results showing that the combination of specific nutritional deficiencies with eCO₂ induces species dependent behaviors, and the significantly higher FCR activity of common bean plants growing in the absence of Fe also suggests that this common bean genotype has higher tolerance to Fe deficiency than the soybean genotype used in this study.

4.4. Elevated CO₂ Reduces the Organic Acid Levels in the Leaves and Roots of Soybean and Common Bean

Organic acids, particularly citric and malic acid, are involved in metal cation uptake. Citrate is known to chelate ferric Fe, facilitating its transportation within the plant for subsequent reduction by a plasma membrane Fe reductase enzyme [23]. In addition, the formation of malic acid in the root is believed to be induced under Fe deficiency to ensure a sufficient energy charge to maintain the required performance of the Fe uptake mechanism.

In soybean leaves and roots, citric and malic acid concentrations decreased significantly under the influence of eCO₂. Similarly, in common bean the levels of these organic acids also tended to be lower in the plants grown under eCO₂ in each Fe condition. This tendency has been previously reported in wheat leaves [75] and in *Arabidopsis* roots [19] of plants grown under eCO₂.

In common bean, Fe deficiency on its own (–Fe/aCO₂) lowered the levels of citric and malic acid in the roots but not in leaves (Figure 3c,d). Under Fe deficiency, the levels of organic acids are generally reported to increase in the roots of a number of crops [22,23,76]. Despite that in soybean roots the levels of these acids tended to increase or remain stable (Figure 3a,b), in common bean roots the levels of citric acid tended to decrease and malic acid significantly decreased (Figure 3c,d). Malate and citrate have been shown to decrease in roots of an Fe tolerant grapevine cultivar grown under Fe deprivation, when compared to a tolerant one [77]. Considering the evidence of common bean tolerance to Fe deficiency, provided by the differences on FCR activity (see section above), the results of our study are in accordance with those previously reported for grapevine.

In addition, when Fe deficiency was combined with eCO₂ the levels of malic acid were totally depleted in the roots of the plants of both species and the levels of citric acid were undetected in the roots of common bean. These results evidence a putative interaction of both effects inducing the extinction of both organic acids, suggesting that beyond the direct effect of eCO₂, an interactive effect of both factors exists determining the depletion of these organic acids or the inhibition of their formation in these crop species.

4.5. The Mineral Levels of Both Soybean and Common Bean Plants Are Highly Affected by eCO₂ and Fe Deficiency on Their Own and in Combination

When looking at the effect of eCO₂ alone, in general, the levels of Fe, Zn, Mg, Mn and K were lower in the leaves and roots of both species when plants were grown under Fe sufficiency. The levels of P were only lower in the roots (Figure 4). Previous works showed that eCO₂ has a significant negative impact on the nutritional status of several crops, decreasing the levels of Zn and Fe in soybean [30]. Loladze (2014, 2002) [13,14] using a meta-analysis approach, unequivocally showed that eCO₂ induces mineral losses in foliar tissues and grains or edible tissues. The mechanism by which mineral concentrations decrease when plants are exposed to eCO₂ is still unknown. Recently, a down-regulation of the expression of genes involved in Fe transport has been suggested [78]. Yet, eCO₂ is also known to: (i) induce photosynthesis, carboxylation and phosphorylation, increasing the levels of sugars and organic acids [18–20], and (ii) reduce stomatal conductance with consequent transpiration reduction [16]. In addition, the two species had a contrasting behavior regarding FCR activation when growing under Fe limitation, which might also explain the different patterns of Fe accumulation. In fact, the increased activity of FCR in common bean, particularly under eCO₂, highlights its higher capacity to counteract Fe deficiency. Another hypothesis is that this genotype is tolerant to Fe deficiency, whereas the soybean genotype may not be. In fact, soybean cv. ‘Williams’, the one used in this study, has been previously reported to be susceptible to develop IDC [73]. The effect of Fe deficiency on mineral accumulation was evident in the leaves and roots of soybean plants with lower Fe levels under Fe deficiency (–Fe/aCO₂) compared to Fe sufficiency (+Fe/aCO₂). A similar pattern was observed in the root of common bean plants but in the leaves the Fe levels increased under Fe restriction (–Fe/aCO₂). These results highlight the behavior differences among legume species when exposed to eCO₂ and Fe restriction individually. These two species had a contrasting behavior regarding FCR activation when growing under Fe limitation, which explains the different patterns of Fe accumulation. In fact, the increased activity of FCR in common bean, particularly under eCO₂, highlights the highest capacity of common bean plants to counteract Fe deficiency. Another hypothesis is that this particular common bean genotype is tolerant to Fe deficiency, being able to efficiently cope with this stress, whereas the soybean genotype may not be. Soybean cultivar ‘Williams’, the one used in this study, has been previously reported to be susceptible to develop IDC (Santos et al., 2013).

Here, an interactive effect of Fe deficiency and eCO₂ led to reduced Fe accumulation in common bean root and further reduced Fe accumulation in the leaves of soybean (Figure 4a,g). Although the Fe levels measured in the leaves of Fe deficient soybean plants under eCO₂ (–Fe/eCO₂) (Figure 4g) were not significantly different from the levels found in the plants exposed to just a single factor, it suggests that the two factors together may further reduce Fe uptake (Figure 4a). Altogether, the results herein presented suggest that the mechanisms involved in reduced Fe accumulation caused by Fe deficiency and eCO₂ are different but may not be independent.

5. Conclusions

The results clearly show an independent effect of eCO₂ and Fe deficiency in decreasing the Fe levels in the leaves and roots of the plants of both legume species. A cumulative effect of these factors leading to a further reduction of Fe levels was observed in common bean roots where the activity of FCR was the highest and the levels of organic acids were the lowest; these plants were also highly repressed photosynthetically. These results suggest that the mechanisms involved in reduced Fe accumulation caused by eCO₂ and Fe deficiency may not be independent, and an interaction of these factors may lead to further reduced Fe levels. If so, it may negatively affect human population diets, particularly of those dependent on Fe from plant sources or those which already suffer from Fe deficiency.

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