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RESEARCH ARTICLE

Combined effect of elevated CO_2 and Fe deficiency on common bean metabolism and mineral profile

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Abstract
Aims Elevated atmospheric CO_2 (eCO_2) and restricted iron (Fe) supply are known to impact plant growth and nutritional quality of food crops. However, studies aimed at understanding how eCO_2 will interact with Fe deficiency are scarce. Changes in the nutritional status of the common bean (*Phaseolus vulgaris* L.) may significantly impact the nutritional status of populations that rely heavily on this crop.
Methods To understand the combined effects of eCO_2 and Fe deficiency on mechanisms relevant to plant nutrient uptake and accumulation, common bean plants were grown under Fe sufficiency (Fe+, 20 mM Fe-EDDHA) and Fe deficiency (Fe-, 0 mM Fe-EDDHA) combined with eCO_2 (800 ppm) or ambient CO_2 (aCO_2, 400 ppm) in hydroponics until maturity.
Results Elevated CO_2, besides stimulating photosynthesis and stomatal closure, highly affected plant Fe metabolism: stimulated root ferric chelate reductase (FCR) activity by 6-fold and downregulated the expression of root *FRO1* and *IRT1* expressions by about 4-fold. In leaves, citrate and oxalate increased, but *ferritin* expression decreased by 9-fold. Such changes may have determined the differences on mineral accumulation patterns particularly the lower levels of Fe in roots (62%), leaves (38%) and seeds (50%). The combination of Fe deficiency and eCO_2 doubled the effect of a single factor on FCR up-regulation, balanced the internal pH of Fe deficient plants, and resulted in the lowest Fe accumulation in all plant parts.
Conclusions These results suggest that eCO_2 directly affects the Fe uptake mechanism of common bean plants, decreasing plant Fe content.

Keywords Elevated CO_2 · Iron (Fe) deficiency · Fe uptake · Mineral nutrition · Organic acids · Photosynthesis · *Phaseolus vulgaris*

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Introduction

Mineral deficiencies are a global public health problem affecting more than two billion people and causing more than two million deaths annually (Black et al. 2008). A diet deficient in essential minerals causes decreased growth in childhood, reduced immunity to infectious diseases, and higher maternal and child death rates. Dietary iron (Fe) deficiency alone leads to nearly 200,000 deaths and the loss of 45 million life years annually (Loladze 2014; Smith et al. 2017; Beach et al. 2019). It is well known that in calcareous soils, which represent about 30% of the arable land in the world, most Fe is unavailable for uptake due

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to its low solubility. Several studies have shown that under Fe restriction, plants are affected by iron deficiency chlorosis (IDC), characterized by early leaf yellowing and necrosis and subsequent reduction of Fe accumulation in legume seeds (Krouma et al. 2003; Santos et al. 2013, 2015; Vasconcelos and Grusak 2014). Dicotyledonous plants such as common beans respond to Fe limitation by inducing the Strategy I mechanism (Römheld and Marschner 1986), which involves: i) the expression of active proton pumps (HA) to increase ferric iron (Fe^{3+}) solubility; ii) a ferric reductase (FRO1) to reduce ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}) making it more soluble; and iii) an Fe transporter (IRT1) to take up Fe from the external medium (Kim and Guerinot 2007; Morrissey and Guerinot 2009; Liang 2022). Under Fe deficiency, the utilization of this mechanism induces proton pumping to the apoplast to acidify the external medium and solubilize Fe, favoring Fe uptake and transportation within the plant (Santi and Schmidt 2009; del Carmen Orozco-Mosqueda et al. 2013; Zhao et al. 2016). Ferritin, as an essential Fe storage protein, also plays a significant role in maintaining cellular Fe homeostasis (Van Wuytswinkel et al. 1999; Ravet et al. 2009; Grant-Grant et al. 2022), preventing free Fe to form highly reactive hydroxyl radicals through the Fenton reaction (Ravet et al. 2009; Goto et al. 2013; Zielińska 2015). Organic acids are also involved in Fe uptake, particularly citrate, which contributes to cytoplasmic acidification and chelates metal cations in the root, transporting them through the plant (Susin et al. 1996; Abadía et al. 2002; López-Millán et al. 2009; Gama et al. 2016; Igamberdiev and Eprintsev 2016).

In addition to the nutritional loss derived from soil Fe deficiency, the earth's climate change poses a fundamental threat to humanity. The rise of atmospheric CO_2 is the main driver for most of these changes. Currently, the global atmospheric CO_2 concentration, as measured in August 2022, is about 417 ppm (CO_2-Earth 2022; Scripps UCSD 2022), but it will reach about 800 ppm by the end of this century (Franks 2013). Beyond its direct effects on global warming, elevated CO_2 (eCO_2) affects plant growth, crop yield, and the nutritional status of agricultural products. eCO_2 induces increased photosynthesis, sugar accumulation, and changes in organic acid concentrations with levels in the shoots tending to increase whereas in the roots, tending to decrease (Li et al. 2007; Jauregui et al. 2015, 2016; Noguchi et al. 2015; Ainsworth and Lemonnier 2018), potentially leading to higher yields. However, photosynthetic acclimation may occur, decreasing quality and productivity (Lambreva et al. 2005; Córdoba et al. 2017; da Silva et al. 2017). Studies based on meta-analyses have shown that eCO_2 reduces mineral or protein concentrations in several crop species (Jablonski et al. 2002; Taub et al. 2008; Loladze 2014; Myers et al. 2014; Medek et al. 2017).

It is now irrefutable that limited soil Fe supply and eCO_2 are independently impacting the nutrition of plant foods (Soares et al. 2019; Deuchande et al. 2021). Given that eCO_2 induces metabolic changes in traits such as stomatal conductance (Ainsworth and Rogers 2007), organic acid release (O'Sullivan et al. 2021), sugar (Jauregui et al. 2015, 2016) and plant biomass accumulation (Ainsworth and Long 2005; Jin et al. 2009; Kumar et al. 2017), these traits directly or indirectly influence Fe uptake. Therefore, we hypothesize that an essential interplay between eCO_2 and Fe deficiency may induce changes in mineral accumulation in crop species. However, investigation on the impact of these two factors is scarce. For instance, we have recently reported an interaction of these two factors in soybean and common bean plants during the first three weeks under hydroponics, leading to reduced growth, photosynthesis, and mineral accumulation (Deuchande et al. 2021). Still, these physiological and molecular mechanisms are not yet understood. Previous studies aimed to understand the effect of eCO_2 on mineral nutrition focused on N and P deficiencies (Leakey et al. 2009; Tawaraya et al. 2014; Jin et al. 2015; Niu et al. 2016; Yilmaz et al. 2017; Dong et al. 2018; Vicente et al. 2018). To the best of our knowledge, there are only a few studies conducted on tomato, soybean, and common bean where these two factors were combined (Jin et al. 2009; Deuchande et al. 2021; Soares et al. 2022). Furthermore, although these studies discuss how these factors affect plant physiology, they do not present results on nutrient accumulation at the grain level.

Legumes provide a large share of the global population's diet, and the common bean (*P. vulgaris*) is the most critical legume produced worldwide for direct human consumption (Blair et al. 2010, 2016). Also, for more than 300 million people in parts of Eastern Africa and Latin America, it is the primary source of protein and micronutrients, especially Fe but also zinc (Zn) and folic acid, among others (Beebe et al. 2000;

Castro-Guerrero et al. 2016; Dissanayaka et al. 2021). This study aimed to investigate the independent and combined effects of eCO_2 and Fe deficiency on plant growth and mineral nutrition, focusing on the impact of these factors in the photosynthetic and stomatal regulation and Fe uptake mechanisms, including: i) root Fe reductase activity; ii) cytoplasmic acidification; iii) organic acid and sugar accumulation; and v) expression of key enzymes and transporters involved in Fe uptake (FRO1, IRT1, ferritin and a plasma membrane H^+-ATPase). The goal is to propose a preliminary model that insight into some of the mechanisms impacted by these environmental factors that may be responsible for the reported nutritional losses under eCO_2 and Fe deficiency.

Materials and methods

Plant material and growth conditions

The *P. vulgaris* cv. "Chocolade Bruine Boon" (PI 477023) was obtained from the United States Department of Agriculture through the Germplasm Resources Information Network (GRIN). This variety was selected considering the results of our previous experiments which showed that it was intermediately tolerant to Fe deficiency (unpublished data) and strong responsive to eCO_2 (Soares et al. 2019). Thus, we ensured that plants would be able to survive in the absence of Fe up to the end of the experiment and a significant effect of CO_2 would be observed. The seeds were placed in germination bags filled with water for six days in the dark, at 25 °C. Germinated seedlings were transferred to 5 L hydroponic vessels (four 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 was maintained at 75% throughout day and night. Plants were grown under ambient CO_2 (aCO_2, 400 ppm) or eCO_2 (800 ppm) in the same chamber in subsequent periods of time. The standard solution for hydroponic growth included: 1.2 mM KNO_3; 0.8 mM $Ca(NO_3)_2$; 0.2 mM $MgSO_4.7H_2O$; 0.3 mM; $NH_4H_2PO_4$; 25 mM $CaCl_2$; 25 mM H_3BO_3; 0.5 mM $MnSO_4$; 2 mM; $ZnSO_4.H_2O$; 0.5 mM $CuSO_4.H_2O$; 0.5 mM MoO_3; 0.1 mM $NiSO_4$. The hydroponic solution was buffered with 1 mM MES, pH 5.5, continuously aerated and changed every two days. Plants were grown for six days under complete solution with 20 µM Fe(III)-EDDHA followed by two weeks under Fe-sufficiency (20 µM Fe(III)-EDDHA (ethylenediamine-N,N bis(o-hydroxyphenyl)acetic acid) or Fe deficiency (0 µM Fe(III)-EDDHA). Under each condition, 15 plants were grown and divided in three sets of five plants each for different purposes. After the three first growing weeks, two sets of plants from each condition were used. In one set of plants morphometric (height), physiological (photosynthetic rates and stomatal conductance) and biochemical (root ferric chelate reductase (FCR) activity) parameters were measured. The roots, stems, and leaves of these plants were dissected and dried at 65 °C until stable weight, for measuring biomass accumulation.

At this time-point another set of plants was sampled for biochemical and gene expression analysis and the remaining set of five plants was used to assess the impact of eCO_2 plus Fe deficiency on grain nutritional composition having these plants been grown until complete pod filling, when the grain yield was also determined.

Sampling

Samples of roots, stems and leaves from five plants of each condition [Fe sufficiency at ambient aCO_2 (Fe+/aCO_2; *control plants*) or eCO_2 (Fe+/eCO_2); and Fe deficiency at ambient CO_2 (Fe-/aCO_2) or eCO_2 (Fe-/eCO_2)], were frozen and grounded into powder with a mortar and pestle under N_2 for biochemical and molecular analyses. The biochemical analyses included the measurement of the concentrations of organic acids, chlorophylls a and b, carotenoids and soluble protein, whereas the molecular analysis included the expression of the genes *ferritin (PvFerritin)*, *ferric reductase oxidase 1-like* (*PvFRO1*), *iron-regulated transporter 1-like* (*PvIRT1*), and plasma membrane *H^+-ATPase 2-like* (*PvHA2*) genes. For mineral analyses, another set of samples of the same organ tissues was dried.

The seeds collected at the end of the experiment were grinded into powder for mineral analyses.

Photosynthesis, gas exchange and pigments accumulation

The photosynthetic rate as well as the stomatal conductance, transpiration rate and intercellular CO_2 were measured using an infra-red gas analyser (IRGA) Li-6400XT Portable Photosynthesis System (LI-COR Inc., Lincoln, USA). The measurements were taken maintaining the following conditions: leaf temperature at 25 °C, photosynthetically active photon flux density at 500 µmol m^{-2} s^{-1}, CO_2 concentration at 400 µmol CO_2 mol^{-1} for plant grown under aCO_2 (aCO_2) and 800 µmol CO_2 mol^{-1} for plants grown under eCO_2, and flow at 500 µmol s^{-1}. The relative chlorophyll levels were assessed using a portable Soil-Plant Analyser Development chlorophyll meter (SPAD-502 Plus; Konica Minolta, Osaka, Japan). The photosynthetic pigments were extracted from leaf samples previously frozen and grinded under N_2 using 10 ml of cold acetone/1 M tris buffer solution [80:20 (v/v), pH 7.8]. The homogenates were incubated at 4 °C for 24 h and then centrifuged for 5 min at 5000 g. The absorbance of the supernatant was measured at: 470, 537, 647 and 663 nm and the concentrations of chlorophylls a and b and carotenoids were calculated as described by Sims and Gamon (2002).

Ferric chelate reductase assay

Root Fe chelate reductase activity was quantified as described by Grusak et al. (1990). The measurements were carried out on intact roots of five individual plants via the spectrophotometric determination of Fe^{2+} chelated to BPDS (bathophenanthroline disulfonic acid) at 535 nm. Roots of each plant were submerged in assay solution containing: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 0.5 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM MoO_3, 0.1 µM $NiSO_4$, 100 µM Fe(III)-EDTA (ethylenediaminetetraacetic acid) and 100 µM BPDS. All nutrients were buffered with 1 mM MES, pH 5.5. Rates of reduction were determined using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}.

Stem internal pH assessment

This analysis has been performed in the roots to assess the effect of Fe deficiency in root cytoplasmic acidification capacity (Tian et al. 2016; Zhao et al. 2016). In this study, the aim was to assess the effect of both factors, restricted Fe supply and shoot exposure to eCO_2, in the internal pH of the plant.

Acidification assays were performed as described by Yi et al. (1994) using 1 cm sections of plant stems frozen under N_2 and stored at −80 °C until analysis. The stem sections of three plants from each condition were transferred to 1% agar plate containing 0.006% bromocresol purple and 0.2 mM $CaSO_4$ (pH adjusted to 6.5 with NaOH) for 24 h. Acidification is indicated by the yellow colour around the stem, whereas alkalinisation is given by the darker purple colour.

Organic acids and sugars

The extraction and analysis of organic acids and sugars was performed as described by Vasconcelos et al. (2014) with slight modifications. Samples were ground in a 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 (Lachrom, Merck Hitachi, Darmstadt, Germany) with an ion exchange Aminex HPX 87E 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, malic, succinic and oxalic acids and glucose, sucrose and fructose were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma (St. Louis, USA). Quantification was made with standards of known amounts of each organic anion and sugar using peak areas.

Protein and minerals

Seeds were analyzed for crude protein concentration (N × 5.28) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA).

Mineral concentrations were assessed in root, leaf, and seed tissues. Dried tissue powder

(200 mg) was mixed with 5 ml of 65% HNO_3 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, Massachusetts, USA). Five biological replicates were analysed in triplicate. Mineral concentrations were expressed in mg kg^{-1} dry weight.

RNA extraction and cDNA synthesis

Five biological replicates of root and leaf samples from each growth condition were grinded with a mortar and pestle, and total RNA was extracted using Qiagen RNeasy Mini Kit (USA, #74904), according to the manufacturer's instructions. RNA quality and quantity were checked by UV spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal) and RNA integrity was verified by agarose gel electrophoresis. Single strand cDNA was synthetized from 750 ng of total RNA, using iScript cDNA synthesis kit (Bio-rad laboratories Inc., CA, USA) in a thermal cycler (VWR, Doppio, Belgium), following the manufacturer instructions.

Primer design and *RT-qPCR*

The transcript levels of genes encoding *ferritin, IRT1, FRO1 and* H^+*-ATPase 2-like (HA2)* were analysed, using *actin-11* (*Act11*) and *Tubulin beta-9* (*β-Tub9*) as reference genes (Borges et al. 2012). Primers were designed using the Primer-Blast tool from NCBI specifying an expected PCR product of 100–200 bp, primer annealing temperatures between 57 °C and 63 °C and selecting span an exon-exon junction (Table 1). The RT-qPCR analyses were performed in a CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories Inc., CA, USA) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, CA, USA). Primer efficiency was determined for all primers by qPCR analysis of a standard curve, constructed by serial dilutions of the synthetized cDNA from one test sample. The amplification protocol was set to cycle as follows: 95 °C denaturation for 10 min; 40 cycles of 95 °C for 15 s followed by 56–60 °C (depending on primers used) for 30 s; followed by melt curve stages to check that only single products were amplified. The stability of the reference genes was evaluated using the ΔCT method and geNorm software. All expression data were normalized against the geometric mean of the expression of the two stable reference genes, using the delta CT method. Five biological replicates were analysed, and two technical repetitions of each biological replicate were performed. Non-template controls (NTC) were included in each plate to discard the presence of primer dimers and/or primer contamination.

Table 1 Primers used for quantitative qPCR analysis

Gene name	Primer pairs	Gene Name /Gene bank Acc. No.	Fragment size (bp)	Reference
PvActin-11	F 5′-TGCATACGTTGGTGATGAGG-3′ R 5′-AGCCTTGGGGTTAAGAGGAG-3′	CV529679.1	190	(Borges et al. 2012)
PvSkip 16	F 5′-CACCAGGATGCAAAAGTGG-3′ R 5′-ATCCGCTTGTCCCTTGAAC-3′	FG231556.1	163	(Borges et al. 2012)
PvFerritin	F 5′-AAGGGATTTGCCAGGTTCTTCA-3′ R 5′-TAACGCATCCCCCTTCTCCA-3′	phavu.Phvul.008G093700 X58274.1	159	–
PvFRO1	F 5′-CATGATCGCTCCGGGGATTT-3′ R 5′-GCACAGGGTAAAATGCGAGC-3′	phavu.Phvul.007G073900 XM_007143400.1	105	–
PvIRT1	F 5′-TACACACCTGTGGTTTGAGC-3′ R 5′-AAGCAGCAGTGCAAACATGG-3′	phavu.Phvul.002G322800 XM_007160383.1	187	–
PvHA2	F 5′-TCCAAAAGAGGCAAGGGCTG-3′ R 5′-GGAGCACCTTTGCTTGAACG-3′	phavu.Phvul.003G143800.1 XM_007154680.1	126	–

Statistical analysis

Data were subjected to analysis of variance (two-way ANOVA) for the effect of Fe and atmospheric CO_2 concentration using the GraphPad Prism version 8.0 (San Diego, CA, USA). Significant differences among plants of all treatments were determined by Tukey's test at $P < 0.05$. For the hierarchical clustering analysis (HCA), data were subjected to square root transformation and auto-scaled. The analysis was conducted using the Euclidean similarity measure and the average algorithm using the MetaboAnalyst version 5.0.

Results

Morphometric, biomass and productivity measurements

Plants exposed to eCO_2 (Fe+/eCO_2) showed the highest root biomass (1.595 ± 0.151 g DW) and height (59.9 ± 8.3 cm) (Table 2). The stem biomass of these plants and those only exposed to Fe limitation (Fe-/aCO_2) was 60% higher than that of control plants (Fe+/aCO_2). Leaf biomass showed no significant differences among the plants of all conditions. The root: shoot ratio was significantly higher in the plants

Table 2 Biomass of root, shoot, unifoliates and trifoliates (g DW), root:shoot ratio, height (cm), grain yield (seeds/plant), average 1-seed weight (g), concentrations of chlorophyll (SPAD units), chlorophyll a and b, chlorophyll (a + b), carotenoids (mmol g^{-1} FW), photosynthetic rates (µmol CO_2 m^{-2} s^{-1}), intercellular CO_2 (µmol CO_2 mol^{-1}), maximum carboxylation rate (V_{cmax}), maximum phosphorylation rate (J_{max}) and maximum CO_2-saturated assimilation rate (A_{max}) (µmol CO_2 m^{-2} s^{-1}), stomatal conductance (mol H_2O m^{-2} s^{-1}), transpiration rate (mmol H_2O m^{-2} s^{-1}) and intrinsic water use efficiency (µmol CO_2 mol^{-1} H_2O) of plants grown under ambient CO_2 (aCO_2, 400 ppm CO_2) or elevated CO_2 (eCO_2, 800 ppm CO_2) and at 0 added (-Fe) or 20 µM Fe-EDDHA (+Fe)

	+Fe, aCO_2	+Fe, eCO_2	-Fe, aCO_2	-Fe, eCO_2
Biomass and height				
Root	0.844 ± 0.078 a	1.595 ± 0.151 b	1.450 ± 0.147 ab	1.439 ± 0.179 ab
Stem	0.643 ± 0.041 a	1.025 ± 0.030 b	1.054 ± 0.111 b	0.744 ± 0.051 a
Unifoliates	0.415 ± 0.061 a	0.643 ± 0.086 a	0.769 ± 0.101 a	0.572 ± 0.087 a
Trifoliates	1.750 ± 0.227 a	1.306 ± 0.167 a	1.440 ± 0.208 a	1.319 ± 0.146 a
Root: shoot ratio	0.359 ± 0.029 a	0.528 ± 0.029 b	0.460 ± 0.029 a	0.621 ± 0.024 b
Height	31.2 ± 1.8 a	59.9 ± 8.3 b	33.0 ± 1.3 a	45.0 ± 5.7 ab
Productivity				
Grain yield (seeds/plant)	53.20 ± 10.78 a	11.20 ± 1.16 b	44.20 ± 5.64 a	12.60 ± 2.77 b
Average 1-seed weight (g)	0.186 ± 0.018 a	0.139 ± 0.024 a	0.175 ± 0.008 a	0.158 ± 0.023 a
Pigments				
Chlorophyll (SPAD)	35.3 ± 0.9 a	31.2 ± 2.1 ab	29.2 ± 1.9 ab	26.0 ± 2.1 b
Chlorophyll a	0.98 ± 0.01 a	0.70 ± 0.04 bc	0.83 ± 0.05 b	0.64 ± 0.03 c
Chlorophyll b	0.39 ± 0.01 a	0.28 ± 0.02 b	0.33 ± 0.02 a	0.25 ± 0.01 b
Chlorophyll (a + b)	1.38 ± 0.02 a	0.94 ± 0.07 b	1.04 ± 0.13 ab	0.80 ± 0.10 b
Carotenoids	0.66 ± 0.01 a	0.46 ± 0.03 b	0.57 ± 0.02 a	0.45 ± 0.02 b
Photosynthesis and gas exchange				
Photosynthetic rate	9.28 ± 0.27 a	10.90 ± 0.38 b	10.40 ± 0.73 ab	10.54 ± 0.18 ab
Intercellular CO_2	253.3 ± 8.8 a	542.0 ± 42.4 b	259.8 ± 18.9 a	558.2 ± 42.3 b
V_{cmax}	63.05 ± 1.28 a	38.50 ± 2.41 b	25.57 ± 1.18 c	25.90 ± 1.21 c
J_{max}	91.49 ± 1.50 a	85.60 ± 0.38 b	51.79 ± 0.25c	62.68 ± 0.61 d
A_{max}	15.00 ± 0.26 a	15.04 ± 0.27 a	8.40 ± 0.28 b	10.74 ± 0.44 c
Stomatal conductance	0.132 ± 0.004 a	0.060 ± 0.011 b	0.126 ± 0.015 a	0.064 ± 0.011 b
Transpiration rate	2.47 ± 0.07 a	1.31 ± 0.20 b	2.92 ± 0.41 a	1.60 ± 0.34 b
Intrinsic Water Use Efficiency	78.3 ± 4.1 a	172.6 ± 19.7 b	78.8 ± 8.1 a	193.5 ± 11.3 b

The results represent the mean of five biologicals replicates ±SE. Different letters in a row indicates significant differences using Tukey test ($p < 0.05$)

exposed to eCO_2 for both Fe treatments (Fe±/eCO_2) (Table 2).

The grain yield of the plants grown under eCO_2 was significantly lower under both Fe conditions: 79% lower in the plants growing under Fe sufficiency and 71.5% lower in the plants growing under Fe deficiency. However, the average single seed weight did not significantly differ among the plants of any of the treatments (Table 2).

Photosynthetic pigments and gas exchange

The levels of chl b and carotenoids were significantly lower in plants grown under eCO_2 regardless of the Fe supply, whereas the photosynthetic rate was significantly higher in the Fe+/eCO_2 plants (Table 2).

The intercellular CO_2 was higher in plants exposed to eCO_2 regardless of Fe supply, but control plants (Fe+/aCO_2) had the highest maximum carboxylation ($V_{cmax} = 63.05 \pm 1.28$ mmol CO_2 m^{-2} s^{-1}) and phosphorylation rates ($J_{max} = 91.49 \pm 1.50$ mmol CO_2 m^{-2} s^{-1}) (Table 2). Fe deficient plants had the lowest rates, with these plants presenting 60% lower carboxylation rates and 44% (Fe-/aCO_2) and 31% (Fe-/eCO_2) lower phosphorylation rates compared with control plants (Fe+/aCO_2). Nevertheless, in the plants exposed to eCO_2 (Fe+/eCO_2), such decreases were slightly attenuated, with V_{cmax} decreasing by 44% and J_{max} by less than 10% compared to control plants (Fe+/aCO_2). The maximum CO_2-saturated photosynthetic rate was significantly lower in the plants growing under limited Fe supply, with the lowest rate measured in the plants growing at aCO_2 (45% lower compared to Fe+/aCO_2 plants). The stomatal conductance was 55% lower in the plants exposed to eCO_2 regardless of Fe supply (Fe±/eCO_2) compared to control plants (Fe+/aCO_2). Accordingly, the transpiration rate of the plants exposed to eCO_2 was 50% lower, and the intrinsic water use efficiency was 50% higher (Table 2).

Ferric chelate reductase

Control plants had the lowest ferric chelate reductase activity, whereas the plants exposed to both perturbations combined (Fe-/eCO_2) had the highest. In these plants, FCR activity was 15-fold higher than in control plants, but in the plants exposed to just a single stress (Fe+/eCO_2 or Fe-/aCO_2), FCR activity was almost similar, being about 6-fold higher than in Fe+/aCO_2 plants (Fig. 1).

Stem internal pH

Plants grown under Fe sufficiency had lower internal pH, particularly Fe+/aCO_2 plants, which showed the lowest internal pH, as shown by the significant yellowing of the medium surrounding these samples (Fig. 2a, b). Plants grown under Fe restriction regardless of CO_2 condition showed higher internal pH, as shown by the dark purple halo surrounding these samples (Fig. 2a–e). Under eCO_2, the differences in internal pH resulting from the different Fe supply regimes were slightly attenuated, with Fe+/eCO_2 showing slightly higher internal pH (Fig. 2c) than Fe+/aCO_2 plants and Fe-/eCO_2 showing slightly lower pH compared to Fe-/aCO_2 plants (Fig. 2).

Organic acids and sugars

In the leaves, the levels of organic acids were generally higher under eCO_2 at both Fe supply regimes (Fe±/eCO_2). In contrast, Fe restriction at both CO_2 conditions showed no significant effect, except for oxalic acid, whose levels were significantly higher (40%) in Fe+/eCO_2 plants compared to Fe-/eCO_2 plants (Fig. 3).

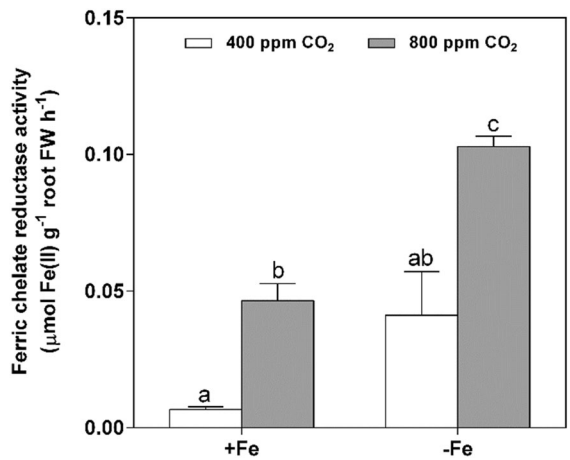

Fig. 1 Ferric chelate reductase activity in the roots of common bean plants grown under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2) and at 0 (-Fe) or 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biologicals replicates ± SE. Different letters above the vertical bars represent significant differences ($p < 0.05$)

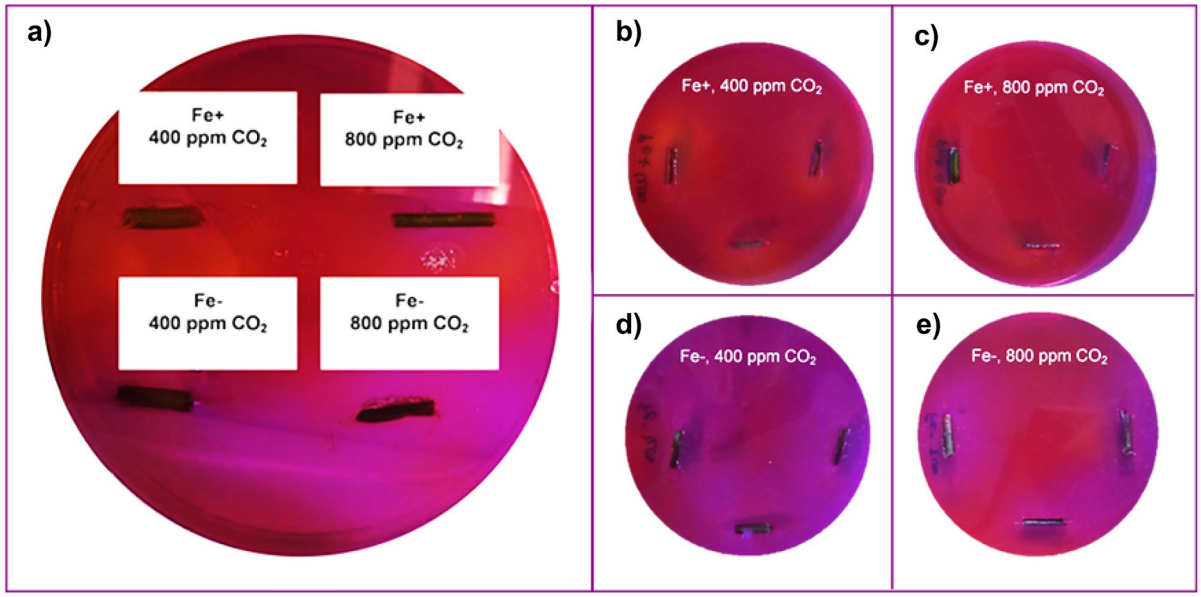

Fig. 2 Stem internal pH of plants grown at Fe sufficiency (Fe+) or Fe deficiency (Fe-) and under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2). Sections of the stem of one plant from each condition (**a**) and sections of the stems of three individual plants from each condition (**b**, **c**, **d** and **e**) were incubated at room temperature for 24 hours. The yellow colour surrounding the stem indicates an acidic pH and the darker purple colour indicates alkaline pH

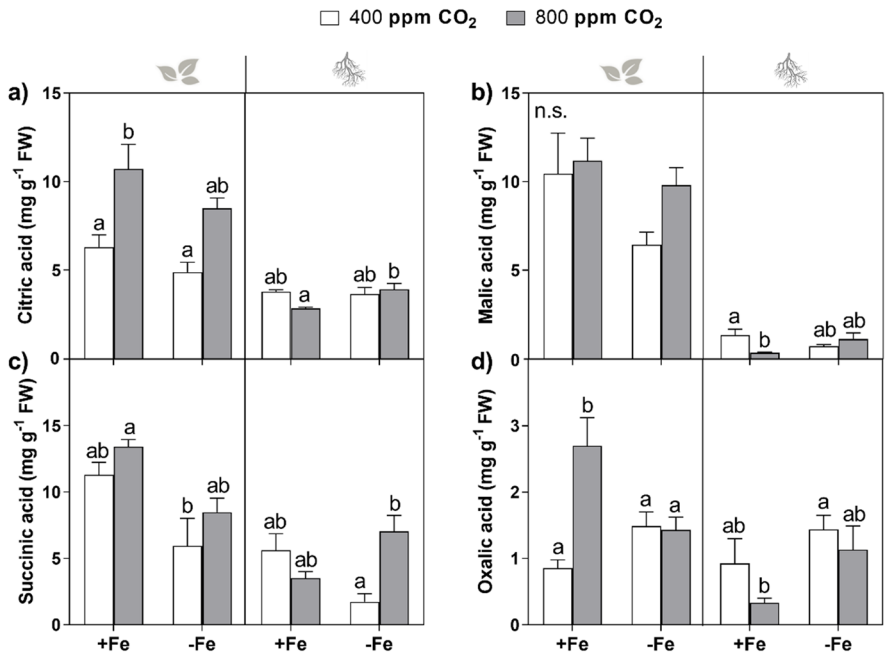

Fig. 3 Concentrations of citric acid (**a**), malic acid (**b**), succinic acid (**c**) and oxalic acid (**d**) in the leaves and roots of common bean plants grown under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2) and at 0 (-Fe) or 20 µM Fe-EDDHA (+Fe). The results represent the mean of five biologicals replicates ± SE. Different letters above the vertical bars represent significant differences ($p < 0.05$)

In the roots, organic acid concentrations varied among plants grown under different conditions. The levels of citric acid significantly increased in plants exposed to both factors (Fe-/eCO_2) compared with Fe+/eCO_2 plants; and the levels of malic acid were significantly lower in the plants exposed to eCO_2 (Fe±/eCO_2) compared to Fe+/aCO_2 plants (Fig. 3a, b). Regarding succinic acid, Fe-/eCO_2 plants had the

highest levels, which were significantly higher (25%) than Fe-/aCO_2 plants (Fig. 3c). Fe-/aCO_2 plants had the highest levels of oxalic acid. In contrast, Fe+/eCO_2 had the lowest levels (Fig. 3d).

Regarding leaf sugars, sucrose levels were not significantly different among the plants of all treatments. However, the levels of glucose and fructose tended to be higher in the Fe+/eCO_2 plants (Fig. 4). Under eCO_2, Fe restriction significantly decreased glucose levels (Fig. 4b). However, fructose levels were not affected by Fe restriction when plants were grown under eCO_2. Still, fructose levels were the lowest in Fe-/aCO_2 plants, significantly lower than in the Fe+/eCO_2 plants (Fig. 4c).

At the root level, Fe+/eCO_2 plants showed the lowest sucrose, glucose, and fructose levels. In contrast, Fe+/aCO_2 plants showed the highest concentrations of these sugars (Fig. 4). Fe deficiency decreased the sucrose levels in the plants growing at aCO_2 but not at eCO_2 (Fig. 4a).

Protein and minerals

The grain protein concentrations were significantly higher under eCO_2 but were not affected by Fe supply (Fig. 5). The protein levels in the grains of plants grown under eCO_2 were about 30% higher than those grown under aCO_2.

The Fe concentration was affected by eCO_2 and limited Fe supply in all plant parts, decreasing Fe in plant roots and leaves. Exposure to eCO_2 (Fe±/eCO_2) determined a decrease of 62% in root Fe and 38% in leaf Fe, whereas Fe restriction decreased by 45% in root Fe and 67% in leaf Fe. Fe concentration in plant seeds was only dependent on the atmospheric CO_2 levels, with seeds of plants grown under eCO_2 presenting Fe losses of more than 50% (from 66.0 ± 4.8 to 32.6 ± 2.76 mg kg^{-1} DW in Fe sufficient plants and from 61.82 ± 5.2 to 26 ± 7.8 mg kg^{-1} DW in Fe deficient plants) (Fig. 6).

The seeds of plants exposed to the combined factors (Fe-/eCO_2) had 20-30% higher Zn concentration compared with the other conditions (Fig. 6). In the roots, Zn concentration was significantly higher in Fe-/aCO_2 plants than in the other conditions, which consistently showed about 50% lower concentration. In the leaves, the exposure to eCO_2 tended to benefit Zn accumulation with plants grown under Fe sufficiency at eCO_2 (Fe+/eCO_2) showing significantly higher concentrations than at aCO_2 (Fe+/aCO_2).

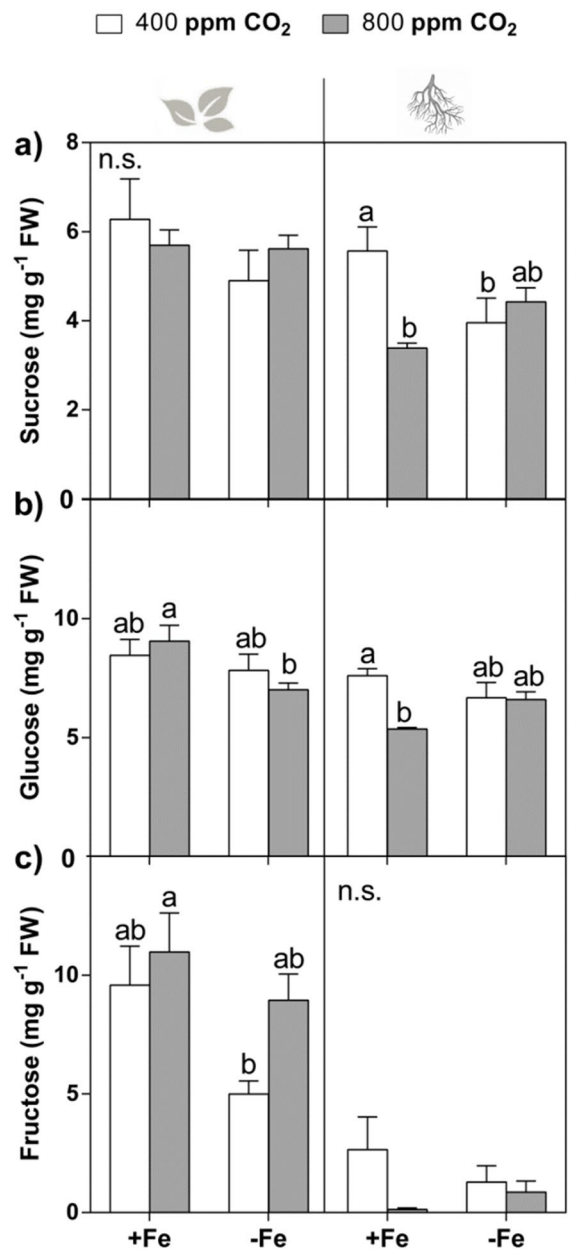

Fig. 4 Concentrations of glucose (**a**), sucrose (**b**) and fructose (**c**) in the leaves and roots of common bean plants grown under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2) and at 0 (-Fe) or 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biologicals replicates ± SE. Different letters above the vertical bars represent significant differences ($p < 0.05$)

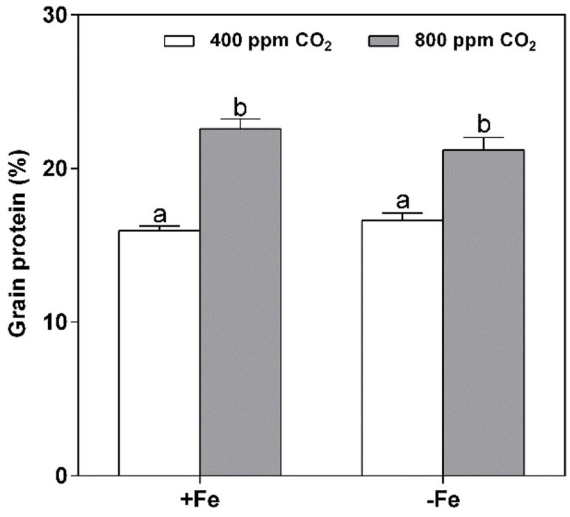

Fig. 5 Concentration of protein in the grains of common bean plants grown under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2) and at 0 (-Fe) or 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biologicals replicates ± SE. Different letters above the vertical bars represent significant differences ($p < 0.05$)

The levels of Mg and K in the roots and leaves were not affected by Fe restriction under both CO_2 conditions, but eCO_2 (Fe±/eCO_2) induced a higher accumulation of these minerals, particularly at the leaf level where the increases were significant (Fig. 6c, f). In the seeds, there were no significant changes.

The concentration of Mn was only significantly different in the roots, being 73% lower in the plants grown under eCO_2 (Fe±/eCO_2) and 54% lower in the Fe-/aCO_2 plants when compared to control plants (Fe+/aCO_2). The latter had 37.9 ± 4.4 mg Mn kg^{-1} root DW (Fig. 6d). In the roots and leaves, the calcium (Ca) concentration was not significantly different amongst the plants of all conditions. However, in the seeds, exposure to eCO_2 led to an increased accumulation of Ca, regardless of Fe supply. Under Fe sufficiency, seed Ca levels were 1.5-fold higher at eCO_2 than at aCO_2; under Fe deficiency, it was 2.5-fold higher at eCO_2 than at aCO_2 (Fig. 6e).

The phosphorous (P) concentration was similar in the roots of plants of all conditions, but in the leaves, significantly higher levels were found in the plants exposed to both factors. In the seeds, the P concentration was only dependent on atmospheric CO_2. The P concentration under eCO_2 was about 30% lower than under aCO_2 (Fig. 6g).

Gene expression

The *PvFerritin* gene was mainly expressed in the leaves, being highly downregulated in the plants exposed to eCO_2, by 9-fold in the plants at Fe sufficiency and by 15-fold in the plants grown at Fe deficiency (Fig. 7a). Plants exposed to Fe restriction at aCO_2 (Fe-/aCO_2) also showed a down-regulation of this gene by 1.8-fold compared to control plants (Fe+/aCO_2).

PvFRO1, *PvIRT1*, and *PvHA2* expression were higher in the roots, and the transcriptional regulation of these genes regarding plant exposure to eCO_2 and Fe restriction followed the same pattern, with the three genes being co-expressed (Fig. 7b–d). In plants exposed to eCO_2 irrespective of the Fe supply, *PvFRO1* was downregulated by 4-fold, *PvIRT1* by 4.6-fold, and *PvHA2* by about 3-fold. In the plants only exposed to Fe restriction (Fe-/aCO_2), *PvFRO1*, *PvIRT1*, and *PvHA2* were down-regulated by about 2-fold (Fig. 7).

Discussion

Biomass, plant height and productivity

Plants grown under Fe sufficiency at eCO_2 showed the highest root biomass and height (Table 2). Previous studies have shown for several plant species, including common bean, that eCO_2 stimulates plant growth, mainly plant height (Ainsworth and Long 2005; Li et al. 2007), but also root development and biomass (Jin et al. 2009; Madhu 2013; Jauregui et al. 2015). Under increased atmospheric CO_2, plants tend to produce more photosynthates which are then directed towards sink organs such as roots, leading to increased root biomass (Pritchard et al. 1999).

Comparatively to plants grown under Fe sufficiency and aCO_2, the root:shoot ratio of plants only exposed to eCO_2 increased by about 25%, whereas in plants grown under Fe deficiency and exposed to eCO_2 it increased about 55% (Table 2).

In general, these results show that under optimal nutritional conditions, eCO_2 stimulates plant growth, whereas, under Fe deficiency, plant growth is slightly

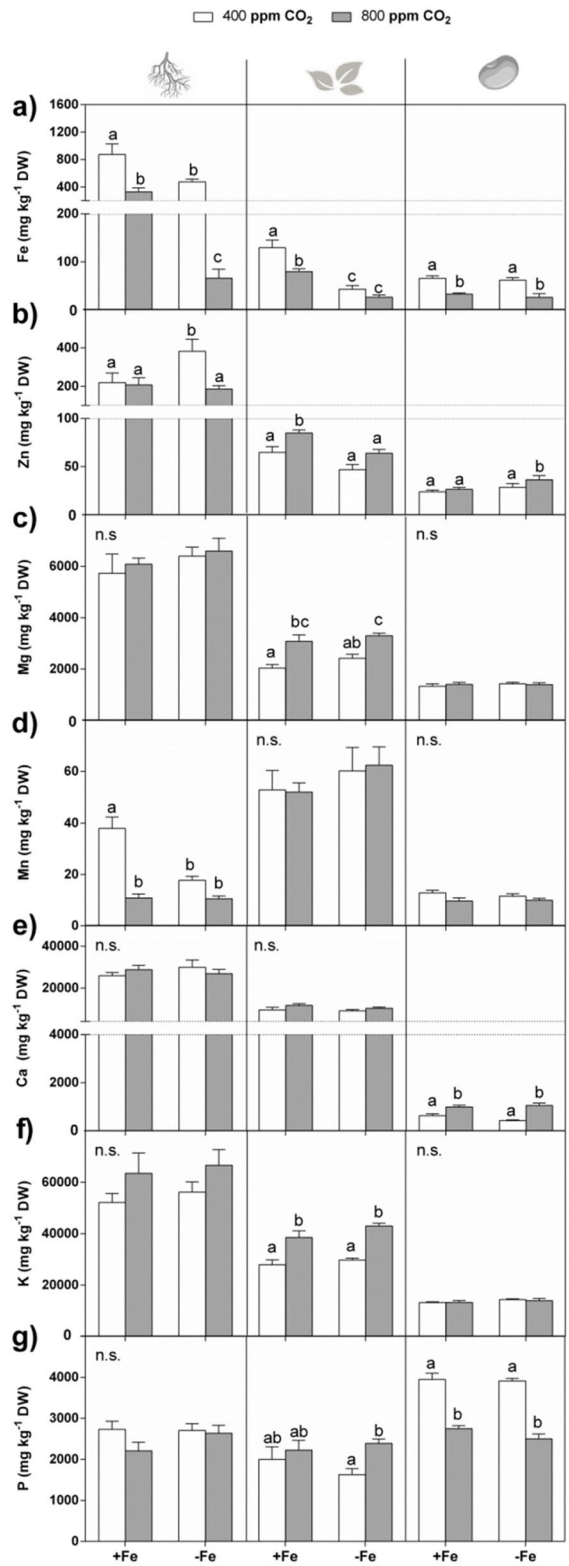

Fig. 6 Concentrations of (**a**) iron (Fe), (**b**) zinc (Zn), (**c**) magnesium (Mg), (**d**) manganese (Mn), (**e**) calcium (Ca), (**f**) potassium (K) and (**g**) phosphorus (P) in the root, leaves and seeds of common bean grown under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2) and at 0 (-Fe) or 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biologicals replicates ± SE. Different letters above the vertical bars represent significant differences ($p < 0.05$)

repressed (Table 2). Although these results suggest a stimulation of growth under eCO_2, the grain yield of these plants was significantly lower (Table 2) which suggests that other processes may have initiated after flowering, leading to decreased productivity.

Chlorophyll, photosynthesis, and gas exchange

It is well known that Fe deficiency causes early leaf yellowing and necrosis in the youngest trifoliate (Brown and Holmes 1955). There is a direct effect of eCO_2 on decreasing photosynthetic pigments accumulation and it was evident for chlorophyll b and carotenoids (Table 2). Plants growing under Fe restriction generally had lower chlorophyll levels than plants growing under Fe sufficiency (Table 2). Also, the photosynthetic rate of plants grown at Fe sufficiency when exposed to eCO_2 was significantly higher than when exposed to aCO_2. This result shows that eCO_2 stimulates photosynthesis of common bean plants under optimal Fe conditions independently of repressing chlorophyll concentrations. Increased photosynthetic rates in C3 plants growing under eCO_2 have been previously reported (Makino and Mae 1999; Teng et al. 2006; Ainsworth and Rogers 2007; Jakobsen et al. 2016; Xu et al. 2016; Faralli et al. 2017; Ainsworth and Lemonnier 2018; Hovenden and Newton 2018; Vicente et al. 2018).

Under eCO_2, the intercellular CO_2 was significantly higher than under aCO_2. However, when comparing control plants (Fe+/aCO_2) with the plants of the other conditions, the maximum carboxylation rate (V_{cmax}) decreased by 40% under eCO_2 and by 60% on Fe deficient plants whereas the maximum phosphorylation rate (J_{max}) decreased by 10% under eCO_2 and by 30-45% on Fe limited plants (Table 2). Concerning Fe restriction, these results were expected, given the importance of Fe on plant metabolic functioning. Regarding eCO_2, these results suggest the initiation of an acclimation process. The down-regulation of photosynthetic activity under

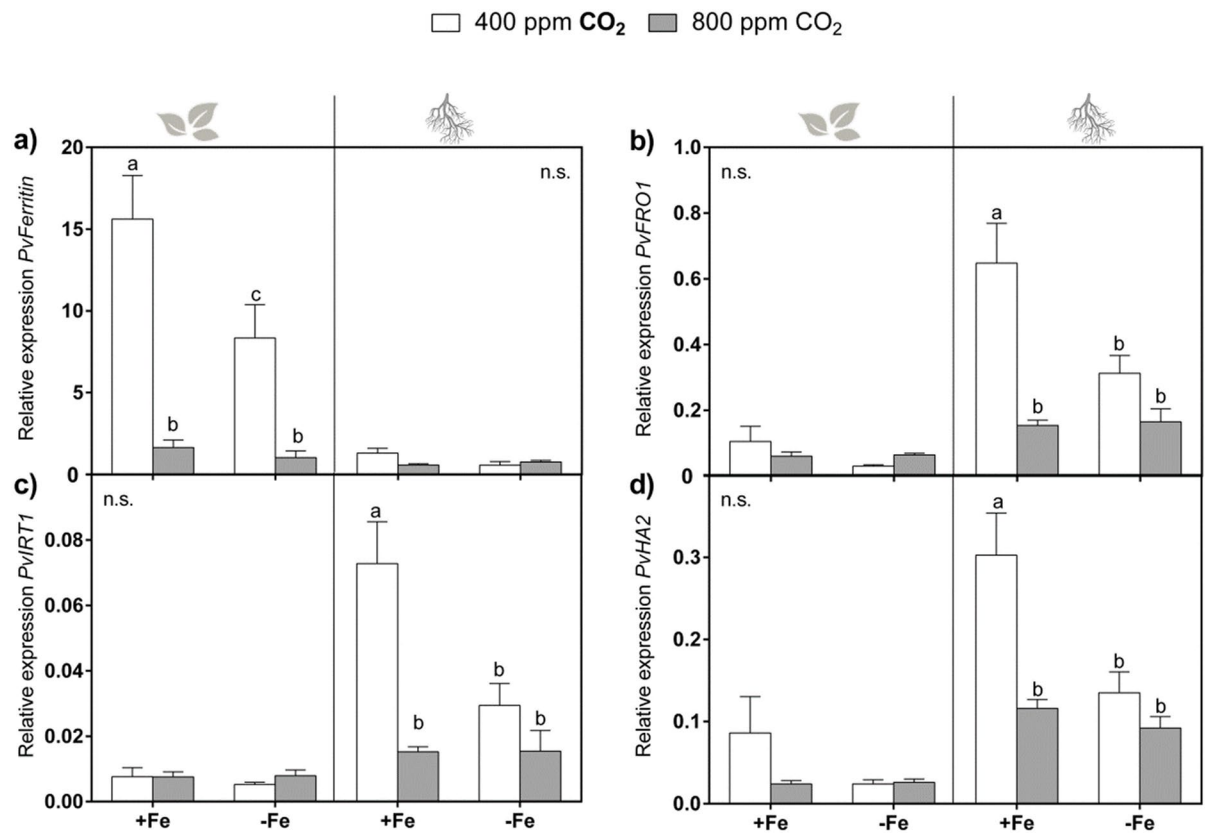

Fig. 7 Relative gene expression of *Ferritin* (*PvFerritin*) (**a**), *Ferric chelate reductase 1-like* (*PvFRO1*) (**b**), *Iron transporter 1-like* (*PvIRT1*) (**c**) and *H+-ATPase 2-like* (*PvHA2*) (**d**) in the leaf and root of common bean plants grown under ambient CO_2 (400 ppm CO_2) or elevated CO_2 (800 ppm CO_2) and at 0 (-Fe) or 20 μM Fe-EDDHA (+Fe). The results represent the mean of five biologicals replicates ± SE. Different letters above the vertical bars represent significant differences ($p < 0.05$)

eCO_2 is characterised by: i) decreased chlorophyll content, as observed in these plants (Table 2), ii) decreased content and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) and these plants had lower V_{cmax}, and iii) limitations in ribulose-1,5-bisphosphate (RuBP) and P regeneration (Urban 2003; Kant et al. 2012), which is supported by the lower J_{max} measured in these plants compared to control plants (Fe+/aCO_2) (Table 2). This acclimation process would explain the lower grain yield of the plants grown under eCO_2. Plants grown under Fe deficiency had lower V_{cmax} and J_{max} than control plants (Fe+/aCO_2), and the maximum CO_2-saturated photosynthetic rate (A_{max}) decreased by 15-30%. Under Fe deficiency, for both CO_2 conditions, the chlorophyll levels were lower. FCR activity was higher, particularly at eCO_2, and these two factors may have determined the lower carboxylation, phosphorylation, and assimilation rates observed in these plants compared to control plants (Fe+/aCO_2). Interestingly, when combined with restricted Fe supply, an interactive effect was observed, leading to slightly higher J_{max} and A_{max} values, suggesting that eCO_2 attenuates the effect of Fe deficiency in these parameters.

Ferric chelate reductase activity and iron transporters

Common bean is a Strategy I plant, i.e., it first reduces Fe from the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form before its uptake by an Fe (II) transporter (IRT1) (Morrissey and Guerinot 2009). Plants exposed to the combination of both factors (Fe-/eCO_2) had the highest root FCR activity, showing almost 15-fold

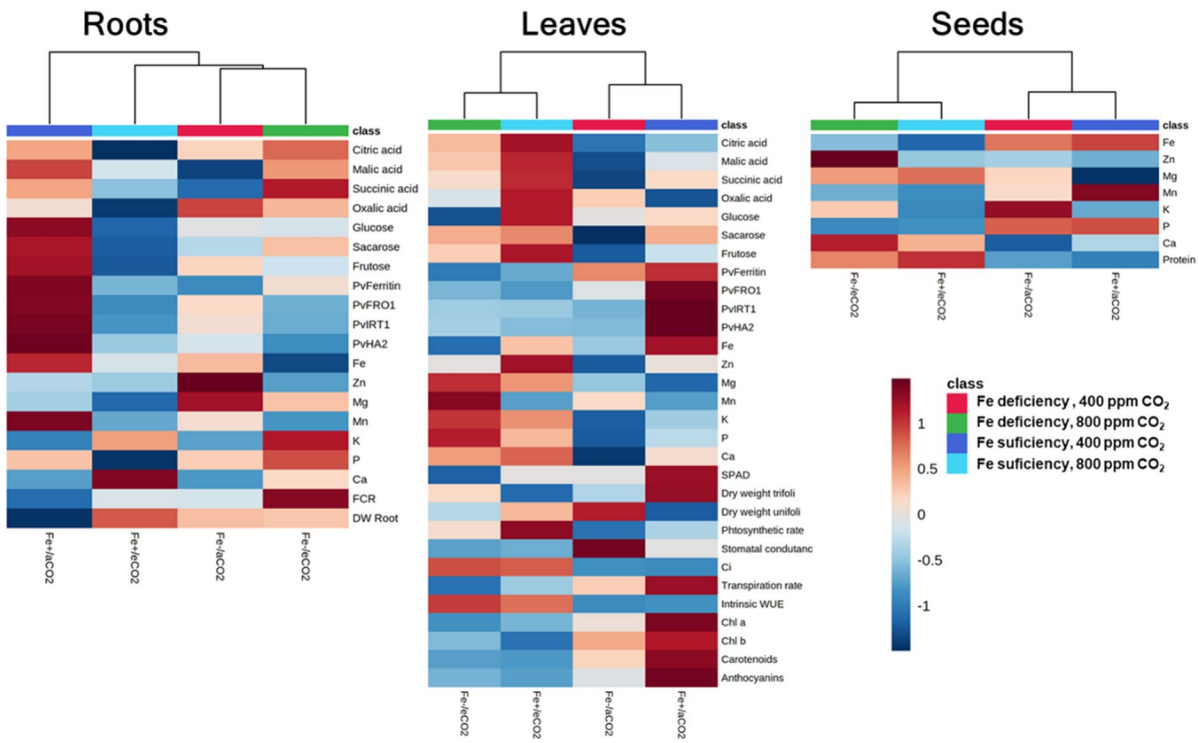

Fig. 8 Heatmap resulting from the hierarchical clustering analysis of samples from roots, leaves and seeds of plants grown under Fe sufficiency (Fe+) or Fe deficiency (Fe-) combined with ambient (400 ppm) or elevated CO_2 (800 ppm) concentrations, as a function of the analyzed variables

higher activity than control plants (Fe+/aCO_2). Fe-/eCO_2 plants had about twice the activity measured in the plants exposed to just a single factor (Fe+/eCO_2 and Fe-/aCO_2 plants) (Fig. 1). These results show that Fe deficiency and eCO_2 act synergistically on the stimulation of FCR activity. Fe deficiency usually stimulates FCR activity in many plant species to increase plants' ability to capture Fe from the nutrient solution (Susin et al. 1996; Robinson et al. 1999; López-Millán et al. 2009; Santos et al. 2016; Vasconcelos et al. 2017). FCR activity has been shown to be stimulated in tomato plants grown at Fe deficiency alone and further when Fe deficiency was combined with eCO_2. However, eCO_2 individually had no effect on FCR activity of tomato plants (Jin et al. 2009). The results of our study show, for the first time, that eCO_2 directly affects FCR activity regardless of the Fe condition and that there is a cumulative effect of both factors affecting the functioning of FCR enzyme, which may affect Fe uptake. The prolonged exposure to eCO_2 may have led to excessive cation uptake by the root with subsequent stem internal alkalinisation (Figs. 2a, d, e and 8). We hypothesize that when plants were exposed to Fe limitation and eCO_2, proton extrusion and organic acid exudation increased, favouring FCR activity. According to our results the concentrations of citric, malic and succinic acids in the roots of the plants exposed to Fe limitation and eCO_2 tended to be higher than in the plants of the other conditions. In addition, the expression of *PvHA2* followed the same pattern as *PvIRT1* and *PvFRO1,* they were significantly downregulated in the roots of these plants (Fig. 7 and 8), supporting a link to changes in pH (Fig. 2). Fe uptake across the plasma membrane requires a sharp pH gradient (Santi and Schmidt 2009) and the co-expression of *PvHA2* with these enzymes contributes to an efficient Fe uptake.

Organic acids and pH gradients

Organic acids concentrations in the leaves of plants grown under eCO_2 (Fe±/eCO_2) were generally higher, whereas under Fe deficiency (Fe-/aCO_2 and Fe-/eCO_2), except for oxalic acid, tended to be lower (Fig. 3). Alkalinisation was slightly mitigated in Fe deficient plants under eCO_2, probably due to the increased organic acids formation in the roots of these plants (Figs. 3 and 8). Fe deficiency increased stem internal pH and eCO_2 seemed to have an attenuating effect (Fig. 2). Under Fe restriction Fe uptake mechanisms of common bean is highly dependent on external pH. An acidification of the nutrient solution is required to increase Fe solubility and subsequent uptake. In contrast to the observed in plants grown under Fe restrictions, in Fe-sufficient plants, eCO_2 did not enhance plant acidification capacity, slightly increasing the stem pH, probably due to the lower levels of organic acids in the roots of these plants compared to control plants (Fe+/aCO_2).

The combination of both factors (Fe deficiency and eCO_2) did not determine significant differences in leaves. However, citric, succinic, and malic acid levels in roots tended to be higher in these plants (Fig. 3). Under eCO_2 carboxylation is generally induced, whereas photorespiration is supressed (Long et al. 2004), with levels of organic acid at the leaf level tending to increase. Organic acids are the transitory or stored forms of fixed carbon which can either be converted back to carbohydrates or further oxidized forming CO_2 and H_2O. Under eCO_2, as organic acid concentrations increase in leaves and as translocation to sink organs occurs, the plant's cytosolic pH decreases, favouring FCR activity and proton extrusion, inducing cation uptake. To counteract the excessive cation uptake induced by the external medium's low pH, the plant produces oxaloacetate (White 2012). The activation of this pathway has been previously reported to occur in some plant species exposed to Fe limitation (Abadía et al. 2002; Rombolà et al. 2005; López-Millán et al. 2009) and under eCO_2 this mechanisms may also be induced which may explain the highest levels of oxalic acid in the leaves of Fe+/eCO_2 plants (Fig. 3d). Oxaloacetate may also convert to malate and other organic acid intermediates, exported to the leaf, or exuded to the external medium. It may explain the higher concentrations of organic acids in the leaves and decreased levels in the roots of Fe-sufficient plants grown under eCO_2.

At aCO_2 the organic acids concentrations were not affected by Fe limitation. The effect of Fe deficiency was apparent only on the increase of root citrate and decreased leaf oxalate of plants exposed to eCO_2. (Fig. 3a). Fe deficiency may have induced the biosynthesis and export of citric acid to the root in order to increase Fe uptake. Within root cells, metal cations like Fe are chelated with organic acids, particularly citrate, and distributed to other plant organs, thus contributing to increased nutrient uptake (Susin et al. 1996; Abadía et al. 2002; López-Millán et al. 2009; Dong et al. 2021).

Sugars

In our study, leaf sucrose levels were not significantly different among the plants of all conditions. However, in the roots, sucrose levels were significantly lower in Fe+/eCO_2 plants than in Fe+/aCO_2 plants (Fig. 4a). Considering the higher root biomass of the plants growing under Fe+/eCO_2 it indicates that sugars are being used for root biosynthesis, but sucrose produced during photosynthesis may be also translocated to the roots, being stored or used as a substrate (Cakmak et al. 1994; Rombolà et al. 2005). Another hypothesis is that as the acclimation process initiates, a decreasing accumulation of sugars in the roots occurs (Thompson et al. 2017).

The levels of glucose and fructose tended to be higher in the leaves of Fe sufficient plants at eCO_2 than at aCO_2 (Fig. 4b, c), supporting the highest photosynthetic rate (Table 2) and sink capacity of these plants until flowering. Studies on Arabidopsis also reported that plants growing under eCO_2 tend to accumulate higher concentrations of leaf sugars (Jauregui et al. 2015, 2016; Noguchi et al. 2015).

Grain protein

The Fe treatment had no effect on productivity (Table 2) and grain protein concentrations (Fig. 5), and this result follows previous studies, including for common bean plants (Shainberg et al. 2000). In contrast, under eCO_2, the productivity was significantly lower and grain protein was significantly higher than in the plants grown under aCO_2 (Fig. 5). Many

studies report that plants grown under eCO_2 produce grains with lower protein levels (Taub et al. 2008; Hampton et al. 2013; Myers et al. 2014; Chaturvedi et al. 2017; Córdoba et al. 2017; Dong et al. 2018; Li et al. 2018). Some studies showed that for legumes, eCO_2 has no impact on grain protein concentrations (Jablonski et al. 2002; Taub et al. 2008; Myers et al. 2014), and, more recently, Soares et al. (2019) reported an increase in grain protein concentrations in several common bean varieties.

Here we show that eCO_2 did not reduce grain protein concentration in common beans but highly decreased productivity. This result supports the hypothesis of the induction of an acclimation process in plants growing under eCO_2 after flowering, leading to decreased photosynthesis and decreased productivity. Due to the lower productivity, even with the purported lower leaf N allocation (Seneweera 2011; Thompson et al. 2022) this effect was not evident on grain protein concentration of plants growing under eCO_2 with the grains of these plants showing significantly higher protein content. This could suggest that the higher protein concentration is due to lower productivity, and a protein "concentration" effect.

Fe accumulation in the leaves, roots and seeds

Fe levels were significantly lower in the root, leaves, and seeds of plants grown under eCO_2, regardless of Fe supply (Fig. 6a).

Root Fe levels were higher in Fe sufficient plants at aCO_2 than in Fe deficient plants at aCO_2 (2-fold) and eCO_2 (12-fold) (Fig. 6a). Restricted Fe supply and eCO_2 individually are known to induce reduced accumulation of Fe in plants (Briat et al. 2006; Högy et al. 2009; Loladze 2014; Myers et al. 2014; Santos et al. 2015). However, few studies are available addressing these factors' interactive effects on plant Fe uptake and accumulation. Jin et al. (2009) showed that the short-term exposure to eCO_2 (7 days) of tomato plants grown under Fe deficiency induces the accumulation of Fe levels in the root and shoot (Jin et al. 2009). Similar results were reported by Soares et al. (2022) regarding the short-term effects of eCO_2 in soybean plants grown under Fe deficiency. Our results highly suggest that the patterns of Fe accumulation differ among species. In bean plants growing under Fe deficiency, the exposure to eCO_2 further exacerbates the low Fe concentrations in roots and shoots. However, the differences observed may also be related with the fact that in the other studies trace amounts of soluble Fe were available in the Fe-limited condition whereas in this study Fe was completely removed from the nutrient solution of the plants growing under Fe deficiency. Although Fe restriction led to evident decreased Fe accumulation in the plant roots and leaves and decreased expression of *PvFerritin* in the leaves, and *PvFRO1* and *PvIRT1* in the roots, this decrease did not reflect on Fe concentration in plant seeds, which were very similar under both Fe conditions (Figs. 6a and 8). The repression of FRO1 in soybean and IRT1in common bean plants growing under Fe deficiency has been previously reported (Santos et al. 2016, 2020; Urwat et al. 2021). In contrast to Fe limitation, eCO_2 led to a significant decrease in seed Fe levels with the lower productivity being correlated with the lower Fe accumulation. In these plants, despite FCR activity was higher, *PvFerritin,* was down-regulated in the leaves and *PvFRO1* and *PvIRT1* were down-regulated in the roots (Fig. 7a). Ferritin is a primary Fe storage protein that plays a significant role in maintaining cellular Fe homeostasis (Van Wuytswinkel et al. 1999; Ravet et al. 2009; Grant-Grant et al. 2022) whereas FRO1 and IRT1 are important Fe transporters. Therefore, the low leaf *Pvferritin* expression and the 60% downregulated expression of *PvFRO1* and *PvIRT1* in the roots of plants exposed to eCO_2 compared to plants grown under Fe sufficiency and aCO_2, may be related to the lower Fe concentrations in the seeds of these plants (Figs. 7a and 8). An acclimation process may have been initiated in eCO_2, contributing to the repression of these genes and subsequent low Fe accumulation. However, the higher FCR activity of these plants grown under eCO_2 and Fe deficiency (Fig. 1) suggests that even with low gene expression, there was already a pool of ready-to-use enzymes for reducing Fe. These results clearly show that eCO_2 leads to decreased accumulation of Fe in all plant parts and Fe deficiency further exacerbates this effect in plant roots and leaves but not in seeds.

Other minerals

The accumulation pattern of seed P and Fe was similar (Fig. 6g), suggesting that seed P and Fe may be related. This correlation was reported before (Briat

et al. 1999; Panda et al. 2012) and seems to be related to the Fe uptake mechanism, which requires ATP and would turn the process less efficient under P limitation. The low P concentrations in the seeds may be the result of an acclimation process at eCO_2 leading to plant's inability to regenerate P (Urban 2003). Therefore, the low levels of P in the seeds of the plants exposed to eCO_2 may be an indirect result of this process.

In contrast, in plants grown under eCO_2, seed calcium (Ca) was significantly higher regardless of Fe supply (Fig. 6e). Ca is transported in the xylem as either Ca^{2+} or complexed with organic acids. However, its mobility in the plant is low, with very limited Ca remobilization from leaves to phloem-fed tissues, such as fruits, seeds, and tubers (White and Broadley 2003). Therefore, it raises the hypothesis of Ca uptake directly to the seed via xylem when plants are grown under eCO_2. Increased Ca uptake in rice and increased concentrations of Ca in cucumber fruit resulting from plant growth under eCO_2 have been reported (Seneweera 2011; Dong et al. 2018).

In the seeds it was also possible to observe an effect of the Fe restriction and eCO_2 leading to increased accumulation of Zn (Fig. 6b). Fe restriction can favour Zn accumulation (Cohen et al. 1998; Kanai et al. 2009). The highest Zn levels in the Fe-limited plants (Fe-/aCO_2) may be related with the highest internal pH of these plants (Fig. 2) since Zn is mainly taken up as Zn^{2+} at high pH (Broadley et al. 2012) or due to the upregulation of the expression of Zn transporter genes which has been previously reported to occur in plants growing under Fe deficiency (Xie et al. 2019). However, eCO_2 can reduce the accumulation of this micronutrient (Loladze 2014; Myers et al. 2014). This result is of interest and clearly shows that there is an interaction of Fe restriction and eCO_2 contributing to increased accumulation of Zn in the grains of these plants.

In the leaves, eCO_2 increased Zn, Mg, and K levels and, to a lesser extent Ca (Fig. 6). eCO_2 can increase Mg, K, and Ca uptake in rice plants (Seneweera 2011), but a meta-analysis has shown decreased levels of these minerals in foliar tissues of plants grown under eCO_2 (Loladze 2014). K is involved in translocation, stomatal regulation, and carbon fixation (Cakmak et al. 1994; Hawkesford et al. 2012). An increase in leaf K may indicate an imbalance of these mechanisms in the plants exposed to eCO_2, since eCO_2 can also promote K uptake, for example, in rice (Jena et al. 2018). However, variations in K are not always consistent since they seem to decrease in soybean leaves (Singh et al. 2017), and in bamboo plants, it is species-dependent (Zhuang et al. 2018).

Proposed mechanism of eCO_2 and Fe restriction action on Fe uptake (Figs. 8 and 9)

Plant exposure to eCO_2 during growth impacted carbon fixation and Fe uptake mechanisms. Elevated CO_2 induced carboxylation and increased production of organic acids and sugars in plant leaves. Sugars were then probably transported to sink organs (e.g., roots), used for biomass biosynthesis, stored as starch, or metabolized. The lowering of cytoplasmic pH due to increased production of organic acids may have prompted organic acid exudation at the root level, creating a pH gradient to increase nutrient uptake. Thus, as organic acids exuded, the pH of the nutrient solution decreased, and Fe became more soluble for subsequent uptake. In addition, the acidification of the external medium may have stimulated FCR activity, which may have contributed to an increased Fe uptake. However, in this process, the cytoplasmic pH highly increased, and to counteract the excessive cation uptake, plants may have produced oxaloacetate, which can be converted to other organic acids. These newly formed organic compounds lead to slight cytoplasmic acidification, balancing internal pH. In addition, under eCO_2, stomatal conductance decreased with a consequent decrease in transpiration rates and Fe uptake through the xylem. This CO_2 effect may have contributed to decreased Fe accumulation in the plants exposed to eCO_2.

Under Fe restriction and eCO_2, plants may have activated two pathways to enhance Fe uptake: i) organic acids exudation and ii) proton extrusion. The activation of these mechanisms doubled FCR activity compared with the plants exposed to just a single factor (Fe-/aCO_2 and Fe+/eCO_2 plants). However, these plants showed the lowest Fe accumulation in plant roots. Under eCO_2, plants exude organic acids decreasing cytoplasmic root pH, which may lead to a reduction of membrane pH gradient, which is necessary for Fe uptake. Besides, during the week

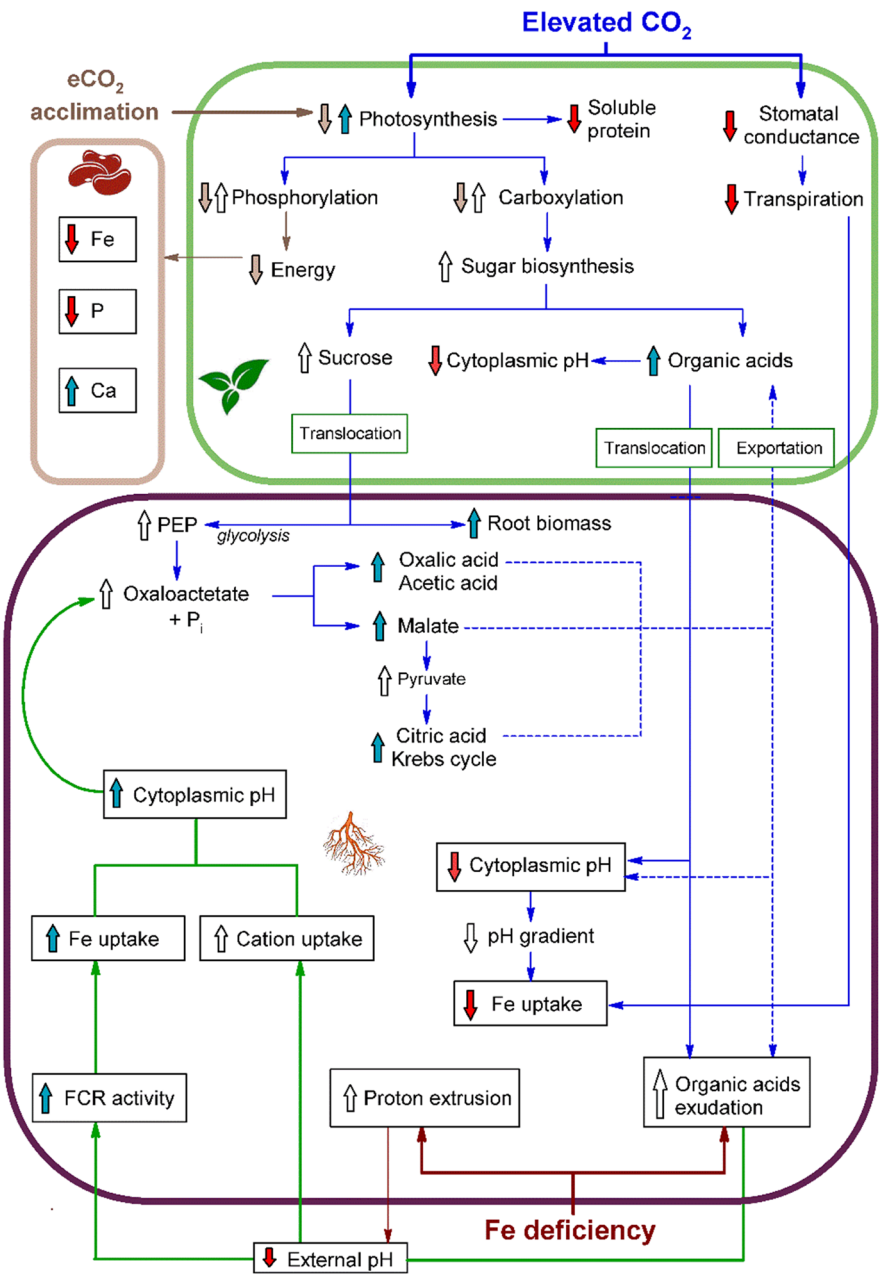

Fig. 9 Schematic representation of the proposed combined effect of elevated CO_2 (eCO_2) and Fe deficiency in common bean plants growing in hydroponics. Plants grew under ambient CO_2 (aCO_2) and eCO_2 for one week with complete solution (20 µM Fe-EDDHA) plus two weeks under Fe sufficiency and Fe deficiency (first sampling date – 21 days) being maintained in these conditions until complete seed filling. The blue arrows represent the effect of eCO_2, the red arrows represent the effect of Fe deficiency, the green arrow the combination of both factors, and the red-grey arrows represent the hypothesised effect of eCO_2 acclimation process. The shaded arrows represent secondary mid-term effect of eCO_2

before the first sampling (end of the third week), just before flowering, an acclimation to eCO_2 may have started, possibly extending up to the end of the seed filling stage. Thus, as the acclimation process begins, it represses photosynthesis, induces decarboxylation, and represses phosphorylation leading to decreased grain yield.

Despite the high FCR activity, the absence of Fe in the nutrient solution and the repression of the expression of Fe transporters limited its transport. These factors may explain the lowest Fe levels in grains of plants subjected to eCO_2 and Fe limitation. Consequently, the plant energy status decreases, and thereby Fe uptake is inhibited, and Pi is not regenerated. Fe restriction exacerbates this effect since, at this stage, there is no Fe in the nutrient solution for uptake. As a result, the expression of proteins involved in Fe uptake was repressed.

Conclusion

Elevated CO_2 highly affects common bean plant metabolism, increasing root FCR activity and highly downregulating root *FRO1* and *IRT1*, and leaf *ferritin* genes expression. In the leaves, eCO_2 increased citric and oxalic acids, whereas sucrose and glucose tended to be lower in the roots. Such molecular and metabolic changes under eCO_2 may have determined the: i) lower levels of Fe and Mn in the roots, ii) lower Fe and higher Zn, Mg, and K levels in the leaves, and iii) the lower P and Fe and higher Ca and protein levels in the seeds (Fig. 8) of the plants grown under eCO_2 compared with the levels found in the plants grown under aCO_2 (Fe±/aCO_2).

When both factors were combined, an interactive effect led to the highest root FCR activity and lowest root, leaf, and grain Fe concentrations. At the seed level, the differences found in mineral concentrations were mainly correlated with eCO_2 and not Fe restriction nor the combined effect of both factors. An exception was the increased Zn levels in the grains of plants exposed to both factors. These results suggest that the bean cultivar used in this study is CO_2-responsive and confirm its tolerance to restricted Fe supply.

The results of this study show that eCO_2 reduces Fe accumulation in all plant parts, including the grains. Although an essential interaction of eCO_2 and Fe deficiency leads to a sharp up-regulation of FCR activity, this regulation was insufficient to ensure an efficient Fe uptake, with plants exposed to eCO_2 having lower Fe levels.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were conducted by Teresa Deuchande. The first draft of the manuscript was written by Teresa Deuchande and both authors commented on previous versions of the manuscript and approved the final manuscript.

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Data availability The datasets generated during the current study are available from the corresponding author, Teresa Deuchande, upon reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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