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ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

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Thesis submitted to Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology, with specialization in Food Science and Engineering

by

José Carvalho Soares

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Under the supervision of Professor Marta Wilton Pereira Leite de Vasconcelos

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Resumo

A concentração elevada de CO_2 na atmosfera e os níveis de ferro no solo são fatores-chave que afetam o crescimento e a qualidade nutricional das plantas. Neste estudo, foram testadas as hipóteses de que 1) a plasticidade fenotípica em variedades de feijão e soja afeta a adaptação a condições de CO_2 elevado; 2) existem mecanismos de resposta contrastantes em plantas crescidas, em ambiente controlado ou em ensaios de campo, a CO_2 elevado; 3) a exposição e adaptação a concentração elevada de CO_2 pode estar envolvida na alteração da expressão genética que origina mudanças nas vias metabólicas das plantas; e 4) a interação de CO_2 elevado com a limitação de ferro deve ter um impacto significativo no crescimento, fisiologia e nas alterações moleculares das plantas. De modo a testar as hipóteses de estudo, vários genótipos de feijão e soja foram crescidos a CO_2 elevado em ambiente controlado (800 ppm) e em ensaios de campo (600 ppm), para avaliar a produtividade e a resposta a nível fisiológico e nutricional. De seguida, no genótipo com maior suscetibilidade ao aumento da produção de sementes e menor redução nutricional a nível do grão, foi avaliado o efeito da concentração elevada de CO_2 na alteração da expressão genética utilizando uma análise de transcritómica. Finalmente, foi avaliado o efeito da interação de CO_2 elevado e da limitação de ferro na resposta das plantas a nível fisiológico e molecular.

A seleção de variedades adaptadas a condições de CO_2 elevado é uma decisão importante, com o intuito de melhorar a produtividade das culturas agrícolas nas concentrações futuras de CO_2. Com o objetivo de estudar a variação intraespecífica em genótipos de feijão e soja, em parâmetros de produtividade e qualidade nutricional, avaliou-se o crescimento das plantas a CO_2 elevado em ambiente controlado. A variação no rendimento de sementes foi de -11.0 a 32.7% em feijão e de -23.8 a 39.6% em soja. A concentração de proteína no grão de feijão aumentou significativamente e não foi afetada na soja. O CO_2 elevado apresentou efeitos positivos e negativos na concentração dos minerais nas sementes. Um aumento da produção de sementes e uma redução da sensibilidade às perdas minerais podem ser parâmetros adequados na seleção de variedades futuras. De seguida, genótipos de soja cultivados anteriormente cresceram em experiências de campo no sistema de enriquecimento de CO_2 ao ar livre (FACE) com o objetivo de avaliar a produtividade e o impacto nutricional no grão em condições naturais. O rendimento das sementes aumentou em média 47.0%. O CO_2 elevado aumentou a taxa de assimilação fotossintética, área foliar, altura e a biomassa das plantas. Os níveis de cálcio, fósforo, potássio, magnésio, manganês, ferro, boro e zinco no grão diminuíram em condições de CO_2 elevado. Os açúcares solúveis e amido aumentaram 9.1 e 16.0%, respetivamente, o ácido fítico aumentou 8.1%, e o teor de proteína no grão diminuiu 5.6% nos genótipos estudados. Além disso, a atividade antioxidante teve um decréscimo de 36.9%, e o conteúdo fenólico total não foi afetado pelas condições de CO_2 elevado. O genótipo de soja *Wisconsin Black* foi selecionado como melhor candidato para avaliar o efeito da exposição a concentrações de CO_2 elevado na análise do transcritoma e nas alterações fisiológicas. Este estudo avaliou a resposta coordenada em tecidos radiculares e foliares em condições de CO_2 elevado. Várias centenas de genes foram expressos diferencialmente pela exposição ao CO_2. Análise das vias metabólicas do KEGG mostraram o enriquecimento em genes presentes na fotossíntese, biossíntese de compostos secundários, metabolismo do azoto, metabolismo dos lípidos e genes relacionados com ritmo circadiano em folhas e raízes. Este estudo revelou potencial para identificar genes e vias metabólicas responsáveis pela adaptação da soja a CO_2 elevado.

Na última parte da tese, plantas de soja foram cultivadas em hidroponia para analisar o resultado da interação entre CO_2 elevado e a limitação de ferro nas respostas morfológicas, fisiológicas e moleculares. A concentração de CO_2 elevado aumentou o crescimento das plantas independentemente da concentração de ferro. No entanto, ocorreu a aclimatização da fotossíntese e um aumento na eficiência do uso da água devido à exposição ao CO_2 elevado. As respostas induzidas pela limitação de ferro nas raízes, sob CO_2 elevado, incluíram o aumento da atividade da redutase férrica e a expressão de genes responsáveis pela captação de ferro. A análise proteómica identificou 705 e 589 proteínas expressas diferencialmente em raízes e folhas, respetivamente, de acordo com as condições observadas. A análise de enriquecimento mostrou que as vias metabólicas relacionadas com a parede celular, metabolismo da glutationa, fotossíntese, proteínas relacionadas com stress e síntese de compostos secundários foram alteradas devido ao stress de ferro nos tecidos radiculares. O crescimento das plantas, com ou sem limitação de ferro, ocorreu a concentrações de CO_2 elevado, assim como, um aumento da expressão de proteínas envolvidas na glicólise, metabolismo de amido e sacarose, síntese de giberelinas, e uma diminuição de proteínas envolvidas na biossíntese proteica. Os resultados revelaram que proteínas e vias metabólicas relacionadas com a limitação de ferro alteram os efeitos do CO_2 elevado e afetam negativamente a produção de soja, e isso pode ter implicações importantes para a sua produção no futuro.

Palavras-chave: CO_2 elevado, gene, limitação de ferro, proteína, soja

Abstract

Elevated CO_2 (eCO_2) and the levels of iron (Fe) in soil are key factors affecting plant growth and the nutritional quality of crop plants. In this study, we tested the hypotheses that 1) phenotypic plasticity in bean and soybean genotypes affects the adaptation to eCO_2 regarding seed yield and nutritional responses; 2) there are contrasting responses between plants grown in a controlled environment and field trials under eCO_2; 3) exposure and adaptation to eCO_2 may be involved in the alteration of gene expression that leads to changes in metabolic pathways of plants, and 4) the interaction of eCO_2 and Fe-limitation in soybean might impact the growth, physiology, and molecular response mechanisms. To test the hypotheses, we exposed several bean and soybean genotypes to eCO_2 in a controlled environment (800 ppm) and in field experiments (600 ppm) to evaluate yield, physiological, and nutritional responses. After that, using the most responsive genotype to CO_2 enrichment in terms of yield improvement and nutritional resilience, we studied the impact of eCO_2 on gene expression using a transcriptomic analysis. Finally, it was assessed how eCO_2 and Fe-limitation affected the physiological and molecular responses of soybean plants.

The selection of varieties adapted to eCO_2 is a crucial decision to improve the yield of crop plants in future CO_2 concentrations. To study the intraspecific variation among bean and soybean genotypes in yield and nutritional quality parameters, plant growth at eCO_2 was evaluated in a controlled environment. The range of seed yield responses was -11.0 to 32.7% in beans and -23.8 to 39.6% in soybean plants. Grain protein concentration increased in beans and was not affected in soybean. Elevated CO_2 had both positive and negative effects on grain mineral concentrations. Variation in seed yield increase and reduced sensitivity to mineral losses might be suitable parameters to select lines for the forthcoming eCO_2 conditions.

Subsequently, the previously cultivated soybean genotypes were grown under free-air CO_2 enrichment (FACE) conditions to evaluate the yield and grain nutritional impact after exposure to CO_2 enrichment under natural conditions. Seed yield increased by 47.0% among soybean genotypes. Elevated CO_2 improved the photosynthetic carbon assimilation rate, leaf area, plant height, and aboveground plant biomass. Moreover, grain concentration of calcium, phosphorus, potassium, magnesium, manganese, iron, boron, and zinc decreased under eCO_2 conditions. Soluble sugars and starch increased by 9.1 and 16.0%, respectively, phytic acid increased by 8.1%, and grain protein content decreased by 5.6%. In addition, antioxidant activity decreased by 36.9%, and total phenolic content was not affected by eCO_2 conditions.

The soybean genotype *Wisconsin Black* was selected as the best candidate to study the effect of eCO_2 exposure on transcriptome analysis and physiological changes. The study evaluated the coordinated response of root and leaf tissues under eCO_2. The transcriptomic analysis showed that several hundred genes were expressed differentially due to CO_2 enrichment in soybean. Further analysis of KEGG pathways showed that differentially expressed genes were enriched in photosynthesis, biosynthesis of secondary compounds, nitrogen metabolism, fatty acid metabolism, and circadian rhythm-related genes in both leaves and roots. Therefore, this study has the potential to discover genes and pathways responsible for adaptation to eCO_2 in soybean plants.

The last part of the thesis included the study of the interaction between eCO_2 and Fe-limitation on soybean plants grown in hydroponics. Elevated CO_2 stimulates plant growth in Fe-limited and Fe-sufficient plants. However, downregulation of photosynthesis and an increase in water-use efficiency occurred at eCO_2. The Fe deficiency-induced responses in roots, including the ferric chelate reductase activity and the expression of Fe-uptake genes, increased by eCO_2. Proteomic analysis identified 705 and 589 differentially expressed proteins in root and leaf tissues, respectively. Pathway enrichment analysis showed that cell wall organization, glutathione metabolism, photosynthesis, stress-related proteins, and biosynthesis of secondary compounds changed to cope with Fe-stress in root tissues. Moreover, plant growth at eCO_2, with sufficient or limited Fe supply, was related to the increased abundance of proteins involved in glycolysis, starch and sucrose metabolism, biosynthesis of plant hormones gibberellins, and decreased levels of protein biosynthesis. Our results revealed that proteins and metabolic pathways related to Fe-limitation changed the effects of eCO_2 and negatively impacted soybean production, which may have important implications for soybean production in the future.

Keywords: elevated CO_2, gene, iron limitation, protein, soybean

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Scope and outline

Preliminary note: The core of this thesis is composed of six papers, five published and one submitted in international scientific journals.

The main objective of the thesis was to study the effects of eCO_2 and Fe-limitation in bean and soybean plants and to understand plant adaptation mechanisms to both abiotic factors. This thesis included four parts divided into eight chapters to systematize the sequence and content of the studies. The different chapters describe how scientific research has progressed over time.

Part I comprises chapter 1, including state of art regarding the preservation of the nutritional quality of crop plants under a changing climate, with a particular focus on the influence of eCO_2. Furthermore, it provides strategies to sustain the nutritional composition of plants in a changing environment.

Part II encompasses chapters 2, 3, and 4. Chapter 2 describes the effects of growing bean and soybean varieties under eCO_2 on the yield and the concentration of grain protein, fat, and mineral elements in a controlled environment. In chapter 3, the previously grown soybean genotypes were evaluated in field trials under FACE conditions to study the yield responses and nutritional impact of eCO_2. The soybean genotype *Wisconsin Black* was selected as the best candidate based on seed yield increase and reduced nutrient losses under eCO_2 and used in further analyses. Then, in a short-term experiment, we studied soybean plants grown under hydroponic conditions at eCO_2 using transcriptomic and physiological analyses in chapter 4.

Part III includes chapters 5 and 6. Herein, we studied the impact of the interaction between CO_2 enrichment and Fe-limitation in the soybean genotype *Wisconsin Black*. In chapter 5, plants were grown in a hydroponic culture system to report the findings of the interaction of both abiotic factors in the growth, mineral status, and Fe-uptake mechanisms. Moreover, we performed proteomic analysis to study differentially expressed proteins influenced by the interaction of both factors on soybean plants, as demonstrated in chapter 6.

In Part IV, concluding research findings were presented in chapter 7, and future perspectives in chapter 8.

PART I: Bibliographic survey

CHAPTER 1
Literature survey

PART II: Growth of bean and soybean genotypes under eCO_2 conditions

CHAPTER 2
Growth and nutritional responses of bean and soybean genotypes to eCO_2 in a controlled environment

CHAPTER 3
Genotypic variation in the response of soybean to eCO_2 (FACE)

CHAPTER 4
Transcriptomic and physiological analyses under eCO_2 conditions in soybean plants

PART III: Interaction between CO_2 enrichment and Fe-limitation in soybean plants

CHAPTER 5
Short-term exposure to eCO_2 stimulates growth and metabolic responses that alleviate early-stage Fe deficiency symptoms in soybean

CHAPTER 6
Effect of the interaction between eCO_2 and Fe-limitation on proteomic profiling of soybean

PART IV: Conclusions and future perspectives

CHAPTER 7
Conclusions

CHAPTER 8
Future perspectives

Most of the scientific data presented here and that constitute this dissertation have been published or submitted to international peer-reviewing via publication in international scientific journals – according to the following list:

Chapter 1:

José C. Soares, Carla S. Santos, Susana M. P. Carvalho, Manuela M. Pintado and Marta W. Vasconcelos (2019). Preserving the nutritional quality of crop plants under a changing climate: importance and strategies. Marschner Review, Plant Soil, 43, 1–26. https://doi.org/10.1007/s11104-019-04229-0

Chapter 2:

José Soares, Teresa Deuchande, Luísa M.P. Valente, Manuela Pintado, and Marta W. Vasconcelos (2019). Growth and nutritional responses of bean and soybean genotypes to elevated CO_2 in a controlled environment. Plants, 8, 465.
https://doi.org/10.3390/plants8110465

Chapter 3:

José C. Soares, Lars Zimmermann, Nicolas Zendonadi dos Santos, Onno Muller, Manuela Pintado, and Marta W. Vasconcelos (2021). Genotypic variation in the response of soybean to elevated CO_2. Plant-Environment Interactions, 2, 263–276. https://doi.org/10.1002/pei3.10065

Chapter 4:

José C. Soares, Manuela Pintado, Conceição Egas, and Marta W. Vasconcelos (2022). Transcriptomic and physiological analyses under elevated CO_2 conditions in soybean plants. Submitted to Frontiers in Plant Science.

Chapter 5:

José C. Soares, Manuela Pintado, and Marta W. Vasconcelos (2021) Short-term exposure to elevated CO_2 stimulates growth and metabolic responses that alleviate early-stage iron deficiency symptoms in soybean. Journal of Plant Interactions, 17, 1, 50–59.
https://doi.org/10.1080/17429145.2021.2011445

Chapter 6:

José C. Soares, Hugo Osório, Manuela Pintado, and Marta W. Vasconcelos (2022). Effect of the interaction between elevated carbon dioxide and iron limitation on proteomic profiling of soybean. International Journal of Molecular Sciences, 23 (21), 13632. Published in the Special Issue Iron and Sulfur in Plants 3.0.

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PART I - Bibliographic Survey

CHAPTER 1 - Literature survey

1.1. Abstract

Background

Global climate is changing more rapidly than ever, threatening plant growth and productivity while exerting considerable direct and indirect effects on the quality and quantity of plant nutrients.

Scope

This review focuses on the global impact of climate change on the nutritional value of plant foods. It showcases the existing evidence linking the effects of climate change factors on crop nutrition and the concentration of nutrients in edible plant parts. It focuses on the effect of elevated CO_2 (eCO_2), elevated temperature (eT), salinity, waterlogging, and drought stresses, and what is known regarding their direct and indirect influence on nutrient availability. Furthermore, it provides possible strategies to preserve the nutritional composition of plant foods under changing climates.

Conclusions

Climate change has an impact on the accumulation of minerals and protein in crop plants, with eCO_2 being the underlying factor of most of the reported changes. The effects are clearly dependent on the type, intensity and duration of the imposed stress, plant genotype and developmental stage. Strong interactions (both positive and negative) can be found between individual climatic factors and soil availability of nitrogen (N), potassium (K), iron (Fe) and phosphorous (P). The development of future interventions to ensure that the world's population has access to plentiful, safe, and nutritious food may need to rely on breeding for nutrients under the context of climate change, including legumes in cropping systems, better farm management practices and utilization of microbial inoculants that enhance nutrient availability.

1.2. Introduction

Although the total global cultivated land area available for farmers has not changed significantly in the last 25 years (O'Mara, 2012), in many regions of the world crop yields have considerably increased. This has been due mostly to agricultural intensification practices and to targeted efforts in plant breeding aiming to increase yield. Nonetheless, human population is growing at a fast pace, with a 33% increase expected to happen in the next 30 years, reaching 9.6 billion by 2050 (Manners and van Etten, 2018, Godfray et al., 2010, Ziervogel and Ericksen, 2010). Consequently, global demands for food will continue to rise throughout this century. Crop yields and nutritional security are extremely dependent on the climatic conditions projected for the future, and consequently, most of the food produced for human consumption is under its menace (Burritt, 2019). Despite efforts to increase global food availability, a key requirement for food and nutrition security, the global burden of malnutrition and micronutrient deficiencies remains alarming and closely linked to climate changes, particularly in low income communities (FAO, 2017). Furthermore, climate changes are also responsible for changing the relationships between crops, pests, pathogens and weeds. It can also aggravate several trends, including decreasing pollinator insects, increasing water scarcity and ozone concentrations at ground level and reducing fishery levels. Therefore, it is imperative to know in more detail the impacts of climate change on food security and undernourishment and its potential implications for nutritional outcomes, reviewed by Myers et al. (2017) and Fanzo et al. (2018).

Climate change is a multifactorial stress (Gray and Brady, 2016), and in the last decades, plants have experienced significant environmental fluctuations, which resulted in the global warming of the planet and in perturbations of the hydrological cycles. These environmental changes are likely to worsen, and their frequency of occurrence is likely to increase in the upcoming decades. Without mitigation and adaptation strategies, these changes will have a cumulative effect as time progresses (Fanzo et al., 2018). Therefore, in the coming decades climate changes will present a major challenge to agriculture, natural ecosystems, and global economies, for producing enough and nutritious food, which has been reflected in a sustainable intensification of agricultural systems (Pretty, 2018).

Climate change has varying effects on plants responses at the level of molecular function, developmental processes, morphological traits, and basic physiological responses. It has been well documented that eCO_2 increases plant growth and yield by enhancing photosynthesis, while oftentimes improving crop water-use efficiency (Guo et al., 2015, Grover et al., 2015, Li et al., 2018b, Dietterich et al., 2015, Han et al., 2015). However, improved plant growth at eCO_2 contrasts with reducing grain quality responses, which are being recognized across a range of plant species (Myers et al., 2014, Dong et al., 2018b). This suggests that eCO_2 changes the equilibrium among plant carbon metabolism and mineral uptake, and nutrient-use efficiency (Nakandalage and Seneweera, 2018). Micronutrient deficiencies are a substantial public health problem, presenting serious health and nutritional consequences (Anandan et al., 2011). A great deal of emphasis has been given in recent decades to zinc (Zn) and iron (Fe) nutritional deficiencies, particularly in developing countries where a considerable proportion of people depend on grains and legumes as main food sources of these elements (Myers et al., 2014). Micronutrient limitation has also an impact on the susceptibility of plants to biotic and abiotic stresses. However, the response largely depends on plant genotype and each mineral element has complex interactions with several changing climate variables (Nakandalage and Seneweera, 2018).

Elevated CO_2 is closely related to increased demands for nutrients and water resulting from increased plant growth (Briat et al., 2015). Extending this knowledge to micronutrients, is also of particular importance because of its role in key biochemical pathways.

Hence, the comprehensive understanding of how the nutrient regulation mechanisms of plants interact with key climate change factors, is a challenge posed to the scientific community that deserves full attention. In order to manipulate the most effective pathways for nutrient provision, it is important to propose predictive models that explain the future response mechanisms of plants to approaching worldwide environmental changes.

Herein we have focused on our current understanding of how climatic changes, with emphasis to eCO_2, impact the nutritional quality of crop plants and associated molecular and physiological response mechanisms. In addition, analysis of the interaction between CO_2 enrichment and low soil nutrient availability will be also addressed. Thus, it will be a pertinent aspect to consider if the concentration of minerals undergoes modifications by climate changes and consequently affects the performance of the plant. The final part of the review will be dedicated to report some of the strategies that can be used to preserve nutrient concentrations in future climates.

1.3. An overview of climate changes as a global problem

The climatic conditions in which our food-producing systems depend on have been shifting quickly and are projected to continue their current pathways unless significant interventions are made. The main cause of climate change is the release of anthropogenic greenhouse gases to the atmosphere, which have intensely increased since the pre-industrial era, determined largely by economic and population growth, and are now higher than ever. Constant release of greenhouse gases has led to atmospheric concentrations of CO_2 at an unprecedented level and will cause further warming and exacerbate changes of the climate system, increasing the likelihood of severe and permanent impacts for people and ecosystems (Myers et al., 2017). These impacts, together with those of other anthropogenic drivers, have been the dominant cause of the observed global warming during the last decades. Thus, significant reductions in greenhouse gas emissions are required and here governmental authorities have a major role to play (IPCC, 2014, Fanzo et al., 2018, Myers et al., 2017). Overall, 1.0 °C increase in global warming since the pre-industrial era has been observed, and is expected to reach 1.5 °C by 2050 if increases at the current rate are maintained (IPCC, 2018). Moreover, it is expected that almost two billion people will be affected by almost complete water deficiency over the course of this century, and that close to 65% of the human population will be affected by circumstances of partial water insufficiency (Nezhadahmadi et al., 2013). Oscillations in the occurrence and intensity of normal precipitation patterns are also increasingly becoming a major global problem for agriculture, with a direct effect on the bioavailability of plant nutrients in soil. The consequences may be a change in soil moisture that is a key factor in nutrient acquisition, as soil water provides the medium in which plants absorb and transport nutrients from, and that can affect nutrient allocation (Fischer et al., 2019).

Even if there will be yield improvements in some crops in different regions of the world, the global impact of climate change on agricultural products is expected to be negative, threatening global food security. Developing countries, which are already vulnerable to food shortage, are likely to be most seriously affected (Nelson et al., 2009). Consequently, our ability to ensure the required amounts of food and nutritional quality in the face of rapidly changing environmental conditions will be an important task for the near coming future.

1.4. The importance of micronutrients in humans and in plants

Micronutrients play a decisive role in maintaining health, because they have an essential role in cognitive growth and development, in reproductive functions and cell metabolism, and also in immune system responses of humans (Nakandalage and Seneweera, 2018). Dietary deficiencies of micronutrients (i.e. hidden hunger) are considered as a global public health problem and it is already estimated to affect around two billion people worldwide (Haddad et al., 2015).

Accordingly, Fe limitation adversely disturbs growth, immune function and is the most common and widespread nutritional disorder in the world causing anemia (Murgia et al., 2012). Millions of people in developing countries are anemic comprising 50 and 40% of pregnant women and preschool children, respectively (Bouis and Saltzman, 2017). Current studies show that Fe-deficit in the first year of life is responsible for permanent effects on brain development, structure and function (Beard, 2008). Furthermore, 0.2% of deaths can be attributed to the Fe deficiency, in children under 5 years of age (Murgia et al., 2012).

Various biological functions have been atributed to Zn, since it cooperates with many enzymes and other proteins and performs critical structural, functional and regulatory roles in the body (Krężel and Maret, 2016). A large consumption of cereal-based foods is considered the main driver to Zn deficiency, since cereals have low concentration and bioavailability of Zn and cannot meet the human demand for Zn (Cakmak and Kutman, 2018). Nowadays, wheat, rice and corn account for about 60% of the world's daily energy consumption, and bread wheat alone is the staple food for about 35% of the world's population (Poursarebani et al., 2014). In Asian countries with a high incidence of Zn and Fe-deficiencies, rice and wheat deliver over 70% of the daily calorie intake in rural areas (Cakmak and Kutman, 2018, Cakmak et al., 2010).

The goal of biofortification is to solve some of these problems by increasing the concentration of micronutrients in the edible parts of crops and improving their bioavailability and absorption in the human body after digestion (Ramzani et al., 2016, Vasconcelos et al., 2017, Carvalho and Vasconcelos, 2013). More than 20 million people in developing countries are consuming biofortified crop products. These include beans and pearl millet fortified with Fe, maize, cassava and sweet potato fortified with provitamin A, and rice and wheat fortified with Zn (Bouis and Saltzman, 2017). Vitamin A enriched rice (golden rice) produced by transgenic approaches has been made available since the beginning of this century (Wesseler and Zilberman, 2014). However, this rice has not been introduced in any country, largely due to the lack of regulatory approval processes. These varieties have enormous nutritional potential and can be an effective economic solution reducing health costs.

Plants require 14 mineral nutrients to achieve for optimal development and growth (Marschner, 2012). These elements are structural components of numerous macromolecules including nucleic acids, phospholipids, certain amino acids, and several coenzymes and play a central role in plant cellular metabolism (Grusak, 2001). In addition, they are beneficial in chlorophyll biosynthesis, redox reactions, plasma membrane integrity and contribute to the osmotic potential of cells (Nakandalage and Seneweera, 2018).

Micronutrient insufficiencies impact plant growth and yield by limiting the biosynthesis or expression of important mechanisms of energy capture and/or metabolism (Grusak, 2001). Therefore, an increase in the vulnerability to abiotic stresses is usually encountered in plants that experienced micronutrient deficiency (Hajiboland, 2012, Bencke-Malato et al., 2019, Jin et al., 2009). A comprehensive understanding of how these abiotic factors affect the regulatory mechanisms of micronutrients in plants is essential, in order to mitigate their negative effects on the nutritional quality of crop plants when grown under a changing climate.

1.5. The influence of eCO_2 on mineral accumulation

The atmospheric CO_2 levels have been progressing from the 280 ppm preindustrial reference levels (Myers et al., 2017, Ainsworth and Long, 2005) to current global levels which are now above 400 ppm (IPCC, 2018). Although the increasing concentration of atmospheric CO_2 is the main driver of harmful anthropogenic climate changes, it can also improve crop performance by increasing rates of photosynthesis and water-use efficiency, particularly in C3 plants. The putative positive effect in agriculture is in fact denoted to as the “CO_2 fertilization effect” (Ainsworth and Long, 2005, Bowes, 1993, Bunce, 2015, Dietterich et al., 2015, Högy and Fangmeier, 2008, Loladze, 2014, Long et al., 2004, Ziska and Bunce, 2007, Myers et al., 2017). This effect has already been observed in crop plants and vegetables, including wheat (Han et al., 2015, Högy and Fangmeier, 2008, Fernando et al., 2012a, Dong et al., 2018c), maize (Zong and Shangguan, 2014), rice (Guo et al., 2015, Yang et al., 2007, Pang et al., 2006), barley

(Haase et al., 2008, Mitterbauer et al., 2017), bean (Bunce, 2008, Ma et al., 2017), soybean (Bunce, 2015, Kumagai et al., 2015), cowpea (Dey et al., 2017), potato (Kumari and Agrawal, 2014), lettuce, carrot, parsley (Dong et al., 2018b, Long et al., 2004, Mortensen, 1994) and tomato (Jin et al., 2009) among others. However, longer treatments with eCO_2 might lead to photosynthetic acclimation, due to increased soluble sugars leading to an imbalanced C:N ratio, accelerated leaf senescence and/or limited growth rate (Ainsworth and Long, 2005, Ainsworth et al., 2004, Ludewig and Sonnewald, 2000, Kaplan et al., 2012).

Future models of climate change for the period of 2000-2100 predicted an overall decrease of the growing season length and crop transpiration, and increase in water-use efficiency, biomass production, and yields, but with considerable variation among crop models (Ahmed et al., 2017, Bassu et al., 2014).

Despite all the compelling evidence, there is still insufficient knowledge on the role of eCO_2 in shifting the nutritional composition of crops and on the direct consequences to humans (Guo et al., 2015, Myers et al., 2014, Loladze, 2002, Högy and Fangmeier, 2008, Dong et al., 2018b, Fernando et al., 2012a, Jablonski et al., 2002, Li et al., 2018b, Duval et al., 2012). Therefore, Figure 1.1 gives an overview of the influence of climatic changes, particularly eCO_2, on grain mineral concentrations and the mechanisms proposed as responsible for changing the plant mineral content.

Using a meta-analysis of FACE and non-FACE studies, Loladze (2014) described a significant reduction in overall mineral concentration (-8%) in C3 plants, including foliar and edible tissues. Precisely, CO_2 enrichment lowered Fe, Zn, and Cu by 6.5–10%, with Mn showing no significant changes. Through a detailed analysis of various plant groups, eCO_2 reduced the mineral concentrations in crops (-7.2%), wild (-9.7%), herbaceous (-7.5%), and woody (-9.6%) plants combining data from foliar and edible tissues. Regarding different tissues, eCO_2 decreased mineral concentrations in foliar (-9.2%) and edible (-6.4%) tissues, including grains (-7.2%). The cereal specific decreases in grains were -7.6, -7.2 and -6.9% for wheat, rice and barley, respectively (Loladze, 2014). Similar findings were obtained by Myers et al. (2014) in the edible portions of C3 grasses and legumes grown under field conditions at eCO_2. A significant reduction in protein concentration in C3 grasses (-6.3% in wheat grains and -7.8% in rice grains), and no significant effects in soybeans or C4 crops were detected at eCO_2. Authors also evaluated phytate concentration that affects mineral bioavailability. The phytate concentration declined significantly at eCO_2 in wheat, which might partly counterbalance the nutritional impact of lower Fe and Zn concentrations in this crop caused by eCO_2, and thus increasing their bioavailability (Myers et al., 2014). Decreases in the concentration of these important micronutrients in such significant food crops will put at risk the populations of the developing world. Iron concentration was also significantly reduced in soybean seeds at fresh edible stage (R6), while Zn and Mn concentrations varied among cultivars (Li et al., 2018b). It was also found that eCO_2 decreased N, Mg, Fe, and Zn concentrations, and not affected P, K, S, Cu, and Mn concentrations in the edible part of vegetables (Dong et al., 2018b).

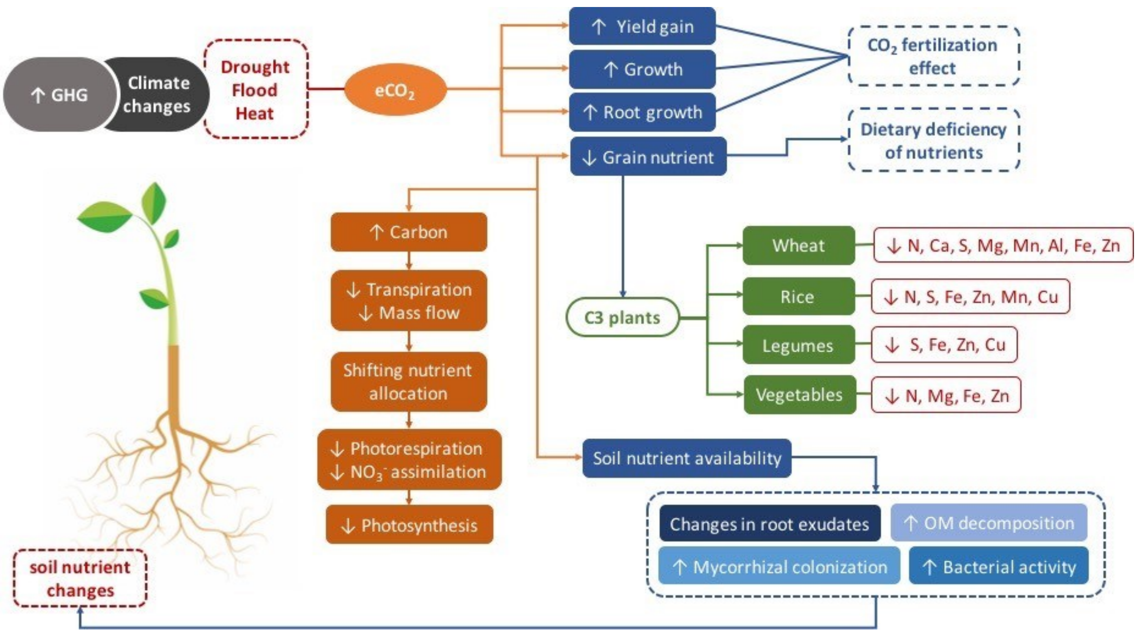

Figure 1.1 - Schematic illustration of the influence of climatic changes, driven by increased greenhouse gas (GHG) emissions, on grain mineral concentrations and their bioavailability in soil. The effects of CO_2 on nutrient concentrations in edible tissues of C3 plants are reflected in FACE and non-FACE studies. The mechanisms proposed as responsible for changing the plant mineral concentration are: "carbohydrate dilution" in which there is an increase in carbon (C) assimilation relative to the mineral concentration, decrease in transpiration rates that reduces mass flow of nutrients, and shifting nutrient allocation by altered biochemical processes between tissues can affect nutrient uptake. In addition, downregulation of photosynthesis and decreased photorespiration have been also expected to elucidate the variations in mineral concentrations. The CO_2 concentration has also a direct effect on the bioavailability of nutrients in soils and, consequently, affecting the quantity of existing microorganisms. Possibly, due to changes in soil pH, eCO_2 improves the exudation processes affecting nutrient availability. Furthermore, the role of eCO_2 increasing mycorrhizal colonization and organic matter (OM) decomposition in the soil, facilitating the availability of several nutrients (Soares et al., 2019b).

Nowadays, there is strong evidence that Zn deficiency is a significant global health problem affecting 17% of the world's population, and that increasing CO_2 levels lower the concentration of Zn in significant food crops (Myers et al., 2017, Myers et al., 2015, Myers et al., 2014). In a meta-analysis with previously published data from FACE and growth chamber experiments, Myers et al. (2014) found a significant reduction in Zn concentration in wheat (-9.1%), rice (-3.1%), barley (-13.6%), field peas (-6.8%), and soybean (-5.0%) grown at eCO_2. Similarly, eCO_2 decreased by 9.4% the Zn concentration in vegetables as described by Dong et al. (2018b). Thus, due to increased concentrations of atmospheric CO_2 it was anticipated that 138 million of people will be placed at new risk of Zn deficiency by 2050, and the most affected populations live in Africa and South Asia, with a particular incidence in India with 48 million people at risk (Myers et al. 2015).

The mechanisms responsible for the overall decline of plant mineral concentrations are not completely deciphered. Despite the “carbohydrate dilution” being a likely cause, it cannot elucidate all the mineral reductions because of the heterogenous response of each mineral tested for a given crop or for different species (Loladze, 2002, Poorter et al., 1997). Moreover, decreases in transpiration rates reduces mass flow of nutrients, and shifting nutrient allocation driven by altered biochemical processes between tissues can both change nutrient uptake (McGrath and Lobell, 2013). In addition, root architecture modification and downregulation of photosynthesis, reviewed in Taub and Wang (2008), and also inhibition of nitrate assimilation by decreased photorespiration (Bloom et al., 2002) have been proposed to elucidate the variations in mineral concentrations. The CO_2 concentration has also a direct effect on the bioavailability of nutrients in soils, and consequently affecting the number and diversity of existing microorganisms. A positive effect on soil nutrient bioavailability was described under CO_2 enrichment conditions (Jablonski et al., 2002, Kimball et al., 2002). However, the increase in plant growth with eCO_2 may have a disadvantage in terms of competition for micronutrient acquisition with microorganisms prevailing in the soil (Guo et al., 2015). Abbas et al. (2009) showed that eCO_2 promoted increases in P, K, Fe, Mn and Zn in the soil. Possibly, eCO_2 -induced changes in soil pH improves some exudation processes which affects the availability of nutrients in the soil. Similarly, it was described that eCO_2 responses averaged across two N treatments increased the concentrations of Ca, Mg, Fe, Zn and Mn at the soil surface by 15.6, 9.5, 23.4, 138.2 and 16.9%, respectively (Guo et al., 2015). In another study, Jin et al. (2019) described the interaction of long-term CO_2 conditions with different soil types (chromosol, vertosol, and calcarosol) on grain nutrient concentrations of wheat, field pea, and canola. At eCO_2, the concentrations of N, P, and Zn decreased by 6, 5, and 10%, respectively, regardless of soil, crop and year. In addition, the concentrations of K, Fe, Mn, and Cu were not affected by CO_2 enrichment in any crop grown in the soils tested. Data concerning the impact of CO_2 on other micronutrients, including selenium (Se), chromium (Cr), and iodine (I), in food crops is still very limited. To our knowledge only two studies specified CO_2 responses to Se and Cr contents (not significantly lowered by eCO_2) in wheat (Högy et al., 2009, Högy et al., 2013), and none report data pertinent on I content. Given that a billion people are I-deficient and I is the primary reason of preventable brain damage, cretinism, and lower IQ in children (Loladze, 2014), so studies focusing on the impact of climate change on I content in food crops would be important. Furthermore, eCO_2 may increase mycorrhizal colonization and protect plants against some stresses, having led to improved P nutrition, particularly on legumes (Jakobsen et al., 2016) and increased soil organic carbon decomposition (Cheng et al., 2012) facilitating the availability of some nutrients. In sum, a clear understanding of the nutrient-related processes that are impacted by climate change will increase our ability to predict responses for diverse crops and could benefit farmers in agronomic management to adapt crops to higher CO_2.

One important final consideration under eCO_2 is the overall effect of lower mineral concentrations while in promoting higher yields. For some situations it has been suggested that regardless of the decrease in grain nutrient concentrations at eCO_2, overall grain content of Fe, Zn, Mn, B, Cu, Ca, N, and other macronutrients on a land area basis was actually enhanced, due to grain yield increase at eCO_2 conditions (Fernando et al., 2012b, Asif et al., 2017a). However, a trade-off effect must be considered because per serving size, the actual amount of minerals provided will still be lower, albeit the higher overall grain yields, which may not be sustainable in the long run due to CO_2 acclimatization effects and faster depletion of mineral nutrients from the soil.

1.6. Impact of eCO_2 on protein concentration

Elevated CO_2 has generally been shown to decrease the concentration of protein in grains of many crops species (Myers et al., 2017, Myers et al., 2014, Dong et al., 2018b, Högy and Fangmeier, 2008, Medek et al., 2017). Consequently, the expected decrease in grain protein concentration associated with eCO_2 will be an important negative factor to be considered in human nutrition (Toreti et al., 2019). Consequently, millions of people may face protein deficiency since a great part of worldwide population depends on plant proteins (Medek et al., 2017). Medek et al. (2017) confirmed that when grown under eCO_2 conditions expected by the middle of this century (500-700 ppm), a lower protein concentration was found in C3 grains (wheat, rice and barley with -7.8, -7.6, and -14.1%, respectively), potato (-6.4%), vegetables (-17.3%), and fruit (-23.0%). The eCO_2 was also responsible for a slight decrease in protein in legume species (-3.5%), and no significant effects were found in oil crops and C4 plants. Accordingly, they anticipated a decrease in protein intake under eCO_2 conditions by >5% in 18 countries predominantly throughout Middle East and India. Moreover, it was highlighted that almost 12% of the world's population is currently at risk of protein deficiency. In the case of constant atmospheric CO_2, they predict that globally, 15% (1.4 billion people) of world population would be at risk of protein deficiency by 2050 due mainly to demographic changes. However, with projections of CO_2 levels above 500 ppm by 2050, it was expected that an additional 148.4 million people will be at risk of protein deficiency compared to the 2050 aCO_2 scenarios.

1.7. The effects of other climate change factors on nutrient accumulation

A permanent state of equilibrium in nutrient concentration is a decisive regulatory factor in maintaining nutritional quality and determining the ability of plants to withstand the impact of climate changes (Nakandalage and Seneweera, 2018). Several studies have shown that climatic changes may disturb the nutritional content of major crops as demonstrated in Table 1.1. Therefore it is important to look at those studies dealing with the nutritional impact of eCO_2 alone (Kumagai et al., 2015, Bunce, 2015, Dietterich et al., 2015, Myers et al., 2014, Jin et al., 2015) or combined with water scarcity (Wu et al., 2004), soil mineral deficiency (Haase et al., 2008, Jin et al., 2009, Asif et al., 2018, Jin et al., 2015, Jin et al., 2014), elevated temperature (eT) (Fernando et al., 2014, Chaturvedi et al., 2017, Bellaloui et al., 2016), or salt stress (Petropoulos et al., 2017, Scagel et al., 2019, Petretto et al., 2019, Chrysargyris et al., 2019). The effect of drought on nutrient accumulation was studied by Fischer et al. (2019) comparing food crops in two different regions of East Africa. Severe drought caused a decrease in nutrients, whereas mild drought caused an increase in nutrient concentrations. This shows that the effects on nutrient accumulation are very depended not only on the type of climatic change but also on its intensity level. Water stress also resulted in significant changes in mineral concentrations of legumes (Hummel et al., 2018, Wijewardana et al., 2019) and vegetables (Sarker and Oba, 2018). In another study, limiting the irrigation in a glasshouse experiment with grapevine cultivars did not result in significant differences in leaf micronutrient concentrations. However, since drought was accompanied by Zn deficiency, authors investigated the effects of Zn pulverization and found that it was effective in providing increases in macronutrients, Zn, Fe and Mn in leaf blades (Sabir and Sari, 2019). Da Ge et al. (2010) reported a field study in order to assess the nutritional quality in maize grains at different soil moisture levels. Severe drought increased N, Ca, Mg, Cu and Zn accumulation by 12%, 28%, 11%, 18%, and 33%, respectively, when compared to control. However, significant decreases in P and K concentration by 17% in both minerals were observed at severe drought. This suggests that the effects of drought on mineral concentration are nutrient specific. The effects of combined water and heat stresses were studied by Velu et al. (2016) in 54 field-grown wheat varieties. Grain Zn concentration was higher under heat and drought stresses, whereas a lower increase of grain Fe was observed in water stress environments. The interaction of drought stress with eCO_2 on wheat was reported by Wu et al. (2004), and the authors found that CO_2 enrichment alleviated the negative effects of drought stress, increasing water-use efficiency. However, grain quality was lowered under eCO_2 as reflected by consistent decreases in mineral nutrients (N, P, K and Zn). Parvin et al. (2019) observed a reduction in Fe, Zn, P, and S concentrations in faba bean and lentil facing drought and eCO_2 conditions. Asif et al. (2017a) reported interaction of eCO_2, drought and soil Zn availability in wheat. It was found that eCO_2 combined with low water and/or Zn availability reduced grain Zn and protein. Asif et al. (2018) found that eCO_2 partially improved the detrimental effect of soil K deficiency on wheat grain yield. Climate changes are also characterized by waterlogging complications due to natural factors or by human activities such as excessive irrigation and low drainage (Smethurst et al., 2005), leading to a decreased O_2 availability in the soil with possible accumulation of phytotoxins, leaf chlorosis, stomatal closure (Wei et al., 2018) and restricted crop performance by decreasing soil mineral nutrient accessibility (Ashraf, 2012).

Table 1.1 - Effects of climate changes on crop nutritional quality

Treatments	Crops	Experimental method	Micronutrients	Protein	Macronutrients	References
D	Maize	Field trial	↑ Cu, Zn		↑ N, Ca, Mg; and ↓ P, K	Da Ge et al. (2010)
D	Maize,	Field trial	= Cu, Mn; and ↑ Fe, Zn		↓ S; = K; and ↑ Ca, Mg, P	Fischer et al. (2019)
	Cassava	Field trial	↓ Mn; = Fe, Cu; and ↑ Zn		= K; and ↑ Mg, P, S, Ca	
	Maize	Field trial	↓ Fe, Mn, Cu; and = Zn		= Mg, P, K, S, Ca	
	Musa acuminata	Field trial	↓ Fe, Mn; = Cu; and ↑ Zn		↓ Mg, P, K, S, Ca	
D	Gravepine	Glasshouse	= Fe, Zn, Mn, Cu, B (leaves)		= P, K, Ca, Mg (leaves)	Sabir and Sari (2019)
D	Common bean	Field trial	↓ Fe; and ↑ Zn	↑		Hummel et al. (2018)
D	Soybean	GC	↑ Fe, Zn, Cu, B		↓N, P, K, Ca; and ↑Mg	Wijewardana et al. (2019)
D	*Amaranthus tricolor*	Field trial	↑ Mn, Cu, Mo, B; and ↓ Fe, Zn (leaves)		↑ Ca, S, Mg, K Na; and ↓ P (leaves)	Sarker and Oba (2018)
eCO_2	Wheat, field peas, and canola	FACE	↓ Zn = Mn, Fe, and Cu	↓	↓ P = K, and Mg	Jin et al. (2019)
eCO_2	Wheat	Meta-analysis	↓ Cd, Cu, Fe, Mn, Zn; and = B	↓	↓ Ca, Mg, P, S; = Na; and ↑ K	Broberg et al. (2017)

eCO_2	Cucumber	OTC	↓ Fe, Zn (low N); and = Cu, Fe, Mn, Zn (moderate and high N)		↑ Ca, K, P, Mg (low N); and = Ca, K, P, Mg (moderate and high N)	Dong et al. (2018a)
eCO_2	C3 plants	Meta-analysis	↓ Fe, Zn, Mn, and Cu	↓	↓ K, P, Ca, and Mg	Loladze (2014)
			= Mn			
eCO_2	Wheat	Meta-analysis	↓ Fe, Zn	↓		Myers et al. (2014)
	Rice		↓ Fe, Zn, Mn, Cu; = Mn; and ↑ B	↓	↓ S; and = Ca, K, P	
	Peas		↓ Zn, Fe, Mn, Cu, S; and =B	↓	↓ P; = Mg, Ca; and ↑ K	
	Soybean		↓ Fe, Zn, Cu, B; and = Mn	=	↓ Mg, Ca, S; and = K, P	
	Maize		↓ Fe		↓ Mg, K, P	
			= Zn, Mn, Cu, B	=	= Ca, S	
	Sorghum		= Fe, Zn, Mn, Cu, B	=	= Mg, Ca, S, K, P	
eCO_2	Soybean	OTC	↓ Fe, Zn, Mn; and = Cu		↑ Mg, Ca, S; and = P	Li et al. (2018b)
eCO_2	Vegetables	Meta-analysis	↓ Fe, Zn; and = Cu, Mn	↓	↓ Mg; = P, K, S; and ↑ Ca	Dong et al. (2018b)
eCO_2	Wheat	FACE	↓ Fe, Cd; = Zn, Se, Cu, Mn, Cr, Ni, Mo, Si; and ↑ Pb	↓	↑ K; and = Na, Ca, P, S, Mg	Högy et al. (2009)
eCO_2	Chickpea	OTC	↓ Zn; and = B, Cu, Fe, Mn	↓	↓ K, Ca; and = P, Mg	Saha et al. (2015)
S	*Amaranthus tricolor*	Pot experiment	↑ Fe, Mn, Cu, Zn (leaves)		↑ Ca, Mg, Na; and ↓ K (leaves)	Sarker et al. (2018)

S	Basil	GH	↓ B, Cu, S; and = Fe, Mn, Zn (leaves)	↓ P; ↑Na, K; and = Ca (leaves)	Scagel et al. (2019)
S	Lettuce	GH	↑Mn, Zn, Cu, Fe (leaves)	↓ K, Ca; and = N, P, Mg (leaves)	Neocleous et al. (2014)
S	Rocket	GH		↓ Ca, K, Mg (leaves)	Petretto et al. (2019)
S	Spearmint	GH	↓ Zn; and = Cu (shoot and leaves) ↑Cu, Zn (roots)	↑Na, P; ↓ N, Mg, K (shoot and leaves) ↑Na; ↓ P, Mg, K; and =N (roots)	Chrysargyris et al. (2019)
S	*Cichorium spinosum*	GH	↑Fe, Mn, Zn (leaves)	↓ K; and↑Na, Ca, Mg (leaves)	Petropoulos et al. (2017)
T	Lettuce	GH	↓ Mn, Mo; and = Fe, B, Zn (leaves)	↓ Mg, K, Ca (leaves)	Sublett et al. (2018)
W	*Medicago sativa*	GH	↓ Zn, B, Cu in leaf and root; and ↑ Fe in root	↓N, P, K, Ca in leaf and root; and ↑ Na in leaf	Smethurst et al. (2005)
W	Barley and wheat	Pot experiment	↓ Zn, Cu, Mn; and = Fe in shoots	↓ N, P, K, Mg; and = Ca in shoots	Steffens et al. (2005)
W	Tomato	Pot experiment	↓	↓K; ↑ Na; and = Ca (fruit)	Rasheed et al. (2018)
W	Wheat	GH	↓ Zn, Cu	↓ P, K	Tarekegne et al. (2000)
D or T	Wheat	Field trial	↑ Zn; and = Fe ↑		Velu et al. (2016)
D and S	Maize	GH	= most ions at D and S (leaves)	= most ions at D and S (leaves)	Hu et al. (2007)
eCO_2 x D	Wheat	GC	↓ Zn	↓ N, P, and K	Wu et al. (2004)

eCO_2 x D	Faba bean and lentil	FACE	↓ Fe, Zn		↓ P, S	Parvin et al. (2019)
eCO_2 x D x Zn	Wheat	GC	↓ Zn	↓		Asif et al. (2017a)
eCO_2 x S	Green leaf lettuce	GC	↓ Fe; = Zn; and ↑ Cu, B, Mn		↓ N, Na, K, Ca, Mg, P	Pérez-López et al. (2015)
	Red leaf lettuce		↑ Zn; and = Fe, Cu, B, Mn		↓ Na; and = N, K, Ca, Mg, P	
eCO_2 x T x AM	Wheat	GH			↑ N, K, Ca, Mg, Na (shoot)	Zhu et al. (2018b)
eCO_2 x T	Wheat	FACE	↑ Fe, Zn	↓	↑ Ca, S	Fernando et al. (2012a)
eCO_2 x T	Soybean	T-FACE	↑ Fe, Zn			Köhler et al. (2018)
eCO_2 x T	Wheat	FACE	↓ Fe, Zn, Cu	↓	↓ P, Ca, Mg, S; and = K	Fernando et al. (2014)
eCO_2 x T	Rice	OTC	↓ Fe, Zn, Mn, Cu	↓	↓ Ca, Mg	Chaturvedi et al. (2017)
eCO_2 x T	Soybean	GH, and GC	↓ Fe, Zn, B, Cu, Mn	↓	↓ N, P, K; Mg	Bellaloui et al. (2016)
eCO_2 x T	Soybean	OTC	↓ Mn; ↑ Fe; and = Zn	=	↑ P, K; and = Ca	Qiao et al. (2019)
	Maize		↑ Fe, Mn; and = Zn	=	↑ K; and = P, Ca	

↑ increase, ＝no significant change, ↓ decrease; D, drought; T, temperature; S, salinity, W, waterlogging; OTC, Open top chamber, GH, Green house, GC, Growth chamber; AM, arbuscular mycorrhizal fungi inoculated in wheat plants. The trend of the concentration of nutrients regards to the seeds, otherwise it will be mentioned in the table.

When *Medicago sativa* was exposed to flooding stress, a marked reduction in leaf and root nutrient composition (B, Cu and Zn) was observed. Similar findings were obtained in barley and wheat shoots indicating that Cu, Zn, and Mn concentrations decreased significantly. The concentration of Fe was not affected (Steffens et al., 2005). Tarekegne et al. (2000) demonstrated that waterlogging-sensitive wheat genotypes appeared to accumulate less Cu and Zn. Conversely, there were higher concentrations of Fe in waterlogged roots of stressed plants (Smethurst et al., 2005). To the best of our knowledge, there are no studies yet that have looked at the combined effect of waterlogging and eCO_2 and their influence on plant nutritional quality, two conditions that are likely to interact in the future.

Sublett et al. (2018) studied the consequence of eT (+8°C) on lettuce in a greenhouse experiment, and found a decrease in leaf Mg, K, Ca, Mn, and Mo concentrations. When looking at temperature and eCO_2, it was shown that the combination of both factors may restore soybean (Köhler et al., 2018) and wheat (Asif et al., 2019) seed Fe and Zn concentrations to levels obtained under ambient CO_2 (aCO_2). However, since there is a strong species and cultivar dependency on these responses, care must be taken to look at these aspects in detail. In a field study, Fernando et al. (2014) observed a cultivar-based response of wheat to eCO_2 with high temperature. In addition, a decreasing trend in phytate concentration was also detected. Perceived genetic variability in terms of grain minerals could be easily combined into future wheat breeding programs to enable adaptation to climate changes.

In a FACE experiment, Fernando et al. (2012a) grew wheat at aCO_2 and eCO_2 in combination with two different sowing dates to mimic high temperature during grain filling. Grain mineral (Ca, S, Fe and Zn) concentrations were lower under eCO_2 conditions. Most of the grain mineral concentrations were significantly increased at late sowing date, suggesting that eT may counterbalance some of the negative effects of eCO_2 on grain mineral concentration. Contrasting findings were obtained in rice (Chaturvedi et al., 2017) and soybean (Bellaloui et al., 2016). Qiao et al. (2019) also investigated the effects of eT in combination with eCO_2 on grain quality of soybean and maize grown in open-top chambers in a Mollisol during five growing seasons. Elevated temperature with eCO_2 increases K, and Fe, whereas Ca and Zn concentrations were not statistically affected in both species. In addition, P and Mn concentrations were species dependent. The metabolic responses to eT (+10ºC) under eCO_2 were studied in tall fescue, a cool-season grass species. Plants showed a significant increase in the quantity of several organic acids, amino acids, and carbohydrates involved in photosynthesis, respiration, and protein metabolism. Consequently, it was determined that eCO_2 could play a role in mitigation of heat stress damage (Yu et al., 2012). In rice, eCO_2 at ambient temperature increased plant growth and led to increased seed yield. However, increasing temperature to 35°C or higher, exceeded the beneficial effects of eCO_2 (Madan et al., 2012). The combination of eCO_2 with eT has a variable effect on growth and photosynthesis, and is dependent on the range of temperature increase, but in general eCO_2 reduced the negative impact of eT (Köhler et al., 2018, Qiao et al., 2019, Yu et al., 2012). This was confirmed in soybean and maize over five-year growing seasons in open top chambers(Qiao et al., 2019).

Soil salinity is a global problem for agricultural production, since almost 20% percent of the total arable land is deteriorated due to high salinity (Scagel et al., 2019, Neocleous et al., 2014). There are several studies assessing the effects of salinity on mineral concentration and nutritional quality on crops plants. These studies have been conducted mainly in vegetables (Neocleous et al., 2014, Scagel et al., 2019, Petretto et al., 2019, Chrysargyris et al., 2019, Petropoulos et al., 2017), with contrasting responses among different species (Table 1.1). Pérez-López et al. (2015) studied the effect of salt stress combined with eCO_2 on the nutritional quality of two differently pigmented lettuce cultivars. The red cultivar was the best adapted to eCO_2 because it better adjusted mineral uptake. They concluded that eCO_2 alone or in combination with short environmental salt stress allows increasing the concentration of some minerals.

Hu et al. (2007) induced short-term events of drought and salinity stress in maize and found that both stresses frequently originate lower nutrient accessibility in soil and low nutrient translocation in plants. However, reduction in the micronutrient concentrations in the grain was not detected.

It is now clear that one of the main nutrient groups being impacted by climate change are minerals, and one of the biggest challenges of today's agricultural sector is to increase crop productivity and maintain nutritional quality of grains in a sustainable way, despite the influences of climate change.

1.8. Plant molecular and physiological responses to climate change factors

Plant responses underlying nutritional losses due to climate change involve complex biological processes that include several physiological and metabolic mechanisms. How plants respond to these changes has been the subject of several studies (Nakandalage and Seneweera, 2018, Hashiguchi et al., 2010, Ahuja et al., 2010, Feng et al., 2014, Dong et al., 2018b, Nakashima et al., 2009, Saeed et al., 2012, Hummel et al., 2018, Hatfield and Walthall, 2014, Newton et al., 2011). In this section, we will discuss current understanding of how plants respond to environmental stresses at the molecular and physiological level.

Vicente et al. (2018) reported the impacts of eCO_2, temperature and N supply on the regulation of C and N metabolism in durum wheat. They found a coordination between C and N metabolisms at biochemical and transcriptional levels. Genes from N uptake and assimilation were co-expressed with genes belonging to the respiratory pathway, highlighting the coordination between the synthesis of organic N compounds and C metabolism. Moreover, included in this coordination were Rubisco and nitrate reductase activities. The combination of eT with eCO_2 in soybean seed composition and transcript levels was also studied. The impact of temperature on seed composition and transcripts level was pronounced, particularly on Gm8, similar to ADR12, and on Gm19, similar to β-glucosidase, but there was no effect of CO_2 concentration (Thomas et al., 2003). Additionally, a network analysis of relationships between biochemical parameters of soybean grains showed that interaction of eCO_2 with eT significantly affect carbohydrate and lipid metabolisms (Palacios et al., 2019).

However, as previously shown, in some cases, a positive interaction of eCO_2 and eT may also occur (Vu and Allen, 2009, Pérez-Jiménez et al., 2019) particularly in biomass accumulation. Parameters such as leaf area, leaf dry weight and stem dry weight of sugarcane was increased under eCO_2 or eT (4.5°C). Such changes were even greater under the combined treatment of eCO_2 and eT (Vu and Allen, 2009).

As previously mentioned, the effects of CO_2 enrichment on plants depend on soil water availability, and plants can greatly benefit from eCO_2 in terms of biomass accumulation when enough water is provided (Wu et al., 2004, Zhao et al., 2006). Most studies confirm that CO_2 enrichment tends to mitigate drought negative effects (Asif et al., 2017a, Bencke-Malato et al., 2019, Sicher and Barnaby, 2012, Li et al., 2018a, Wang et al., 2018a, Yuhui et al., 2017) by improving plant water relations, reducing stomatal opening and transpiration, increasing photosynthesis, shortening crop growth period and increasing the antioxidant activity (Kumar et al., 2019). In rice, both treatments have no interactive effects on maximal quantum yield of PSII photochemistry, intrinsic efficiency of PSII and non-photochemical quenching. However, in soybean eCO_2 reduced the negative effects of drought on effective quantum yield of PSII photochemistry and photochemical quenching coefficient (Wang et al., 2018a), showing that these responses are very species specific.

Drought and temperature induce oxidative damage in legumes and grasses. These effects may be mitigated by eCO_2, more extensively in legumes, due to lowered photorespiration and reduction of NADPH oxidase activity. The increased antioxidant activity (flavonoids and tocopherols) possibly also contributes to the stress mitigation effect of CO_2 enrichment (Abdelgawad et al., 2015). In another study looking at drought and eCO_2, in maize, transcript levels of 14 genes encoding stress responsive proteins were monitored. All the transcripts were induced by drought except for rbcS1, but this response was delayed by CO_2 enrichment. Accordingly, eCO_2 had a larger impact on maize responses to drought at the beginning rather than at the end stages of water stress (Sicher and Barnaby, 2012), showing that the importance of the developmental stage on understanding these responses.

Li et al. (2018a) used a metabolomics approach to search for metabolites that were affected by eCO_2 under drought stress in cucumber leaves. The results showed that under severe drought, eCO_2 changed several metabolic pathways related to the metabolism of several amino acids and carbohydrate synthesis. In eCO_2 plants accumulated more amino acids and carbohydrates, 1,2,3-trihydroxybenzene, pyrocatechol, glutamate, and L-gulonolactone, for better tolerating drought stress. The improved root growth and mitigation of drought stress under eCO_2 was also described by Burgess and Huang (2014). This could be associated with alteration in proteins involving nitrogen metabolism (glutamine synthetase), energy metabolism involving respiration (glyceraldehyde-3-phosphate dehydrogenase), and stress defense by increasing antioxidant metabolism (ascorbate peroxidase, superoxide dismutase, and catalase) and chaperone protection (HSP81-1).

Elevated CO_2 and salinity, individually, seem to affect plant growth in opposite directions (Shahbaz and Ashraf, 2013, Zaghdoud et al., 2016). Salt stress, in contrast to eCO_2, is generally considered a negative driver for growth of crop plants, especially in arid and semi-arid zones (Zaghdoud et al., 2016). This was confirmed by Kazemi et al. (2018) in rice who described a significant cultivar-dependent response to eCO_2 under different salinity concentrations. Both treatments, besides influencing secondary metabolism, can disturb the oxidative system while acting in different directions, with salinity provoking, and eCO_2 alleviating, oxidative stress (Sgherri et al., 2017). Similarly, Kanani et al. (2010) investigated the effects of eCO_2 with salinity on the transcriptional and metabolic responses of *Arabidopsis thaliana*. The observed metabolic differences suggest that eCO_2 mitigates the metabolic effect of the salinity stress. However, a strong similarity was observed between the transcriptional responses to salt stress and combination of eCO_2 with salt stress (Kanani et al., 2010). In lettuce, both treatments originated a higher concentration of phenolic compounds, in particular luteolin, and increased plant growth and nutritional quality comparing to salinity alone (Pérez-López et al., 2015). Broccoli treated with 90 mM NaCl and eCO_2, had higher photosynthetic rate and water-use efficiency. These factors led to greater leaf area and biomass as well as to increased abundance of aquaporins in roots and leaves at eCO_2, in comparison with aCO_2 (Zaghdoud et al., 2013). In Bermuda grass, salinity stress induced a reduction in leaf water content, leaf photosynthetic rate, transpiration rate, stomatal conductance, and cellular membrane stability. Elevated CO_2 mitigated the depression of those physiological parameters and promoted osmotic adjustment by accumulation of soluble sugars, proline, and glycine betaine under salinity stress (Yu et al., 2015).

Recently, a transcriptome combined with proteome study discovered key factors involved in alfalfa waterlogging-based responses. The authors identified genes-related to beta-amylase, ethylene response factor, calcineurin B-like interacting protein kinases, glutathione peroxidase, and glutathione-S-transferase with key roles in conferring alfalfa tolerance to waterlogging (Zeng et al., 2019). Elevated CO_2 is also capable of reducing the negative effects of waterlogging as demonstrated in several species (Arenque et al., 2014, Shimono et al., 2012, Lenssen et al., 1995, Pérez-Jiménez et al., 2017, Pérez-Jiménez et al., 2017). Hence, waterlogging affected sweet cherry cultivars severely by reducing photosynthesis, stomatal conductance, transpiration, chlorophyll fluorescence and plant growth (Pérez-Jiménez et al., 2017). Proline accumulation, to cope with oxidative damage, was also observed. Nevertheless, increasing CO_2 concentration not only mitigated all these effects but also induced the production of soluble sugars and starch in the leaf (Pérez-Jiménez et al., 2017). In woody plants, Lawson et al. (2017) found species-specific effects of CO_2 concentration and waterlogging on plant growth, gas exchange, and functional traits, and no evidence for an overall effect of eCO_2 in mediating plant responses to flooding. In pea and soybean, an association among tolerance to hypoxia (induced by flooding), the rate of reactive oxygen species production, and antioxidant enzyme activities were recognized. The environment with the higher CO_2 concentration induced higher changes in the processes of reactive oxygen species accumulation and activities of lipoxygenase and antioxidant enzymes (Ershova et al., 2011).

In fact, the observed nutritional impacts due to climate change may also be linked to alterations in below ground interactions between plants and microorganisms. In flooded soils, rice plants responded more positively to the CO_2 enrichment than the non-flooded soil. The results advocate that in tropical rice soils, eCO_2 amplified C accumulation in the soil, which possibly stimulates growth of N fixing bacteria and thereby higher available N (Das et al., 2011). In wheat, a mutualistic symbiosis with arbuscular mycorrhizal increased carbohydrate and nutrient accumulation in plants exposed to eCO_2 and salinity (Zhu et al., 2018b). Thus, looking at the impact of climate change on below ground traits and linking these with processes of nutrient absorption and accumulation seems like a promising line of research for future studies. However, care must be taken at factoring in issues of low nutrient availability in the soil.

1.9. Effects of eCO_2 under low soil nutrient availability

The availability of nutrients in the soil has a significant influence on the effects of eCO_2 on plant nutritional quality. Usually, a low nutrient availability limits the eCO_2 effect on plant photosynthetic rates, possibly resulting in less carbon available for producing secondary compounds (Dong et al., 2018b).

Under different levels of N supply, rice grain quality was not significantly affected by eCO_2 in a FACE experiment (Yang et al., 2007). In addition, soil mineral bioavailability was enhanced by eCO_2 in rice, both under high and low N supply. It was also demonstrated that eCO_2 can favor the translocation of Ca, Mg, Fe, Zn, and Mn from the soil to the stem, leaf, and panicle but decreased in grains (Guo et al., 2015). However, Haase et al. (2007) observed symptoms of N deficiency in common bean plants grown under eCO_2 conditions. The authors suggest that this is possibly due to improved root exudation and a related stimulation of soil microbial growth causing enhanced plant microbial N competition. Asif et al. (2019) studied the interactive effects of predicted climate (eCO_2 and +3°C) and N and Zn supply on the growth and yield of wheat plants. In both predicted climate and environmental conditions, a low supply of N significantly decreased straw and grain yield by affecting the number of spikes per plant and the number of grains per spike.

The bioavailability of soil P to plants is often restricted due to their strong bonding in insoluble forms. However, plants generally develop potential adaptive mechanisms that enhance P uptake in plants under P starvation (Wissuwa et al., 2005). The photosynthetic inhibition caused by low P supply could be overcome by eCO_2. Furthermore, photosynthetic downregulation at eCO_2 could be reversed by increasing P supply. This might be due to the availability of sufficient P to support increased protein degradation and metabolic rates at eCO_2, enhancement in Rubisco activation, ribulose-1,5 bisphosphate regeneration and global energy demands (Pandey et al., 2015). In addition, in cotton plants grown under P stress, an apparent limitation of CO_2 diffusion across stomata and mesophyll was observed (Singh et al., 2013, Singh and Reddy, 2014). Under limited P supply, eCO_2 increased acid phosphatase activity, responsible for hydrolyzing insoluble P in the soil (Barrett et al., 1998, Niu et al., 2012). However, there are some studies reporting no increase in root phosphatase activity in response to P shortage caused by eCO_2 (Norisada et al., 2006, Wasaki et al., 2003). Elevated CO_2 with low P supply also results in increased root dry matter. Changes in the processes at the soil level including root morphology, exudation and mycorrhizal association are also influenced by eCO_2 and P bioavailability (Pandey et al., 2015, Pang et al., 2018, Watts-Williams et al., 2019).

Potassium is an important plant nutrient with a significant role in key physiological processes as described by Cakmak (2005). Yilmaz et al. (2017a) determined growth performance and antioxidant response in wheat plants grown at different K levels under different CO_2 conditions. In low and deficient K plants, biomass was either not affected or even decreased by eCO_2. Additionally, the limitation of K induced oxidative stress, and eCO_2 had no significant impact on the antioxidant system. Similarly, K deficiency clearly limited the effects of eCO_2-induced biomass enhancement in both well-watered and drought-stressed plants (Asif et al., 2017b). Under severe K limitation, several photosynthesis-related parameters were downregulated in common bean. However, eCO_2 also stimulated carbon assimilation and K utilization efficiency when K deficiency was not severe (Singh and Reddy, 2018).

Magnesium has a determinant role in biosynthesis of proteins, nucleic acids, ATP and chlorophyll by activating several enzymatic reactions involving carboxylases, polymerases, kinases, and phosphatases (Cakmak and Kirkby, 2008). To our knowledge, there is only one published study dealing with interaction of eCO_2 with low and adequate Mg treatments on durum wheat growth and nutrient composition. Low Mg plants responded to eCO_2 by decreasing biomass, particularly in roots and eCO_2 increased photosynthesis in adequate-Mg plants, but not in low Mg plants. Leaf carbohydrate concentration was increased 2-fold by low Mg at aCO_2 and 3-fold at eCO_2 (Yilmaz et al., 2017b) suggesting that low Mg and eCO_2 decreased carbohydrate transport from source to sink tissues.

In plants, Zn is associated with the activity of several enzymes, it has a structural role in cell metabolism and is implicated in DNA replication and transcription as an intrinsic component of Zn metalloproteins. Zinc deficiency is related to the suspension of photosynthetic activity since this mineral is required for the activity of carbonic anhydrase and because the availability of substrate for carboxylation is limited under Zn limitation (Broadley et al., 2012). Asif et al. (2017a) noticed that eCO_2 increased wheat grain yield, number of spikes per plant, and straw yield under sufficient and low Zn conditions. Furthermore, Zn efficiency (yielding ability under low Zn supply) was positively affected by eCO_2 which also reduced grain Zn concentration.

Iron metabolism in crop plants as well as its influence on productivity are already well referenced in the literature (Briat et al., 2015, Kobayashi and Nishizawa, 2012, İNCESU et al., 2015, López-Millán et al., 2009, Zocchi et al., 2007, Li et al., 2008a). Therefore, similar to eCO_2 it is very likely that restricted Fe availability will impact on the nutritional quality of foods, which we will consume in the future (Vasconcelos et al., 2014, Vasconcelos et al., 2017). So far, researchers have been assessing these issues independently and studies linking these two important aspects are scarce.

Expanding this knowledge to Fe is of particular interest because of its major role in the photosynthetic process and because its bioavailability to plants is often limited, particularly in calcareous soils, which represent 30% of cultivated soils (Robin et al., 2008, Briat et al., 2015). Several papers described organic acid concentration increases, mainly citrate and malate, in xylem sap, leaf apoplastic fluid and whole leaves of plants with Fe deficiency (Larbi et al., 2010, López-Millán et al., 2000, López-Millán et al., 2009, Abadía et al., 2002). Further changes include shifts in the redox state of the cytoplasm and increases in the activity of phosphoenolpyruvate carboxylase and several enzymes of the Krebs cycle and the glycolytic pathway. For a detailed description of the metabolic changes induced in Fe-stressed plants, see Zocchi (2006) and Zocchi et al. (2007). Transcriptomic (Thimm et al., 2001), proteomic and metabolomic studies

(Brumbarova et al., 2008, Li et al., 2008a, Rodríguez-Celma et al., 2016, Rellán-Álvarez et al., 2010) in Fe-deficient plants have also reported increases in root transcript and protein abundances, respectively, of enzymes-related to the glycolysis, Krebs cycle, anaerobic respiration, stress-related and metabolism-related proteins, among others. In barley, CO_2 enrichment increased biomass production in Fe-deficient and Fe-sufficient plants, both in hydroponics and soil experiments. Higher Fe accumulation in shoots (+52%) of barley grown in soil without Fe supply under eCO_2 conditions was achieved, demonstrating an improved Fe-use efficiency (Haase et al., 2008). Similar findings were obtained in tomato (Jin et al., 2009). Plant biomass and root-to-shoot ratio were greater under eCO_2 conditions than in plants grown in aCO_2. Root and shoot Fe concentration significantly increased in Fe-deficient plants under eCO_2 attenuating the symptoms of chlorosis, and were not changed in the Fe-sufficient plants. The authors also suggested some involvement of nitric oxide in enhancing Fe deficiency responses (increased ferric chelate reductase activity, and expression of FRO1, IRT1, and FER genes in roots) when Fe limitation and eCO_2 occurred together. The nutrient supply and, accordingly, the nutrient status of plants should be a critical issue-defining growth response to the eCO_2. It was observed that eCO_2 treatments significantly increased the Fe concentrations in tomato leaves and alleviated the Fe deficiency-induced chlorosis when grown in a Fe-limited medium (Jin et al., 2009).

The data concerning the interaction of eCO_2, and Fe deficiency are very scarce in the literature despite the importance of this micronutrient in plant metabolism. Thus, a better understanding of the interaction of these two factors and how they affect metabolic pathways in plants is required.

1.10. Strategies to preserve the nutritional content in future climates

As described in the previous sections, the combination of different climate change factors will impact precipitation patterns, plant physiology and the functioning of the ecosystem, ultimately resulting in environmental constraints which limit nutrient uptake and accumulation. Several practices are under way to mitigate and/or adapt to climate change consequences (Figure 1.2), but the high variability in research regarding their real environmental impact impair the definition of a strategy to be successfully implemented in supply chains (Parajuli et al., 2018).

The practices associated to agricultural production are a main target for climate change mitigation. Agricultural planning and farmers' sensitization might contribute not only for climate change mitigation, but also to climate change adaptation. For example, one strategy frequently mentioned in adaptation frameworks is the relocation and protection of farms, namely by moving crop production to promote food security from extreme weather events (Prior et al., 2018).

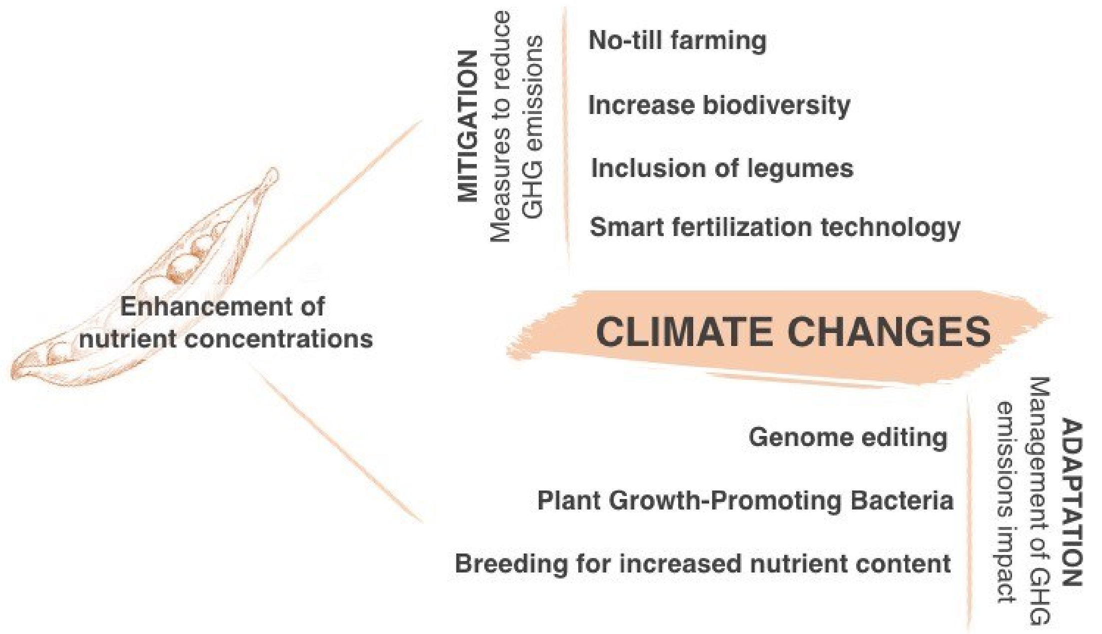

Figure 1.2 - Strategies for mitigation and adaptation to climate changes (Soares et al., 2019b).

With climate change, soil quality declines, mostly because soil microbial communities are deeply affected, negatively impacting the degradation of organic pollutants (Ai et al., 2018) and soil organic matter (Chen et al., 2016b) or nitrogen fixation (Lobo et al., 2018). In order to prevent nutritional losses of crops farmers must work towards yield optimization and smart fertilization decisions. Currently, fertilization decision support systems can be accessed via online platforms. These are based on algorithms that include experimental data on soil nutrition and crop nutritional requirements and provide farmers fertilizer recommendations and professional fertilization information specific to their farming conditions (Elia and Conversa, 2015, He et al., 2011). This allows utilizing a controlled amount of fertilizer, achieving optimum yields and increasing nutrient-use efficiency. Nowadays, the development of technologies for non-invasive nutritional estimation in plants is growing. With these, crop monitoring and diagnosis are improved and targeted, once again facilitating the optimum fertilizer application for desirable production outcomes (Zheng et al., 2018). Examples of these techniques include hyperspectral imaging, successfully utilized in discriminating N nutritional levels in tea plants (Wang et al., 2018b), as well as N, P, K, S, Cu, Zn, Fe and Mn levels in maize and soybean plants (Pandey et al., 2017); unmanned aerial vehicle based multispectral imagery applied in the estimation of plant nitrogen concentration and management of N fertilizer application in rice (Zheng et al., 2018) and in wheat (Zhu et al., 2018a); and reflectance spectroscopy through which authors were able to characterize Fe deficiency symptoms in grapevine and prospect the possibility of detecting in field Fe deficiency conditions (Rustioni et al., 2017).

Additionally, sustainable alternatives to synthetic fertilizers which may sustain plant nutrition in a changing climate include plant growth-promoting bacteria (PGPB) inoculants, which can be used as biological fertilizers (Olanrewaju et al., 2017). These are associated with many mechanisms that improve not only plants' health, but also soil conditions, such as, phosphate solubilisation, nitrogen fixation, siderophore and phytohormone production, ethylene regulation and biological control (Lobo et al., 2018). For example, the inoculation of *Pseudomonas fluorescens* in a grass species was able to aid in the decomposition of the increased plant C inputs associated with eCO_2, while promoting plant productivity (Nie et al., 2015). In another study, the best performing PGPB under drought stress conditions was selected from field-grown sorghum (Silva et al., 2018). Also, under drought stress conditions, the application of N fixing bacteria reduced the requirement of chemical fertilizer and enhanced macro and micronutrient concentrations in *Medicago scutellata* (Shabani et al., 2015). Even in poor soil strata, a mixture of five PGPB was found to have a significant impact on nutrient availability, alongside with the capacity to rehabilitate the soil (Radhapriya et al., 2018). With this strategy, it is possible to perform a pre-selection of the PGPB to optimize plant growth for each specific field condition (Silva et al., 2018). This might be optimal for small farming conditions, but in a wider scale, challenges still reside regarding stability and economic feasibility (Lobo et al., 2018).

Another efficient strategy for climate change mitigation is the inclusion of legumes in farming systems, since it allows to naturally reduce the amount of inorganic N fertilizer, reduce CO_2 emissions, amend soil physical properties, maintain soil fertility and decrease pest susceptibility, as recently reviewed by Karkanis et al. (2018). A predictive model that included climate data from the last 80 years demonstrated that the inclusion of a legume in a crop rotation system would decrease 25% of the greenhouse gas emission (Ma et al., 2018). Besides decreasing denitrification, the inclusion of legumes in intercropping systems has also contributed to improve P-fertilizer-use efficiency and led to increased plant availability of P, Fe and Zn (Xue et al., 2016). Although the use of legumes or legume-residues as cover crops (green manure) might be associated with some disadvantages, such as, lack of persistence or excess N supply in high vigor crops, they have much lower environmental impact than non-leguminous crops, lower energy demand per unit area and lower global warming potential (Tani et al., 2017). This strategy is key in the modern paradigm of 'sustainable intensification' (Mungai et al., 2016, Pretty et al., 2018), where we find it urgent to shift agriculture practices to be more sustainable and efficient, while also meeting rising human needs.

Amongst the most common promising sustainable agricultural practices, reduced or no-till farming decreases decomposition rates of organic matter and enhances recycling of nutrients, soil structure and water infiltration (Barão et al., 2019). Although this technique was used in ancient agriculture, during Europe's agricultural revolution, tilling was largely adopted, which in the long run, resulted in soils which are eroded, nutritionally poor and deprived of microbial activity (Gomiero, 2016, Lal et al., 2007). Lately, the benefits of no-till farming have been discussed in the context of sustainable agriculture, however, due to its fall into disuse, information on the implementation and economics of this strategy is still scarce in certain agricultural areas, which prevents its successful and widespread implementation (Bavorova et al., 2018). Nowadays, technologies for no-till farming are more effective and require less efforts from the farmers (Rafiq et al., 2017). Evidence has shown that these practices also reduce methane emission (Zhao et al., 2016), contribute to the reduction in dissolved-P loss when combined with other land management strategies (Daryanto et al., 2017) and influence the distribution of pesticides between the soil solution and the solid phase (Elias et al., 2018).

While limited research focus has been put on understanding the regulatory mechanisms of differential gene expression under climate change conditions, newest genetic technologies will most certainly impulse sustainable practices in agriculture. However, the limiting conditions to transfer strategies from the research field to crop improvement impair further advances (Kromdijk and Long, 2016). Historically, the major focus of plant breeders has been on yield and resistance, but as plants' nutrient content decrease is progressively evident, reversing this problem should also be a priority. Hence, some approaches are under development and are based, for example, on the identification of QTLs for nutrient efficiency under eCO_2 and warmer temperatures (Pilbeam, 2015) or on the study of differentially expressed genes in response to eCO_2 (Zhang et al., 2018a). With this information, predictive models can be designed and selection for increased food quality can be made (Dwivedi et al., 2018). This can be achieved with the application of gene editing tools (Haque et al., 2018) such as the CRISPR/Cas9 system, which has been successful in developing rice lines with improved seed longevity, high amylase content and resistant starch (Mishra et al., 2018).

1.11. Concluding Remarks

Several studies have been conducted in recent decades on the effects of climate change on plant productivity and yield parameters. However, there is a lack of knowledge dedicated to the nutritional dynamics, particularly, on micronutrient-use efficiency under climate changes, which impacts crop nutrient uptake, transport, and remobilization. Rising CO_2 has been shown to affect the nutritional value of not only cereals and legume crops, but also fruits and leafy vegetables, but few studies point out that this is not a generalizable phenomenon. Nowadays, we are facing a compromise between the necessities for higher yields and the prevention of food quality loss. Knowing that nutrient deficiencies are one of the major causes of quality and production losses around the world, understanding the interaction of these stresses with eCO_2 is of paramount importance. It seems clear that we can no longer underestimate the effect of eCO_2 on food mineral composition and that the likelihood of growing less nutritional food in the future is a real threat both to agriculture and human health.

In this way, a better understanding of the genetic mechanisms, and of the physiological and molecular processes determining mineral nutrient absorption is essential and will help to improve the nutritional performance of grains subject to climate change. In addition, germplasm screening to identify varieties that have a better use of nutrients will provide the tools for biofortification strategies. In summary, this doctoral thesis aimed to bring new insights into the topic of projected climate change, concentrating on the response mechanisms of bean and soybean genotypes after exposure to CO_2 enrichment and Fe-limitation.

PART II - Growth of bean and soybean genotypes under eCO_2 conditions

CHAPTER 2 - Growth and nutritional responses of bean and soybean genotypes to elevated CO_2 in a controlled environment

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2.1. Abstract

In the current situation of a constant increase in the atmospheric CO_2 concentration, there is a potential risk of decreased nutritional value and food crop quality. Therefore, selecting strong-responsive varieties to elevated CO_2 (eCO_2) conditions in terms of yield and nutritional quality is an important decision for improving crop productivity under future CO_2 conditions. Using bean and soybean varieties of contrasting responses to eCO_2 and different origins, we assessed the effects of eCO_2 (800 ppm) in a controlled environment on the yield performance and the concentration of protein, fat, and mineral elements in seeds. The range of seed yield responses to eCO_2 was - 11.0 to 32.7% (average change of 5%) in beans and -23.8 to 39.6% (average change of 7.1%) in soybeans. There was a significant correlation between seed yield enhancement and aboveground biomass, seed number, and pod number per plant. At maturity, eCO_2 increased seed protein concentration in beans, while it did not affect soybean. Lipid concentration was not affected by eCO_2 in either legume species. Compared with

ambient CO_2 (aCO_2), the concentrations of manganese (Mn), iron (Fe), and potassium (K) decreased significantly, magnesium (Mg) increased, while zinc (Zn), phosphorus (P), and calcium (Ca) were not changed under eCO_2 in bean seeds. However, in soybean, Mn and K concentrations decreased significantly, Ca increased, and Zn, Fe, P, and Mg concentrations were not significantly affected by eCO_2 conditions. Our results suggest that intraspecific variation in seed yield improvement and reduced sensitivity to mineral losses might be suitable parameters for breeders to begin selecting lines that maximize yield and nutrition under eCO_2.

2.2. Introduction

With the worldwide population predicted to increase to almost 9.5 billion by 2050, a larger portion of the essential nutrients for humans will be provided by plant-based sources (Preece et al., 2017, Myers et al., 2014). The regular consumption of plant proteins, including that of grain legumes, can reduce the risk of diet-related diseases like obesity, diabetes, cardiovascular problems, hypertension, stroke, and cancers that have been increasing in previous decades (Ebert et al., 2017). Consequently, legumes could be considered an important part of the human diet, as they are a good source of minerals, proteins, vitamins, and bioactive compounds (Singh et al., 2017). Among the grain legumes cultivated, dry beans and soybeans are regarded as important crops, and the European Union highlighted the importance of increasing their production to reduce external requirements, and decrease possible negative impacts associated with intensive cereal production (Cernay et al., 2015), thus improving farming sustainability. An overview from 2000 to 2017 reported an increase from 500 Kt to 1.1 Mt, and from 1.9 Mt to 10.7 Mt in dry bean and soybean production in Europe, respectively (FAOSTAT, 2019). However, among European countries, Portugal has a diminutive production of beans equivalent to 1.7 Kt, and in the case of soybean, the production is practically non-existent. Plant growth is dependent on some resources, including water, mineral nutrients, light, and CO_2 (Ma et al., 2017). The effects of elevated CO_2 (eCO_2) on plant responses are an important topic and has been the subject of scientific research. Nevertheless, there is a lack of information about the genotypic variation of eCO_2 responses on yield and grain quality parameters, particularly in legume species. The atmospheric CO_2 concentration has raised almost 12%, from nearly 370 ppm in 2000 to almost 413 ppm in 2019 (Earth System Research Laboratory, 2019), surpassing anything that plants had to deal with millions of years ago. In this manner, eCO_2 is typically considered as either a positive or a negligible driver of photosynthesis, growth, and yield, mainly on C3 plants (Resco de Dios et al., 2016). However, differences in the range of yield stimulation are usually detected (Kimball et al., 2002), and a significant intraspecific variation in responses to eCO_2 has been found in rice (Moya et al., 1998, Baker, 2004, Ziska et al., 1996), cowpea (Ahmed et al., 1993), wheat (Ziska et al., 2004), common bean (Bunce, 2008), and soybean (Ziska and Bunce, 2000, Ziska et al., 2001). These variations in eCO_2 responsiveness suggest that selecting and breeding genotypes that respond positively to eCO_2 may ensure sustained productivity and improve food security in an upcoming high CO_2 world (Kumagai et al., 2015).

Simultaneously, this trend of increasing ambient CO_2 (aCO_2) levels, which are projected to reach 550 ppm by the middle of this century, is possibly threatening human nutrition, even if further actions are taken to reduce emissions (IPCC, 2014). Consequently, the concentration of various grain mineral elements is influenced to a great extent by eCO_2 conditions (Li et al., 2018b). Myers et al. (2014), in a meta-analysis, evaluated the response of several crops grown at aCO_2 and eCO_2 in free-air CO_2 enrichment (FACE) conditions. Elevated CO_2 was associated with significant decreases in the concentration of zinc (Zn) and iron (Fe) in the edible parts of rice, wheat, field peas, and soybeans. In another study, a decrease in the overall mineral concentrations (a change of −8%) was observed in several C3 crops, reflecting foliar and edible tissues, FACE and non-FACE studies (Loladze, 2014). Other studies also reported decreased nutritional value in edible parts of C3 crops due to eCO_2 conditions (Högy and Fangmeier, 2008, Singh et al., 2016, Dong et al., 2018a). Furthermore, eCO_2 was associated with lower protein concentration in the edible parts of rice, wheat, barley, potato, field peas (Myers et al., 2014), and vegetables (Dong et al., 2018b), but not in soybean, combining FACE and growth chamber data (Myers et al., 2014). Further characteristics of seed quality are also maintained at eCO_2 in legumes, such as grain crude fat on beans, mung bean, and soybean (Ziska et al., 2007, Thomas et al., 2009, Palacios et al., 2019). So, there is still a need to explore genotypic variability, among legume species, which reveal an improved seed yield and nutritional responsiveness to eCO_2 levels. In the present study, we focused on the intraspecific variation of two legume species on yield responses under eCO_2 in a controlled environment, simultaneously assessing aspects associated with the nutritional quality.

2.3. Materials and Methods

2.3.1. Plant material

In this study, we used bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.) varieties, which were obtained either from CIAT (Cali, Colombia) or from USDA-ARS via Germplasm Resources Information Network (Washington, USA). Varieties of both species were chosen based on a preliminary experiment (aCO_2, 400 ppm and eCO_2, 600 ppm) conducted under FACE conditions at Campus Klein (Altendorf, Germany) to find out the performance under eCO_2. The seed yield response (strong-responsive with >25% vs. weak-responsive with <25% of yield increase) at eCO_2 was based on average seed yield responses under eCO_2 and reported by (Bunce, 2008, Ziska et al., 2001, Kumagai et al., 2015, Bunce, 2015, Bishop et al., 2015, Ziska and Bunce, 2007, Ainsworth and Long, 2005). In the selected varieties, the growth and yield performance at eCO_2 were assessed in a controlled environment (Table 2.1).

2.3.2. Growth conditions

The experiment was conducted from January to May in 2017, at the Grow to Green facility (Castelo Branco, Portugal). Seeds were sown on phenolic foam plugs, and seven days after sowing (DAS), seedlings were transplanted to the growth chamber. Plants were grown in a thin nutrient film solution in polyvinyl chloride-coated gullies and placed with 0.20 m in between. Irrigation was performed through 10 min ON/15 min OFF during light period; and 10 min ON/30 min OFF during night period. Plants grew with a photoperiod of 16/8 h (day/night) at an average light intensity expressed as photosynthetic photon flux density of 350 µmol m^{-2} s^{-1} at canopy level. Light conditions were provided by LED lamps with peak emissions of 650, 540, and 460 nm for Red/White/Blue (80:6:14) light, with ratio representing the contribution of red, white, and blue light to total intensity. The temperature was kept at 25/20 °C (day/night) and relative humidity at 75%. Electric conductivity and pH in the nutrient solution were registered by sensors and automatically readjusted to 0.60 mS m^{-1} and 5.5, respectively. The composition of the nutrient solution for hydroponic growth included: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4.7H_2O$, 0.2 mM $NH_4H_2PO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 0.5 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.1 µM $NiSO_4$, and 20 µM FeEDDHA. The experiment was conducted at eCO_2 (800 ppm) and aCO_2 (400 ppm) concentrations until maturity in two independent growth chambers. There were two replicates, with five plants per replicate, in each treatment arranged in a randomized block design.

Table 2.1 - List of bean (*n* = 18) and soybean (*n* = 17) genotypes grown at aCO_2 (400 ppm) and eCO_2 (800 ppm). Performance at eCO_2 was obtained from a preliminary FACE experiment to find out the strong-responsive (>25% yield increase) and weak-responsive (<25% yield increase) varieties against eCO_2.

Crop	Accession Number	Growth Habit	Common Name	Origin	Performance at eCO_2
Bean [a]	PI 203929	D	G1274	Mexico	Strong-responsive
Bean [a]	PI 458586	D or I	NHB	Netherlands	Strong-responsive
Bean [b]	PI 169920	D	Kazak	Turkey	Weak-responsive
Bean [a]	PI 324691	D	ZK	Hungary	Weak-responsive
Bean [a]	W6 9628	I	Dama	Czechoslovakia	Weak-responsive
Bean [a]	W6 12428	NS	PP 63	Bulgaria	Strong-responsive
Bean [a]	PI 550128	I	Trend	Netherlands	Weak-responsive
Bean [a]	PI 550038	NS	Garnet	United States	Weak-responsive
Bean [b]	PI 212027	D	G1378	Iran	Weak-responsive
Bean [a]	PI 598287	I	PV1-4	Japan	Weak-responsive
Bean [a]	PI 368715	D or I	Rosomanska	Macedonia	Strong-responsive
Bean [a]	PI 550035	D	Agate	United States	Weak-responsive
Bean [b]	PI 149484	D	Logan	United States	Weak-responsive
Bean [a]	PI 136687	D	Yamal	Canada	Weak-responsive
Bean [a]	PI 165933	D	Shimi	India	Weak-responsive
Bean [a]	PI 550037	D	Dandy	United States	Strong-responsive
Bean [b]	G 8853	D	Medra	Germany	Strong-responsive
Bean[a]	PI 477023	D or I	CBB	Netherlands	Strong-responsive
Soybean [a]	PI 361085 A	I	L.117	Romania	Strong-responsive
Soybean [a]	PI 437413	I	Ussurijscaja	Russia	Weak-responsive
Soybean [a]	PI 424194	D	ISZ-II	Hungary	Weak-responsive
Soybean [a]	PI 445823	I	Tubinger	Germany	Weak-responsive
Soybean [a]	PI 378676 A	I	Primorskaja	Russia	Strong-responsive
Soybean [a]	PI 561302 A	I	BMS	China	Weak-responsive
Soybean [a]	PI 437101	I	DV-0197	Russia	Weak-responsive
Soybean [a]	PI 319537 A	I	Tono	China	Strong-responsive
Soybean [a]	PI 437224	I	CSchi 675	Moldova	Strong-responsive
Soybean [a]	PI 319534 A	I	Honshu	China	Strong-responsive
Soybean [a]	PI 437676 A	I	MTTPDH	China	Weak-responsive
Soybean [a]	PI 445829 A	I	Dunayka	Romania	Strong-responsive
Soybean [a]	PI 361097 A	I	Novosadska	Serbia	Strong-responsive
Soybean [a]	PI 360952	I	Amurskaja	Russia	Weak-responsive
Soybean [a]	PI 417554	I	EM	Poland	Strong-responsive
Soybean [a]	PI 538409	D	Shironomai	Japan	Strong-responsive
Soybean [a]	PI 153271	I	WB	Belgium	Strong-responsive

[a] Obtained from GRIN; [b] obtained from CIAT; D, determinate; I, indeterminate; NS, not specified; NHB, North Holland Bruine; ZK, Zlaty Knot; CBB, Chocolate Brown Bean; BMS, Bai mao Shuang¸ MTTPDH, Man-tsan tszinxPhin-di-Huan; EM, Early Mandarin; WB, Wisconsin Black.

2.3.3. Growth and yield measurements

For all genotypes, SPAD values were determined at 54 DAS at the pod formation stage. Following senescence of the foliage and discoloration of the pods between 9–10 weeks, irrigation was discontinued, and plants allowed to dry in situ. Pods were hand harvested at maturity between 79–99 DAS depending on the variety. At maturity, aboveground dry weight (sum of the weights of stems, pods shells, and seeds), plant's height, number of pods per plant, number of seeds per plant, and the average weight of 100 seeds were performed for all varieties in both treatments. Seed yield per plant was obtained from ten plants (n = 2 replicates) and adjusted to a 15% moisture content.

2.3.4. Nutritional analysis

Seeds from independent plants (n = 4 replicates) were collected and analyzed for minerals, protein N, and total lipid concentration. Mineral analysis determination was performed as described by Santos et al. (Santos et al., 2015). The minerals analyzed were Zn, Fe, manganese (Mn), phosphorous (P), magnesium (Mg), calcium (Ca), and potassium (K). Briefly, 200 mg of the seed material was mixed with 5 mL of 65% HNO_3 (v/v) and 1 mL of H_2O_2 30% (*v/v*) in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was conducted in five steps, consisting of different temperature and time sets: 130 °C/10 min, 160 °C/15 min, 170 °C/12 min, 100 °C/7 min, and 100 °C/3 min. The resulting clear solutions of the digestion procedure were then brought to 50 mL with ultrapure water for further analysis. Mineral concentration determination was performed using the ICP-OES Optima 7000 DV (PerkinElmer, USA) with radial configuration.
Seeds were analyzed for crude protein concentration (N x 5.28 and N x 5.5 in bean and soybean, respectively) using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, St. Joseph, USA), and crude fat concentration was measured by petroleum ether extraction (40–60 °C) using a Soxhlet fat extraction system (Gerhardt, Germany). All chemical analyses followed AOAC (2006) methods.

2.3.5. Statistical analysis

To test for significant differences between CO_2 treatments and among varieties, and for significant interactions, plant data were analyzed as a completely randomized design using a two-way ANOVA. The correlations among seed yield and agronomic traits were performed using Pearson's product-moment correlation (*r*). All statistical analyses were performed with version 25.0 of the SPSS statistics software.

2.4. Results

2.4.1. Genotypic variation of yield responses to eCO_2

A significant increase in seed yield due to eCO_2 was observed in beans, with a mean response of 5.0% ($p < 0.05$), as demonstrated in Figure 2.1 and Table 2.2. The rank of seed yield improvement was greatest for Chocolate Brown Bean (CBB, 32.7%), followed by Medra (30.3%), Dandy (28.0%), and Shimi (25.0%) varieties. These were considered strong-responsive varieties under eCO_2 conditions (see section 2.3.1). Besides, no significant differences were observed among the remaining varieties due to eCO_2. Agate had the highest seed yield at both CO_2 concentrations. The extent of seed yield improvement due to eCO_2 differed significantly among the varieties ($p < 0.0001$), with a significant CO_2 x variety interaction ($p < 0.05$), as demonstrated in Table 2.2.

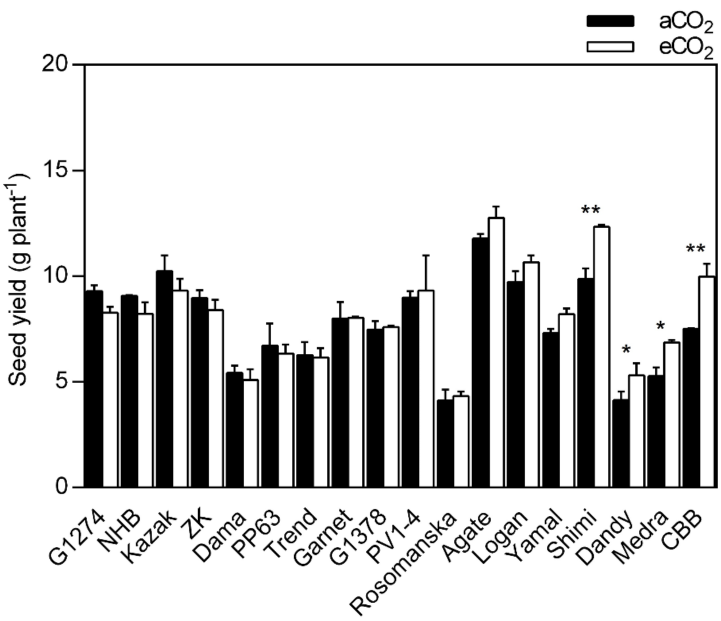

Figure 2.1 - Seed yield of bean grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). Data are means ± SE ($n = 10$ plants). From left to right, varieties are classified in order of increasing seed yield responsiveness to eCO_2. * $p < 0.05$; ** $p < 0.01$ significance level.

Table 2.2 - Growth and reproductive characteristics at maturity of 18 bean varieties grown at aCO_2 (400 ppm) and eCO_2 (800 ppm), and correlations (Pearson's *r*) and their statistical significance for the relationship between the relative increase in bean seed yield due to eCO_2 (value at eCO_2/value at aCO_2) and values of other parameters measured under the same conditions. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. C x V, CO_2 x variety interaction; ns, not significant.

Parameter	Mean CO_2 Effect	CO_2	Variety	C x V	Correlation	
Aboveground dry weight, g plt^{-1}	5.8%	*	****	ns	0.747	*
Height, cm plt^{-1}	4.8%	*	****	ns	0.593	**
Seed yield, g $plant^{-1}$	5.0%	*	****	*	–	–
Harvest index, g g^{-1}	−0.2%	ns	**	ns	0.096	ns
No. of pods, plt^{-1}	2.9%	ns	****	*	0.736	*
No. of seeds, plt^{-1}	3.8%	ns	****	***	0.838	**
No. of seeds, pod^{-1}	7.5%	**	****	ns	0.314	ns
100-seed weight, g	−13.1%	****	****	*	−0.108	ns

The aboveground biomass (sum of the weights of stems, pod shells, seeds) at maturity was significantly increased by eCO_2 ($p < 0.05$), and there was a significant intraspecific variation associated with eCO_2 ($p < 0.0001$) without a significant CO_2 x variety interaction ($p > 0.05$). The biomass response was strongly correlated with yield increase to eCO_2 ($r = 0.747$, $p < 0.01$). On the other hand, the harvest index, which was expressed as the ratio of seed yield to aboveground biomass, was not changed by eCO_2 ($p > 0.05$). Further, there was no significant correlation between harvest index and yield enhancement due to eCO_2 conditions (Table 2.2).

The relative increase in height in response to eCO_2 was 4.8% ($p < 0.05$; Table 2.2), and the magnitude of this increase differed significantly between varieties ($p < 0.0001$), without a significant CO_2 x variety interaction ($p > 0.05$). Further, we observed a strong correlation between yield response to eCO_2 and relative increase in height ($r = 0.593$, $p < 0.01$).

Of the yield components, exposure to eCO_2 resulted in a significant stimulation on the number of seeds per pod (mean CO_2 effect of 7.5%, $p < 0.01$; Table 2.2), and the magnitude of this increase differed significantly among the varieties ($p < 0.0001$), without a CO_2 x variety interaction ($p > 0.05$). Moreover, a correlation between increased seed yield and an increased number of seeds per pod was not observed ($p > 0.05$).

Elevated CO_2 resulted in seed mass reduction by −13.1% ($p < 0.0001$), but there was no significant correlation between seed mass reduction and yield improvement ($p > 0.05$).

No significant differences were observed in the number of pods (mean CO_2 effect of 2.9%, $p > 0.05$) and in the number of seeds per plant (mean CO_2 effect of 3.8%, $p > 0.05$) due to eCO_2. However, a significant intraspecific variability was observed ($p < 0.0001$) with a significant CO_2 x variety interaction ($p < 0.05$) for both yield components. There was a strong positive correlation between the number of pods ($r = 0.736$, $p < 0.01$) and the number of seeds per plant ($r = 0.838$, $p < 0.01$) with seed yield enhancement (Table 2.2).

Concerning soybean, CO_2 enrichment significantly stimulated seed yield by an average of 7.1% ($p < 0.05$; Figure 2.2 and Table 2.3). This magnitude of seed yield enhancement differed significantly among the varieties ($p < 0.0001$), and there was a significant CO_2 x variety interaction ($p < 0.01$). The largest seed yield increase at eCO_2 was observed in Wisconsin Black (WB, 39.6%), Shironomai (28.5%), and Early Mandarin (24.5%), which were considered strong-responsive varieties, followed by Amurskaja (18.4%). No significant differences in seed yield were observed among the remaining cultivars, except for L.117 ($p < 0.05$), which showed a significant decrease in seed yield under eCO_2. At aCO_2, WB with Tubinger had the highest seed yield, which was consistent at eCO_2, whereas WB significantly surpassed all other varieties (Figure 2.2).

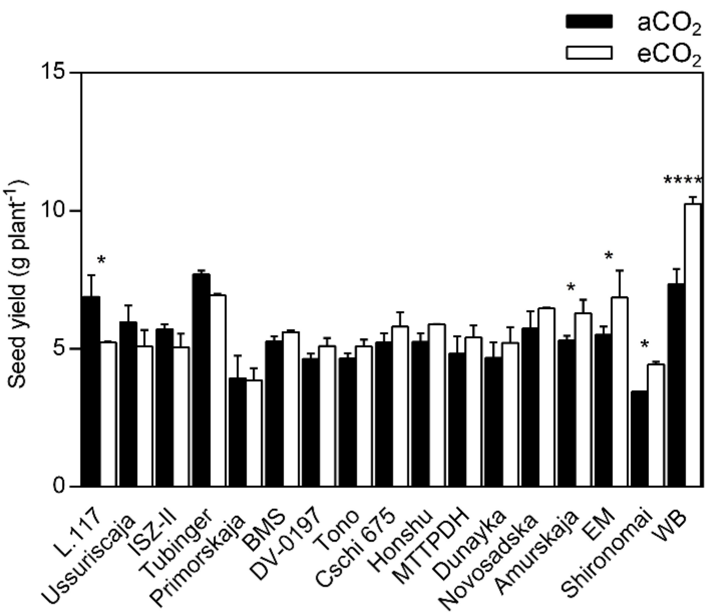

Figure 2.2 - Seed yield of soybean grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). Data are means ± SE (n = 10 plants). From left to right, varieties are classified in order of increasing seed yield responsiveness to eCO_2. * $p < 0.05$; **** $p < 0.0001$ significance level.

Table 2.3 - Growth and reproductive characteristics at maturity of 17 soybean varieties grown at aCO_2 (400 ppm) and eCO_2 (800 ppm), and correlations (Pearson's *r*) and their statistical significance for the relationship between the relative increase in bean seed yield due to eCO_2 (value at eCO_2/value at aCO_2) and values of other parameters measured under the same conditions. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Parameter	Mean CO_2 Effect	CO_2	Variety	C x V	Correlation	
Aboveground dry weight, g plt^{-1}	6.9%	*	****	ns	0.625	**
Height, cm plt^{-1}	3.6%	*	****	****	0.119	ns
Seed yield, g plt^{-1}	7.1%	*	****	**	–	
Harvest index, g g^{-1}	−1.0%	ns	****	ns	0.396	ns
No. of pods, plt^{-1}	7.2%	**	****	ns	0.784	**
No. of seeds, plt^{-1}	5.5%	*	****	****	0.600	*
No. of seeds, pod^{-1}	5.9%	*	*	*	0.665	**
100-seed weight, g	−12.3%	****	****	**	−0.280	ns

The aboveground biomass was significantly increased by 6.9% due to eCO_2 ($p < 0.05$, Table 2.3), and there was a significant difference among the varieties ($p < 0.0001$), without a CO_2 x variety interaction ($p > 0.05$). This increase in biomass was significantly correlated with seed yield enhancement at eCO_2. ($r = 0.625$, $p < 0.01$). The harvest index was not affected by eCO_2 ($p > 0.05$), with a significant intraspecific variation ($p < 0.0001$). Further, there was no significant correlation between harvest index and yield enhancement due to eCO_2 conditions (Table 2.3).

On the other hand, a significant increase in height due to eCO_2 was observed, with an average response of about 4%. The magnitude of this enhancement due to eCO_2 differed significantly among the varieties ($p < 0.0001$), with a significant CO_2 x variety interaction ($p < 0.0001$, Table 2.3).

Of the yield components, eCO_2 had significant effects on pod number per plant (mean CO_2 effect of 7.2%, $p < 0.01$), seed number per plant (mean CO_2 effect of 5.5%, $p < 0.05$), seed number per pod (mean CO_2 effect of 5.9%, $p < 0.05$), and 100-seed weight (mean CO_2 effect of −12.3%, $p < 0.0001$). The extent of all reproductive parameters differed significantly among the varieties ($p < 0.05$), with a significant CO_2 x variety interaction ($p < 0.05$), except on the number of pods per plant ($p > 0.05$, Table 2.3). Moreover, there was a strong and positive correlation between seed yield improvement and pod number per plant ($r = 0.784$, $p < 0.01$), seed number per plant ($r = 0.600$, $p < 0.05$), and seed number per pod ($r = 0.665$, $p < 0.01$), as described in Table 2.3.

2.4.2. Variation of grain nutritional composition due to eCO_2

Elevated CO_2 did not influence Zn, P, or Ca concentrations in bean seeds at maturity ($p > 0.05$, Figure 2.3). However, the concentrations of the other minerals (viz. Mn, Fe, Mg, and K) responded differently to eCO_2. Under eCO_2, the Mn concentration was significantly decreased by 25.2% ($p < 0.0001$). The decrease was significant in 9 out of 18 varieties, whereas it increased in Garnet ($p < 0.05$), and in Kazak, Dama, PP63, G1378, Rosomanska, Yamal, Dandy, and CBB, no changes were observed at eCO_2 (Figure 2.4).

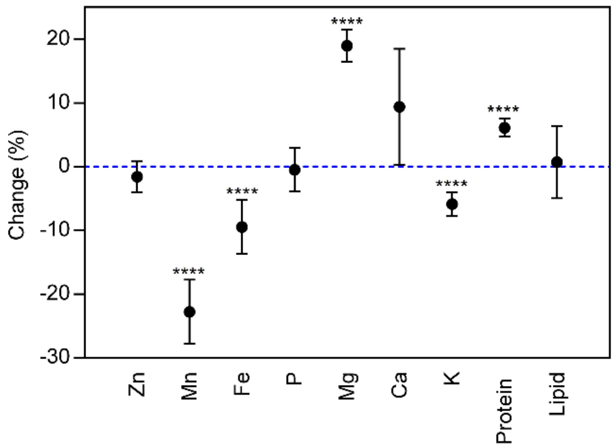

Figure 2.3 - Mean response change (%) of the seed mineral, protein, and lipid concentrations of 18 bean varieties grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). **** $p < 0.0001$ significance level.

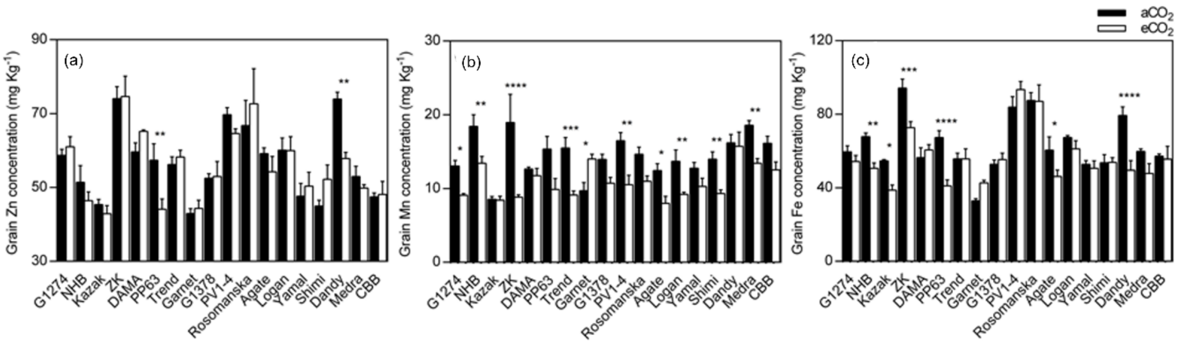

Figure 2.4 - Grain micronutrient (a–c) concentrations of bean grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). Each bar represents the mean ± SE (n = 10 plants). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ significance level.

The Fe concentration was decreased by 39.1%, 37.6%, 29.0%, 25.4%, 23.7%, and 22.9% ($p < 0.001$) in PP63, Dandy, Kazak, North Holland Bruine (NHB), Agate, and Zlaty Knot, respectively (Figure 2.4).

Grain Mg concentration increased under eCO_2 for G1274, NHB, Dama, Trend, G1378, PV1-4, Rosomanska, Logan, Yamal, Dandy, and Medra and remained unchanged in the rest of the varieties (Figure 2.5). Significant changes in K concentration were observed in G1274, Kazak Logan, and Medra (Figure 2.5), which showed a decrease in grain K concentration (mean CO_2 effect of −6.0%, $p < 0.05$, Figure 2.3), while no changes were demonstrated in the remaining varieties. The extent of change in all grain mineral concentrations in response to eCO_2 varied between varieties (Table 2.4, $p < 0.01$), implying a significant CO_2 x cultivar interaction ($p < 0.01$).

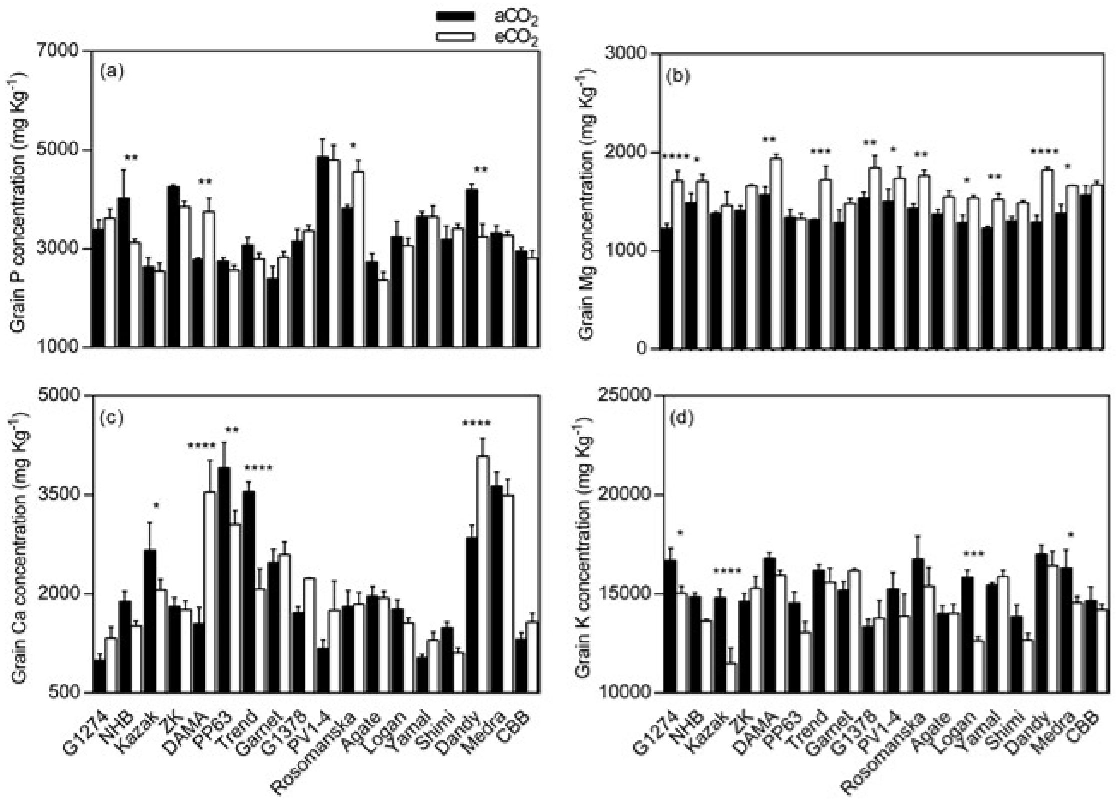

Figure 2.5 - Grain macronutrient (a–d) concentrations of bean grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). Each bar represents the mean ± SE (n = 10 plants). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ significance level.

Table 2.4 - Significance levels of main effects and interactions of CO_2 and varieties on bean grain nutrient, protein, and lipid concentrations at maturity. ns, not significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Seed Element	CO_2	Variety	C x V
Zn	ns	****	ns
Mn	****	****	***
Fe	****	****	****
P	ns	****	**
Mg	****	****	ns
Ca	ns	**	****
K	****	****	**
Protein	****	****	****
Lipid	ns	**	ns

Exposure to eCO_2 significantly increased protein concentration when compared to aCO_2 (mean CO_2 effect of 6.0%, $p < 0.0001$, Figure 2.3). The increase was significant in 12 out of 18 varieties, while decreased in Kazak ($p < 0.05$), and in Agate, CBB, Dandy, PP63, and Shimi, the concentration remained unchanged (Figure 2.6). A significant effect of CO_2 x variety interaction on protein concentration was observed ($p < 0.0001$, Table 2.4). Elevated CO_2 had no influence on fat concentration in all bean varieties at maturity when compared to aCO_2 (Figure 2.3).

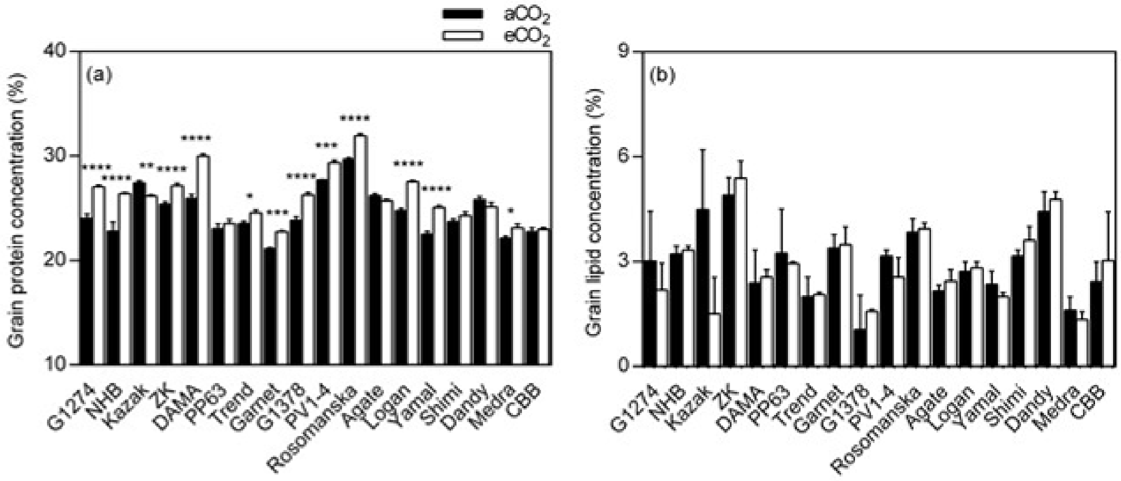

Figure 2.6 - Influence of eCO_2 on bean seed protein and lipid concentrations. Each bar represents the mean ± SE (n = 10 plants). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ significance level.

In soybean, eCO_2 did not influence Zn, Fe, P, or Mg concentrations in seeds ($p > 0.05$, Figure 2.7). On the other hand, eCO_2 significantly decreased grain Mn concentration by 23.2% ($p < 0.0001$). The concentration of this element decreased in Tubinger, Primorskaja, Bai Mao Shuang, DV-0197, Tono, Cschi675, Man-tsan-tszinxPhin-di-Huan (MTTPDH), Dunayka, and Novosadska, and no significant differences were observed in the remaining varieties (Figure 2.8).

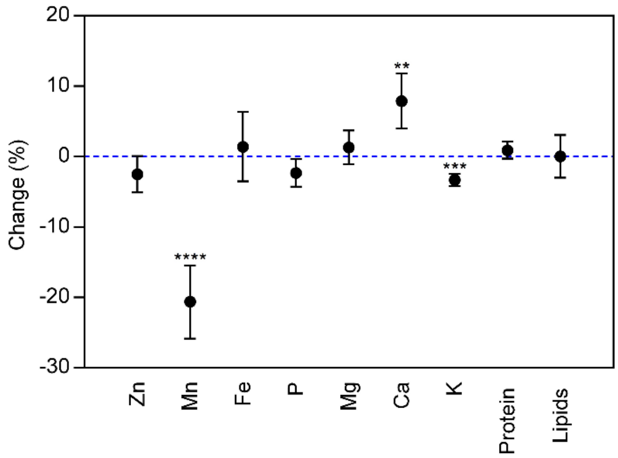

Figure 2.7 - Mean response change (%) of the seed mineral, protein, and lipid concentrations of 17 soybean varieties grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ significance level.

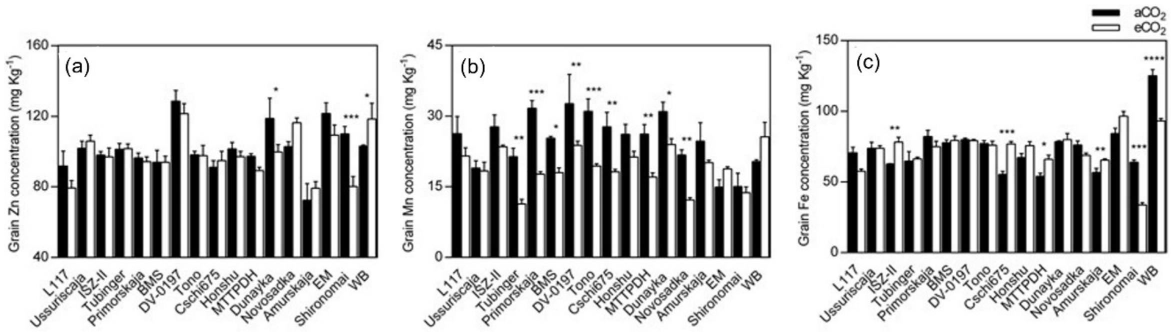

Figure 2.8 - Grain micronutrient (a–c) concentrations of soybean grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). Each bar represents the mean ± SE ($n = 10$ plants). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ significance level.

Elevated CO_2 significantly increased grain Ca concentration by 36.3%, 34.9%, 25.3%, and 24.3% in ISZ-II, Amurskaja, Ussuriscaja, Tubinger, respectively, decreased by 21.5% in Primorskaja, and was not affected in the remaining varieties (Figure 2.9).

Furthermore, eCO_2 decreased K concentration by 3.5% ($p < 0.001$) when compared to aCO_2. The response of grain mineral concentrations to eCO_2 varied between varieties (Table 2.5, $p < 0.01$), implying a significant CO_2 x cultivar interaction ($p < 0.01$), except for P concentration. Also, eCO_2 had no influence on the grain protein and lipid concentrations ($p > 0.05$, Figure 2.7) in soybean. However, the extent of change in grain protein and lipid concentrations in response to eCO_2 varied between varieties ($p < 0.001$, Figure 2.10 and Table 2.5).

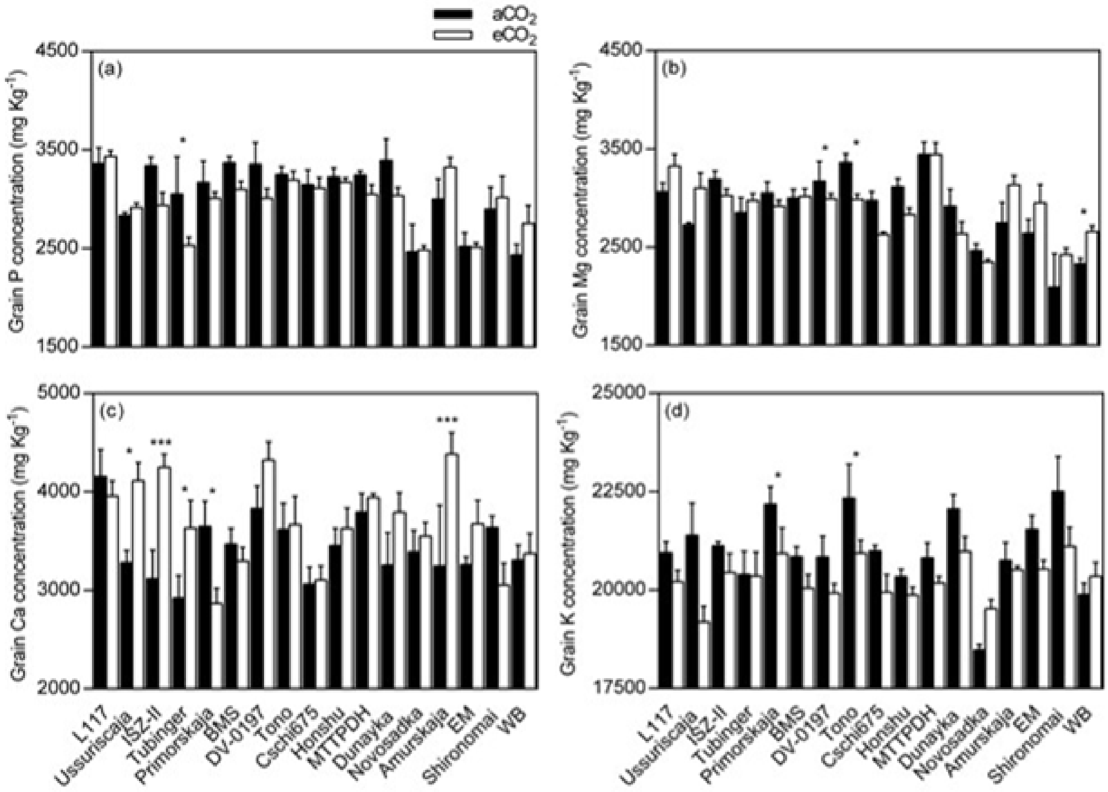

Figure 2.9 - Grain macronutrient (a–d) concentrations of soybean grown under aCO_2 (400 ppm) and eCO_2 (800 ppm). Each bar represents the mean ± SE (n = 10 plants). * $p < 0.05$; *** $p < 0.001$ significance level.

2.5. Discussion

Strong-responsive genotypes to eCO_2 have been crucial and might support significant yield increases in a future eCO_2 environment. The increased performance must encompass not only productivity at the whole-plant level but must also nutritional resilience to future climate conditions.

Table 2.5 - Significance levels of main effects and interactions of CO_2 and varieties on soybean grain nutrient, protein, and lipid concentrations at maturity. ns, not significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Seed Element	CO_2	Variety	C x V
Zn	ns	****	**
Mn	****	****	**
Fe	ns	****	****
P	ns	****	ns
Mg	ns	****	**
Ca	**	***	**
K	***	****	**
Protein	ns	****	**
Lipid	ns	***	ns

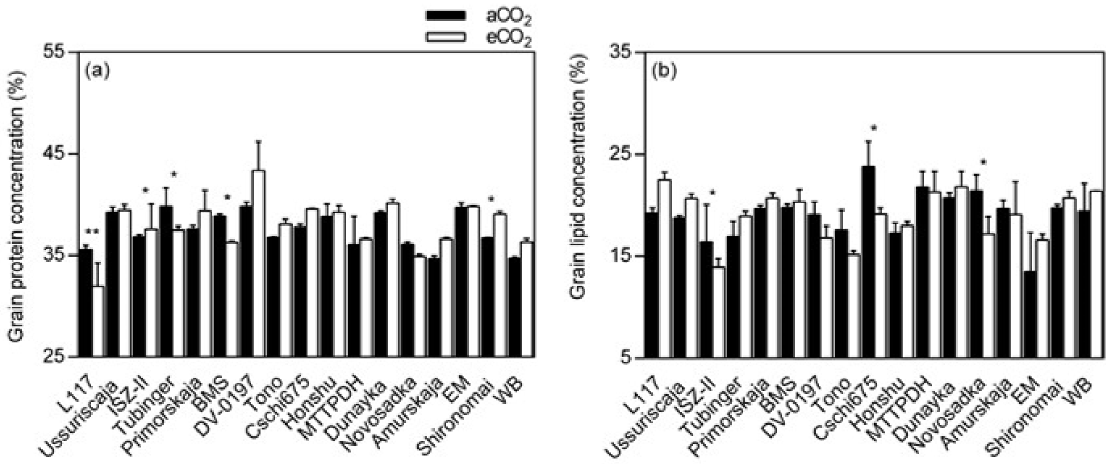

Figure 2.10 - Influence of eCO_2 on soybean seed protein and lipid concentrations. Each bar represents the mean ± SE ($n = 10$ plants). * $p < 0.05$; ** $p < 0.01$ significance level.

The current study demonstrated that under eCO_2, seed yield differed substantially among the varieties tested ($p < 0.0001$), ranging from −11.0 to 32.7% in bean, and from −23.8 to 39.6% in soybean (Figures 2.1 and 2.2), suggesting a considerable genetic background for genomic improvement. It was also previously demonstrated that yield responses to increasing CO_2 varied greatly, among varieties and between species, ranging from −10 to 80% for soybean (Ziska et al., 2001, Kumagai et al., 2015, Bunce, 2015, Bishop et al., 2015) and from −11 to 39% for common bean (Bunce, 2008, Prasad et al., 2002). Nevertheless, eCO_2 increased the seed yield but failed to improve the harvest index; however, decreases in harvest index due to CO_2 enrichment can occur in soybean (Ainsworth et al., 2002). Similar results have been reported in lupin (Palta and Ludwig, 2000), where exposure to eCO_2 did not decrease the harvest index, because the effect of CO_2 was mainly an increase in biomass and, consequently, an increase in the number of pods that reached maturity and the number of pods with filled seeds. Herein, the seed yield increase was 5.0% (bean) and 7.1% (soybean), which is relatively lower than other reports (Bunce, 2008, Ziska et al., 2001, Kumagai et al., 2015, Bunce, 2015). This failure of seed yield increase is possibly associated with the physical restriction to root growth, since the volume of the containers for root growth was <2 L. It is widely accepted that the pot size significantly affects seed yield responses to eCO_2, since plants grown in larger pots (>9 L) have greater stimulation compared to those grown in small pots (Ainsworth et al., 2002). In addition, the CO_2-induced reduction in seed mass, which may be a consequence of the restriction of nutrient production, mobilization, and translocation to the seeds during seed filling, is probably also associated with the physical restriction of root growth.

However, the driving force in the yield-enhancing strategy was linked to the response of biomass to eCO_2 and, subsequently, to the number of pods and seeds production, and these were probably useful indicators of the intraspecific variation (Tables 2.2 and 2.3). This is in agreement with Kumagai et al. (2015), who reported the growth of soybean in a greenhouse at eCO_2. The authors showed that cultivars with the strongest responsiveness of biomass to eCO_2 produced more pods and greater seed yield. Bunce (2016) also demonstrated seed yield improvement under eCO_2, among common bean varieties, and a high correlation with stimulation of pod and seed numbers. Therefore, it was proposed that a genotype with higher sink formation due to eCO_2 would be a promising candidate for higher yield responses to eCO_2 (Ziska and Bunce, 2000).

However, it is important to understand whether the characteristics that lead to higher responsiveness to eCO_2 are also manifested under aCO_2 for the development of effective plant breeding strategies (Bunce, 2008). In the current study, the highest yielding variety at aCO_2 was the highest yielding variety at eCO_2 in both species. Therefore, Agate (bean) and WB (soybean) have a higher yield at both concentrations. This suggests that varieties best adapted to current CO_2 levels may also have the characteristics best adapted to future CO_2 concentrations, providing good genetic support for future studies.

The impact of eCO_2 on the grain nutritional quality has also been studied, since CO_2 enrichment can lead to a decrease in plant nutritional status, and pose a potential challenge to human health (Li et al., 2018b). Elevated CO_2 significantly reduced the grain nutritional value in terms of Mn, Fe, and K in bean, and Mn and K in soybean (Figures 2.3 and 2.7). Similar results for Mn and K have been reported by Loladze (2014) in a wide range of C3 crops, reflecting foliar and edible tissues, FACE and non-FACE studies, and by Myers et al. (2014) in field peas. The reduction in grain Fe content due to eCO_2 has also been reported in rice, wheat, barley, peas, and soybeans (Myers et al., 2014, Li et al., 2018b).

Furthermore, exposure to eCO_2 increased Mg and Ca concentrations in bean and soybean, respectively. Similar results were obtained by Li et al. (2018b) in soybean seeds at the fresh edible and mature stages. On the other hand, grain Zn and P concentrations were not influenced by eCO_2 in either species. Dong et al. (2018b) in vegetables and Li et al. (2018b) in soybean also found that P concentration was not affected by eCO_2.

The mechanisms responsible for reducing the concentration of nutrients associated with eCO_2 have not yet been fully clarified. Many studies attribute this to the carbohydrate dilution effect, where increasing plant biomass under eCO_2 conditions dilutes the rest of the grain components (Li et al., 2018b, Wu et al., 2004, Parvin et al., 2019). Our findings were contradictory, with carbohydrate dilution functioning alone since we found that mineral changes within the same species are distinct from each other, suggesting that the mechanism is more complex than carbohydrate dilution alone. For example, in bean (Figure 2.3), the decrease in Mn concentration was significantly different from the decrease in Fe concentration or K concentration, and the increase in Mg concentration. It also seems that the mechanisms causing these changes function distinctly in different species. Consequently, we found Mg concentration to be significantly increased in bean ($p < 0.0001$), whereas it was not changed in soybean grains ($p > 0.05$, Figure 2.7). Therefore, eCO_2 has both positive and negative effects on the nutritional quality of legume seeds. Inhibition of photorespiration and malate production (Bloom, 2015), carbohydrate dilution, and decreased mass flow due to reduced transpiration may all be relevant to explain this phenomenon of decreased grain nutritional value under eCO_2 conditions (Pleijel et al., 2000, Gifford et al., 2000).

We also examined the effects of eCO_2 on mineral concentrations as a function of variety. Both crops showed significant differences across varieties among all minerals studied (Tables 2.4 and 2.5). Such changes among varieties suggest a basis for breeding varieties whose reduced nutrient levels are less responsive to eCO_2.

Legumes are a major source of proteins and oil, particularly soybean, containing essential free amino acids and fatty acids (Li et al., 2018b). Concerning grain protein concentration, it was demonstrated that eCO_2 increased grain protein in bean ($p < 0.0001$) and had no influence in soybean seeds ($p > 0.05$, Figures 2.3 and 2.4), with significant differences among varieties (Tables 2.4 and 2.5). These findings that protein concentration was less affected are also associated with the competence of leguminous crops to counteract the stimulation of photosynthetic C gain at eCO_2, with better nitrogen fixation for preserving tissue C:N ratios (Qiao et al., 2019). Our results are in agreement with those of Jablonski et al. (2002), in a meta-analysis of several crops and wild species, showed that seed protein was not affected by high CO_2 concentrations in legumes, but declined significantly in most non-legumes. Similarly, Taub and Wang (2008) indicated that eCO_2 did not affect soybean seed protein concentration.Myers et al. (2014) also found that eCO_2 was associated with lower protein concentration in wheat and rice grains, and a non-significant effect of eCO_2 was demonstrated in soybeans or C4 crops grown under FACE conditions.

Few studies dealing with the effects of eCO_2 on plant lipid metabolism have been carried out. In this study, it was demonstrated that eCO_2 had no effect on lipid concentration in bean and soybean grains ($p > 0.05$, Figures 2.3 and 2.7). Similar results were reported in *Arabidopsis thaliana* (Ekman et al., 2007), wheat (Högy and Fangmeier, 2008), and soybean grains (Li et al., 2018b) at the fresh edible stages and grown at eCO_2.

It was previously demonstrated that eCO_2 decreased the concentrations of Fe and Zn in grains of most C3 plants (Li et al., 2018b, Högy and Fangmeier, 2008, Dong et al., 2018b, Fernando et al., 2014), and usually, C3 crops other than legumes also have lower concentrations of protein (Myers et al., 2014). These dietary deficiencies are considered a global public health problem, as it is estimated that two billion people worldwide are affected by these nutritional deficiencies (Myers et al., 2014). Therefore, strong-responsive cultivars (i.e., CBB, Medra, and Shimi in bean, and EM in soybean) in terms of seed yield enhancement and that maintain or even increase Fe, Zn, and grain protein concentrations at eCO_2 might be considered as promising varieties for future studies.

2.6. Conclusions

In summary, our results indicate that consistent and significant variation in the response of seed yield to eCO_2 under controlled conditions does exist among legume species, and that the response of pod and seed numbers are suitable for predicting their responsiveness to future eCO_2. Moreover, Mn and K concentrations were significantly decreased by eCO_2 in both species. The protein concentration in bean seeds was significantly increased. Lipid concentrations were not influenced by eCO_2 in the present study Thus, it is important to develop specially designed programs to increase seed yield while avoiding or reducing some of the important nutritional losses that may arise under eCO_2 conditions.

CHAPTER 3 - Genotypic variation in the response of soybean to elevated CO_2

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3.1. Abstract

The impact of elevated CO_2 (eCO_2) on soybean productivity is essential to the global food supply because it is the world's leading source of vegetable proteins. This study aimed to understand the yield responses and nutritional impact under free-air CO_2 enrichment (FACE) conditions of soybean genotypes. Here we report that grain yield increased by 46.9% and no reduction in harvest index was observed among soybean genotypes. Elevated CO_2 improved the photosynthetic carbon assimilation rate, leaf area, plant height, and aboveground biomass at vegetative and pod filling stages. Besides the positive effects on yield parameters, eCO_2 differentially affected the overall grain quality. The levels of calcium (Ca), phosphorous (P), potassium (K), magnesium (Mg), manganese (Mn), iron (Fe), boron (B), and zinc (Zn) grain minerals decreased by 22.9, 9.0, 4.9, 10.1, 21.3, 28.1, 18.5, and 25.9% under eCO_2 conditions, respectively. Soluble sugars and starch increased by 9.1 and 16.0%, respectively, phytic acid

accumulation increased by 8.1%, but grain protein content significantly decreased by 5.6% across soybean genotypes. Furthermore, the antioxidant activity decreased by 36.9%, but the total phenolic content was not affected by eCO_2 conditions. Genotypes, such as Winsconsin Black, Primorskaja, and L-117, were considered the most responsive to eCO_2 in terms of yield enhancement and less affected in the nutritional quality. Our results confirm the existence of genetic variability in soybean responses to eCO_2, and differences between genotypes in yield improvement and decreased sensitivity to eCO_2 in terms of grain quality loss could be included in future soybean selection to enable adaptation to climate change.

3.2. Introduction

In the 20 million years preceding the industrial revolution, atmospheric CO_2 concentration in the atmosphere was below 280 ppm but continued to increase since then and reached almost 410 ppm (https://gml.noaa.gov/ccgg/trends/) by 2020. In the future, with current trends, it will probably exceed 550 ppm by 2050 (IPCC, 2014). Therefore, plants are facing unprecedented levels of CO_2 concentration, and it is highly questionable that they could adapt to this change so quickly (Bishop et al., 2015). Several changes in terms of growth, physiology, biochemical and genetic traits takes place in plants exposed to elevated CO_2 (eCO_2) conditions (Palit et al., 2020b). Thus, eCO_2 has been reported to stimulate plant growth, and photosynthesis of several crops, and to reduce stomatal conductance (g_s), leading to a greater transpiration efficiency (Hajiboland, 2012, Soares et al., 2019b, Singh et al., 2016, Asif et al., 2018, Bourgault et al., 2017, Palit et al., 2020b). The extent of the response varies between and within species, experimental conditions (Ainsworth and Long, 2005), and according to the interactions with climate changes and soil conditions (Bishop et al., 2015). The effects of eCO_2 could also mitigate the damaging effects on yield due to other aspects of climate change such as rising temperature, increased frequency and intensity of droughts, and increased contact of vegetation to atmospheric water vapor pressure deficit (Bishop et al., 2015, Abdelgawad et al., 2015, Bencke-Malato et al., 2019).

Soybean is an important crop consumed globally and the most extensively grown legume worldwide providing an essential source of protein and oil (Ainsworth et al., 2012, Kumar and Pandey, 2020). Nowadays, there is a growing demand for the consumption of legumes due to their high protein content, low in calories and glycemic index, and because they provide various health benefits (Kumar and Pandey, 2020). Global soybean production has steadily increased growing its production from 161 to 348 million tons in the last two decades (http://www.fao.org/faostat/en/#data), due to improved agronomy techniques and selection of cultivars suited to a wide range of environments (Ainsworth et al., 2012). CO_2-based responses in soybean have been extensively investigated, either in controlled and field experiments (Leakey et al., 2009, Ainsworth et al., 2002, Kimball, 2016). However, under FACE conditions, most studies have been carried out with one or a few genotypes (Hao et al., 2014, Hao et al., 2016, Rosenthal et al., 2014, Bunce, 2014, Bunce, 2016), and to gain more knowledge about the adaption process to eCO_2 it is essential to consider the intraspecific variability in yield responses. Bishop et al. (2015) investigated the intraspecific variation in the response of 18 soybean genotypes to increased CO_2 (550 ppm) under FACE conditions. On average, there was an increase in biomass by 22%, and seed yield by 9%, partially because most genotypes showed a reduction in the partitioning of energy to seeds. In a controlled environment, Ziska et al. (2001) investigated nine soybean genotypes, and observed significant differences in the magnitude of the yield response under eCO_2 conditions (710 ppm). Similar findings were observed by Soares et al. (2019a) studying 17 soybean genotypes in a controlled environment, and the range of yield responses to eCO_2 (800 ppm) was -23.8 to 39.6%. Considering the effects of eCO_2 on soybean grain quality, results from a previous study suggest that eCO_2 decreased soybean grain protein in open-top chambers (Li et al., 2018b). Myers et al. (2014) also found that eCO_2 was associated with reduced protein content in C3 grasses, wheat, and rice grains, and with a small decrease in field pea although there was no significant effect in soybean under FACE conditions. Besides, the concentration of several minerals are significantly influenced by eCO_2 which could affect the human nutrition in the upcoming future (Köhler et al., 2018). Using a meta-analysis, Loladze (2014) showed that eCO_2 declines the overall mineral concentrations by 8% in a range of C3 plants, reflecting foliar and edible tissues, using FACE, and non-FACE studies. It was also reported that C3 grains and legumes have reduced content of zinc (Zn) and iron (Fe) under FACE conditions (Myers et al., 2014). In another study, exposure to eCO_2 during consecutive seasons decreased nitrogen (N), potassium (K), calcium (Ca), protein, and total amino acid concentrations in wheat grains, even though the starch concentration was not significantly affected (Li et al., 2019). Moreover, it is also important to consider phytate which is a phosphate storage molecule present in most plants, and a strong inhibitor of Fe, Zn, and Ca absorption (Gibson et al., 2010). Myers et al. (2014) measured phytate in plants grown under eCO_2 and observed a significant reduction in wheat, but there was no decrease in phytate concentration in rice, field peas, soybeans, maize, and sorghum. Still, the combined analysis of minerals and phytate could provide a more thorough understanding on the impact of eCO_2 on mineral bioavailability. There is even less information about the responses to eCO_2 in terms of sugar concentration and on the antioxidant capacity in the grain of legumes. Dong et al. (2018b) conducted a meta-analysis suggesting that eCO_2 increases the concentration of total soluble sugar, total antioxidant capacity, total phenols, total flavonoids, and ascorbic acid in the edible part of vegetables. In contrast, Zheng et al. (2020) proposed that the content of soluble sugars in soybean grains was not affected by eCO_2, but the levels of natural antioxidants decreased. In another study, conducted using open-top chambers, the total phenolic content (TPC) of two rice varieties decreased at eCO_2 (Goufo et al., 2014). Therefore, most studies looking at the effects of eCO_2 have focused on either the physiological or the nutritional responses, and very few have combined these two components to explain the basis for the impacts of eCO_2 on nutrient accumulation. We therefore hypothesized that genetic selection towards CO_2-based responses for yield and grain quality are likely to involve a range of characteristics that balance sink and source associations. In this study, we analyzed the genotypic variation in soybean yield responses under field conditions. At the same time, we assessed leaf photosynthesis parameters, and grain quality, specifically, protein concentration, minerals, sugar, starch, phytic acid, phenolics content, and antioxidant activity.

3.3. Materials and Methods

3.3.1. Research site and experimental design

This study was conducted at the FACE facility from the experimental station of the University of Bonn located at Campus Klein-Altendorf (50°37'30.5"N 6°59'15.8"E, 160 m above sea level) in Germany. The soil is a loamy-clay silt soil (luvisol) with a pH of 6.6 (1:5 soil:water), organic carbon of 1.84%, and a total N of 1.07 g/kg. During the growing season in 2018, the average precipitation and daytime temperature in June, July, August, and September was 44.7, 29.4, 19.1, and 37.1 mm and 17.8, 21.0, 19.8, 14.9 °C, respectively. The soil was not irrigated or fertilized, only receiving water through rainfall. Soybeans were planted on 30 May 2018. The FACE facility, consisted of two blocks, each containing two 17.5 m diameter octagonal plots. The CO_2 concentration at the center of the ring was frequently monitored, and CO_2 was released from the peripheral emission tubes at 0.5 m above the canopy. The emission source was chosen based on the current wind direction to maintain CO_2 concentration within the ring at a level of 200 ppm above that in the ambient CO_2 (aCO_2) plots. The experimental design was a split-pot model design (main plot = CO_2 and split-pot = genotypes) with two replicates. Within each block, one plot was at current CO_2 concentration of 400 ppm, and one plot was fumigated with CO_2 to 600 ppm using the FACE system. Each plot was divided into 52 of 1.5 m × 3 m subplots, and plants were sown in rows with 0.45 m spacing at a sowing density of 20 plants/m^2. One side of the ring was subdivided into 26 subplots and planted with common bean, and the other side was planted with a range of soybean genotypes described in Table 3.1 and used in the current study. Each genotype occupied the same position in each ring and was randomly replicated in two subplots of each ring. Plots were fumigated with eCO_2 during daylight from emergence to maturity using the FACE system.

3.3.2. Crop growth and yield

All soybean genotypes, but one (VDGY), were previously grown in a growth chamber experiment (Soares et al., 2019a). Sampling points were determined at vegetative (V3-V4), and pod filling (R4) stages (Fehr et al., 1971). Three plants from each subplot were harvested for determination of leaf area (LI-3100C area meter, LI-COR, Lincoln, USA), plant height, and aboveground dry weight after drying to constant weight at 60 ºC in a forced-air oven. Moreover, Soil and Plant Analyzer Development (SPAD) readings were conducted with a portable chlorophyll meter (Konica Minolta SPAD-502 Plus; Minolta, Osaka, Japan), using the first expanded trifoliate leaf from three plants. At maturity (R8), ten plants from each subplot were taken to assess the number of pods per plant, the number of seeds per pod, number of seeds per plant, the average mass of 100 seeds, harvest index, and grain yield.

Table 3.1 - Description and ranks of yield response to eCO_2 in soybean genotypes grown in growth chamber (Soares et al., 2019a), or in FACE plots, where 1 is the rank of the most responsive and 13 is the least responsive.

Accession no	GH	Common name	Origin	Growth chamber	Yield stimulation	FACE	Yield stimulation	Average rank
PI 437101	I	DV-0197 [a]	Russia	9	-	13	-	11
PI 417554	I	EM [a]	Poland	3	+	12	-	7.5
PI 437413	I	Ussurijscaja [a]	Russia	11	-	11	-	11
PI 361097 A	I	Novosadska [a]	Serbia	4	-	10	+	7
PI 319537 A	I	Tono [a]	China	8	-	9	+	8.5
PI 538409	D	Shironomai [a]	Japan	2	+	8	+	5
PI 319534 A	I	Honshu [a]	China	6	-	7	+	6.5
PI 445829 A	I	Dunayka [a]	Romania	5	-	6	+	5.5
PI 153271	I	WB [a]	Belgium	1	+	4	+	2.5
PI 361085 A	I	L-117 [a]	Romania	12	-	5	+	8.5
PI153245	I	VDGY [a]	Germany	nd	nd	2	+	-
PI 437224	I	Cschi675 [a]	Moldova	7	-	3	+	5
PI 378676 A	I	Primorskaja [a]	Russia	10	-	1	+	5.5

Abbreviations: D, determinate; EM, Early Mandarin; GH, growth habit; I, indeterminate; VDGY, Van Dieckman Green-Yellow; WB, Wisconsin Black. (+) significant grain yield stimulation; (-) no significant grain yield stimulation; (nd) not determined.
[a] Obtained from USDA-ARS via Germplasm Resources Information Network (Washington, USA).

3.3.3. Gas exchange measurements

Gas exchange parameters were performed from each subplot in the last fully expanded leaves of three plants, at vegetative and pod filling stages. Rates of photosynthesis were determined between 10 and 16 hr on clear sunny days. Leaf photosynthetic carbon assimilation rate (A_{sat}), transpiration rate (T_r), and g_s were measured with a portable gas exchange system incorporating an infrared CO_2 and water vapor analyzers (LI-COR 6400, LI-COR, Lincoln, USA). The CO_2 concentration in the leaf chamber was controlled by the LI-COR CO_2 injection system, and irradiance of 1500 µmol photons/(m^2 s) supplied by a built-in LED lamp (red/blue). The temperature in the leaf chamber configured to 25 ºC, and CO_2 concentration to 400 or 600 ppm for each treatment. Instantaneous water-use efficiency was calculated as A_{sat}/g_s.

3.3.4. Light-induced fluorescence transient (LIFT) device

The LIFT method is a distinctive approach to probe photosystem II from a distance under natural conditions (Muller et al., 2018). The LIFT instrument (Version LIFT-REM, Soliense Inc., New York, USA) was equipped with a blue light-emitting diode (LED) (445 nm), an STS-VIS spectrometer (Ocean Optics, Florida, USA), and two

RGB cameras (FLIR Integrated Imaging Solutions Inc., British Colombia, Canada). Subsaturating actinic LED flashlets in fast repetition rate (FRR) induce the maximum fluorescence yield and monitor its relaxation with decreasing repetition rates. Chlorophyll fluorescence is detected at 685 (±10) nm. The FRR flash was used with an excitation phase of 0.75 ms consisting of 300 flashlets. The relaxation phase included 127 flashlets triggered at decreasing repetition rate and lasted for 200 ms. Fluorescence measurements were performed in the last fully expanded leaves of three plants from each subplot with five measurements per plant at vegetative and pod filling stages. The LIFT instrument was fitted to a phenotyping bike with a track width of 3 m allowing top canopy measurements from 60 to 80 cm. The operational procedures of the system were described in a previous experiment (Keller et al., 2019).

3.3.5. Grain nutritional analysis

Ten seeds from independent plants at each subplot were pooled together and used for subsequent nutritional analysis. The mean values for each plot were treated as one replicate.

3.3.5.1. Mineral analysis

Grain mineral analysis was performed as reported by Soares et al. (2019a). The seed material (200 mg) was mixed with 5 mL of HNO_3 65% (v/v), and 1 mL of H_2O_2 30% (v/v) in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was achieved as follows: 130 °C/10 min, 160 °C/15 min, 170 °C/12 min, 100 °C/7 min, and 100 °C/3 min. Each solution of the digestion procedure was brought to 50 mL with ultrapure water, and determination of mineral concentrations performed using the ICP-OES Optima 7000 DV (perkinElmer, USA). The assays were performed in duplicates and mean values calculated.

3.3.5.2. Determination of protein concentration

For each sample a total of 75 mg of flour was collected and analyzed for protein concentration (N x 5.5) using a Leco N analyzer (Model FP-528, Leco Corporation, St. Joseph, USA). The assays were performed in duplicates and mean values calculated.

3.3.5.3. Phytic acid determination

The colorimetric Wade reagent method was used for detecting phytic acid as described by Gao et al. (2007) with some adjustments. A total of 50 mg of flour was mixed with 1 mL of 0.8 N HCl:10% Na_2SO_4, shaken at 220 rpm during 16-24 h, and centrifuged at 3000 g for 20 min at 10 ºC. The extract was stored at 4 ºC in the dark for further analysis. Then, 30 µl of extract was mixed with 720 µl of distilled water and 250 µl of Wade's Reagent, vortexed for 10 sec, and an aliquot (200 µl) was read at 540 nm using a microplate reader (Synergy H1, Vermont, USA). The assays were performed in duplicates and mean values calculated.

3.3.5.4. Determination of total sugars and starch

The sugar extraction was determined based on the protocol of Chow and Landhausser (2004). For each sample, 100 mg was extracted three times with 5 ml of 80% ethanol (v/v), by boiling the samples in a 95 ºC water bath for 10 min. After each extraction, the tubes were centrifuged at 3000 rpm for 5 min, and supernatants combined for sugar analysis. Sugar quantification followed the microplate phenol–sulfuric acid assay developed by Masuko et al. (2005). Total starch was determined with kit from Megazyme according to AOAC method 996.11 (AOAC, 2006). The assays were performed in duplicates and mean values calculated.

3.3.5.5. Extraction of phenolic compounds

For the preparation of the phenolic extract, 500 mg of each sample was mixed with 10 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v), and the extract was shaken overnight at 300 rpm in the dark using an orbital shaker (Zhou et al., 2017). Then, the extract was centrifuged at 1600 rpm for 10 min, and the supernatant stored at 4 °C in the dark until further use.

3.3.5.6. Total Phenolic Content

The TPC assay was performed using the Folin-Ciocalteu colorimetric method as described by Ramos et al. (2019), with slight variations. In a 96-well plate, 150 µL of Folin–Ciocalteu reagent, and 75 µL of sodium carbonate solution (75 g/L) were added to 30 µL of soybean extracts. The mixture was incubated at room temperature in the dark and the absorbance was measured after 60 min at 750 nm, in a Thermo Scientific MultiskanTM FC microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). TPC in each sample was determined using a standard curve prepared by gallic acid (0.025–0.5 mg/mL). The result was expressed as mg of gallic acid equivalent per gram (mg GAE/g) of soybean. The assays were performed in duplicates and mean values calculated.

3.3.5.7. Antioxidant Activity – ABTS radical cation scavenging effect

The phenolic extract was used for measuring the antioxidant activity by the ABTS radical scavenging assay according to Goncalves et al. (2009). Daily, the concentration of ABTS working solution was adjusted to an initial absorbance of 0.7 at 734 nm. Then, in a 96-well plate, 280 µL of ABTS solution was added to 20 µL of sample or Trolox or solvent. After that, the mixture was allowed to react for 5 min in the dark, and the absorbance was immediately recorded at 734 nm, using a Thermo Scientific MultiskanTM FC microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Trolox was used as the reference antioxidant, and the result was expressed as mmol of Trolox equivalent per gram (mmol TE/g) of soybean. The assays were performed in duplicates and mean values calculated.

3.3.6. Statistical analysis

The 13-genotype experiment was analyzed with a split-plot mixed model analysis of variance, where CO_2 was treated as the main factor, and genotype as the split factor, using the general linear model procedure of SPSS (28.0 SPSS Inc., Chicago, IL, USA). Where significant differences were found, means were compared using Tukey's Test at 0.05 significance level. For some dependent variables, the variance was heterogeneous and, so a transformation was performed before the statistical analysis. The correlations among seed yield and agronomic traits were performed using Pearson's product-moment correlation (*r*) at 0.05 significance level. Thus, mean response of each of the genotypes exposed to eCO_2 was used to investigate how seed yield response to eCO_2 (eCO_2/aCO_2) correlated with different yield parameters. Principal component analysis (PCA) was performed on grain nutritional analysis and yield data using PAST 4 (paleontological statistics software package for education and data analysis, version 4.03.

3.4. Results

3.4.1. Yield responses to eCO_2

Growth at eCO_2 significantly stimulated yield by 46.9% ($p < 0.001$; Figure 3.1a and Table 3.2) averaged across soybean genotypes under FACE conditions. The extent of yield improvement due to eCO_2 differed significantly among the genotypes ($p < 0.001$), with a significant CO_2 x genotype interaction ($p < 0.01$). The seed yield increase of Primorskaja (89.7%) was greatest, followed by Cschi675 (75.4%), VDGY (75.0%), and WB (55.7%), whereas in DV-0197, EM, and Ussuriscaja no stimulation in seed yield was observed. WB evidenced the greatest seed yield at both CO_2 concentrations used in this study.

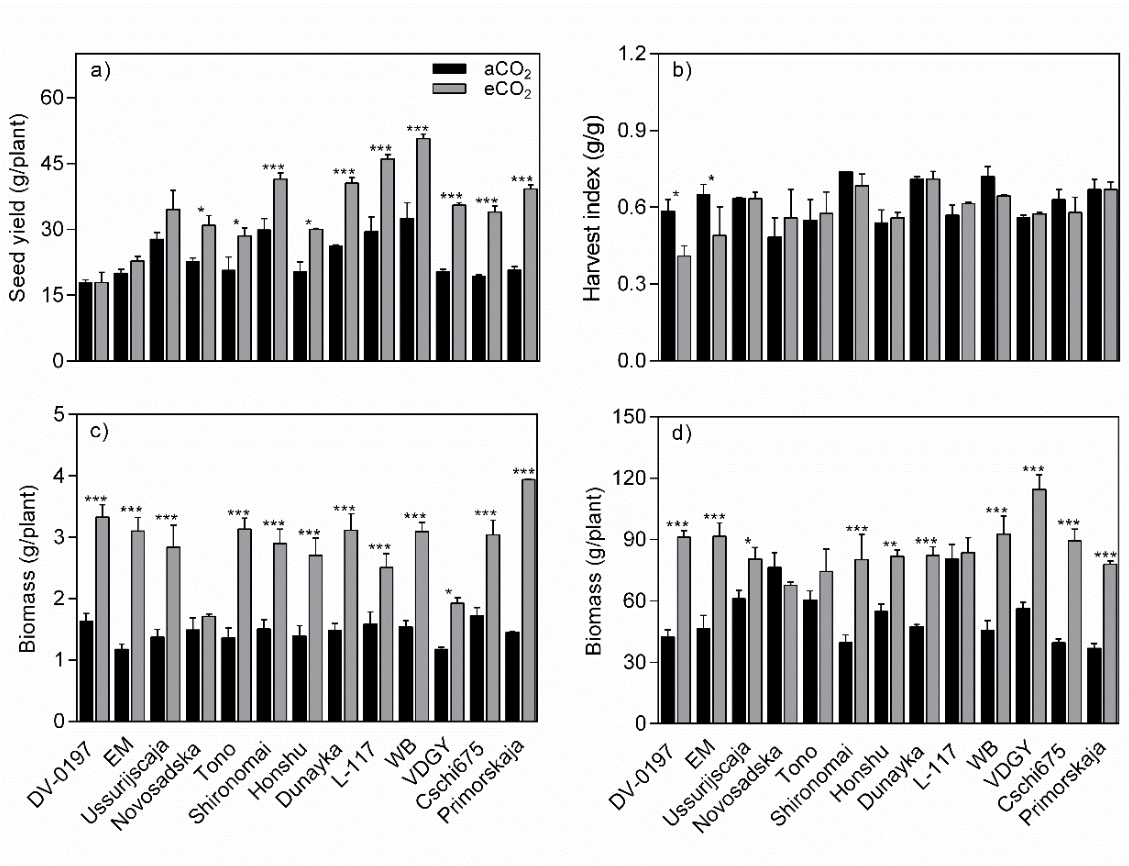

Figure 3.1 – Genotypic variation in (a) soybean seed yield, (b) harvest index, and aboveground biomass at (c) vegetative and (d) pod filling stages under eCO_2. Bars show the mean value of each variable ± standard error in 13 genotypes grown at the FACE facility in 2018. Bars with asterisk(s) indicate significant effects of CO_2 for each genotype tested. Ten plants from each subplot were sampled to assess the grain yield and harvest index, and three plants from each subplot were sampled to assess the aboveground biomass. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

Exposure to eCO_2 slightly decreased the harvest index by 4.0% ($p > 0.05$), with a significant difference among genotypes ($p < 0.05$, Figure 3.1b and Table 3.2). Moreover, the genotypes with the highest decrease in harvest index were genotypes with no yield improvement. ANOVA results showed that the aboveground dry weight was highly significant ($p < 0.05$) for CO_2, growth stage, CO_2 × genotype, CO_2 × growth stage, genotypes × growth stage and interaction of CO_2 × genotype × growth stage (Table 3.4). Under eCO_2 aboveground biomass was stimulated by 97.2% at the vegetative stage ($p < 0.05$, Figure 3.1c and Table 3.3) among soybean genotypes, while the increase in biomass was not statistically significant ($p > 0.05$) in Novosadska genotype. At the pod filling stage, eCO_2 increased aboveground biomass by 61.2% ($p < 0.05$, Figure 3.1d and Table 3.3) averaged among soybeans. This increase was significant ($p < 0.05$) in ten out of 13 genotypes. SPAD readings were highly significant ($p < 0.01$) for genotype, growth stage, CO_2 × growth stage, CO_2 × genotype, genotype × growth stage and interaction of CO_2 × genotype × growth stage. Exposure to eCO_2 increased height by 11.1 and 23.9% at vegetative and pod filling stages, respectively, and there was a significant effect for CO_2, genotype, growth stage, CO_2 × growth stage, CO_2 × genotype, genotype × growth stage and interaction of CO_2 × genotypes × growth stage. Moreover, leaf area increased by 88.5 and 59% at the vegetative and pod filling stages, respectively, due to the exposure to eCO_2 conditions. There was a significant effect for CO_2, growth stage, CO_2 × growth stage, CO_2 × genotype, genotype × growth stage and interaction of CO_2 × genotypes × growth stage (Table 3.4).

The yield parameters including the number of pods per plant (mean CO_2 effect of 63.3%, $p < 0.001$), number of seeds per plant (mean CO_2 effect of 60.3%, $p < 0.001$), and one hundred seed weight (mean CO_2 effect of -11.9%, $p < 0.001$) were significantly affected by eCO_2 conditions. However, the number of seeds per pod was not significantly ($p > 0.05$) changed by eCO_2 conditions. ANOVA showed that these yield parameters were ighly significant ($p < 0.05$) for genotype, and interaction of CO_2 × genotype (Table 3.2).

Table 3.2 – Analysis of variance of yield parameters in soybean genotypes exposed to aCO_2 (400 ppm) and eCO_2 (600 ppm), and correlations (pearson's *r*) and their statistical significance for the relationship between the relative increase in yield due to eCO_2 (value at eCO_2/value at aCO_2) and values of other parameters measured under the same conditions.

Variables	CO_2 effect	CO_2			Genotype			CO_2 x G			Correlation	*P*
	(%)	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*	*F*	*P*	*d.f.*		
Seed yield, g/plant	46.94	**181.88**	**<0.001**	1	**21.40**	**<0.001**	12	**3.33**	**0.005**	12	-	-
No pods/plant	63.27	**297.48**	**<0.001**	1	**16.85**	**<0.001**	12	**3.43**	**0.004**	**12**	**0.668**	**0.013**
No of seeds/plant	60.25	**204.48**	**0.001**	1	**12.70**	**0.001**	12	**3.02**	**0.009**	**12**	**0.865**	**<0.001**
No of seeds/pod	-3.33	1.60	0.218	1	**2.51**	**0.024**	12	**3.68**	**0.003**	12	0.492	0.088
100-seed weight, g	-11.86	**44.26**	**0.001**	1	**35.15**	**0.001**	12	**2.81**	**0.013**	12	-0.082	0.789
Harvest index, g/g	-4.03	1.61	0.217	1	**4.01**	**0.001**	12	1.13	0.381	12	0.497	0.084

Note: Results from the analysis of variance with degrees of freedom (d.f.), F ratios and probabilities (*p*) for some plant parameters. Significant effects are shown in boldface.

Table 3.3 – Analysis of the response characteristics in soybean genotypes exposed to aCO_2 (400 ppm) and eCO_2 (600 ppm) at the vegetative and pod filling stages.

Growth stage	[CO_2]	ADW	SPAD	Height	Leaf area	A_{sat}	g_s	T_r	A/g_s	Fq′/Fm′‡
Vegetative	Ambient	1.45	30.89	15.39	182.28	18.67	0.30	4.99	62.02	0.38
	Elevated	2.87	33.09	17.09	343.67	24.69	0.28	4.97	86.18	0.36
	Mean change (%)	97.23	7.12	11.05	88.54	32.24	-6.67	-0.1	39.3	-5.26
Pod filling	Ambient	52.87	35.99	48.93	4234.08	11.66	0.10	2.28	123.50	0.24
	Elevated	85.21	34.33	60.63	6732.70	14.75	0.10	2.51	156.38	0.17
	Mean change (%)	61.17	-4.61	23.91	59.01	26.50	2.04	10.1	27.1	-29.17

Note: ADW, aboveground dry weight; Fq′/Fm′‡, photosynthetic light-use efficiency. ADW (g/plant), height (cm), leaf area (cm^2/plant), A_{sat} (μmol/(m^2 s)), g_s (mol/(m^2 s)), T_r (mol/(m^2 s)), A_{sat}/g_s (μmol/mol).

Table 3.4 – Analysis of variance of the response characteristics in soybean genotypes exposed to aCO_2 (400 ppm) and eCO_2 (600 ppm)

Effect	d.f.	ADW	SPAD	Height	Leaf area	A_{sat}	g_s	T_r	A/g_s	Fq′/Fm′‡
CO_2	1	**497.8, <0.01**	3.57, 0.06	**364.29, <0.01**	**206.26, <0.01**	**317.0, <0.01**	**7.97, <0.01**	**8.87, <0.01**	**475.7, <0.01**	**49.42,<0.01**
Genotype (G)	12	1.28, 0.25	**6.46, <0.01**	**10.15, <0.01**	1.69, 0.086	2.05, 0.023	**4.87, <0.01**	**2.35, <0.01**	**4.40, <0.01**	**4.70, <0.01**
Stage (S)	1	**6663.2,<0.01**	**316.85, <0.01**	**3065.94,<0.01**	**4286.02,<0.01**	**929.6, <0.01**	**1396.3, <0.01**	**1850.1, <0.01**	**3298.8, <0.01**	**97.32, <0.01**
CO_2 x G	12	**8.28, <0.01**	**5.85, <0.01**	**4.94, <0.001**	**4.12, <0.01**	**3.24, <0.01**	**4.96, <0.01**	**2.48, <0.01**	**2.26, 0.011**	**3.09, <0.01**
CO_2 x S	1	**15.01, <0.01**	**115.54, <0.01**	**362.00, <0.01**	**169.59, <0.01**	**27.19, <0.01**	**4.77, 0.03**	**8.45, <0.01**	**339.5, 0.01**	**23.00, <0.1**
G x S	12	**7.97, <0.01**	**6.79, <0.01**	**10.14, <0.01**	**1.99, 0.036**	**3.22, <0.01**	**4.97, <0.01**	**2.53, <0.01**	**4.16, 0.01**	**2.62, <0.01**
CO_2 x G x S	12	**3.93, <0.01**	**4.21, <0.01**	**4.94, <0.01**	**3.42, <0.01**	**2.95, <0.01**	**4.13, <0.01**	**2.48, <0.01**	**2.31, <0.01**	**4.19, <0.01**

Note: ADW, aboveground dry weight; Fq′/Fm′‡, photosynthetic light-use efficiency. ADW (g/plant), height (cm), leaf area (cm2/plant), Asat (µmol/(m2 s)), gs (mol/(m2 s)), Tr (mol/(m2 s)), Asat/gs (µmol/mol). Results from the mixed model analysis of variance with degrees of freedom (d.f.), F ratios and probabilities (p) for some plant parameters. Significant effects are shown in boldface

3.4.2. Correlations between yield responses to eCO_2

The relationships between the relative increase in grain yield at eCO_2 (i.e. the value at eCO_2/value at aCO_2) were used to investigate how seed yield responses to eCO_2 correlated with different variables affecting yield. Consequently, the number of pods per plant were positively and significantly correlated ($r = 0.67, p < 0.05$) with the magnitude of seed yield response to eCO_2 (Table 3.2). The number of seeds per plant had also a strong positive correlation ($r = 0.87, p < 0.001$) with yield responses. These results indicate that genotypic variation in CO_2-based responses could be explained primarily by the higher pod production and consequently by the increased number of seeds per plant. Although no other parameters were significantly correlated with yield responsiveness to eCO_2, the plasticity in pod production seems to play an essential role in soybean yield improvement.

3.4.3. Photosynthetic assimilation rate and gas exchange parameters

ANOVA results showed that gas exchange parameters (A_{sat}, g_s, T_r, and A_{sat}/g_s) were significantly ($p < 0.05$) affected by CO_2, genotype, growth stage, CO_2 × genotype, CO_2 x growth stage, genotype × growth stage, and interaction of CO_2 × genotype × growth stage (Table 3.4). The average of A_{sat} across the soybean genotypes and the growing stages varied from 9.1 to 22.7 μmol/(m^2 s) under aCO_2 and from 13.4 to 27.8 μmol/(m^2 s) under eCO_2 (Figure 3.2). Elevated CO_2 increased significantly ($p < 0.05$) A_{sat} in all genotypes, except for L-117, at the vegetative stage, while this stimulation was only significant in seven genotypes at the pod filling stage (Figure 3.2a and b). When plants were at the vegetative stage, g_s decreased by 6.7% on average across genotypes, A_{sat}/g_s increased by 39.3%, and T_r slightly decreased by 0.1%. At the pod filling stage, g_s increased by 2.0%, T_r by 10.1%, and A_{sat}/g_s by 27.1% (Table 3.3).

3.4.4. Chlorophyll fluorescence transients

The photosynthetic light-use efficiency (Fq′/Fm′) was investigated using the automated LIFT system. ANOVA results showed that Fq′/Fm′ was significantly ($p < 0.01$, Table 3.4) affected by CO_2, genotype, growth stage, CO_2 × genotype, CO_2 x growth stage, genotype × growth stage, and interaction of CO_2 × genotype × growth stage. The

Fq′/Fm′ values ranged from 0.28 to 0.44, and from 0.05 to 0.35 at the vegetative and pod filling stages, respectively (Figure S3.1). When plants were at the vegetative stage, a significant decrease in Fq′/Fm′ was observed in EM, Ussurijscaja, Novosadska, and Tono. At the pod filling stage, the fluorescence measurements were delayed one week (late pod filling stage), regarding to the measurements of A_{sat}, due to climatic conditions. Therefore, under eCO_2 a decrease of 29.2% in Fq'/Fm' values was observed (Table 3.3). This reduction was significant in EM, Tono, Shironomai, Honshu, WB, and L-117 genotypes and was not changed in the remaining genotypes.

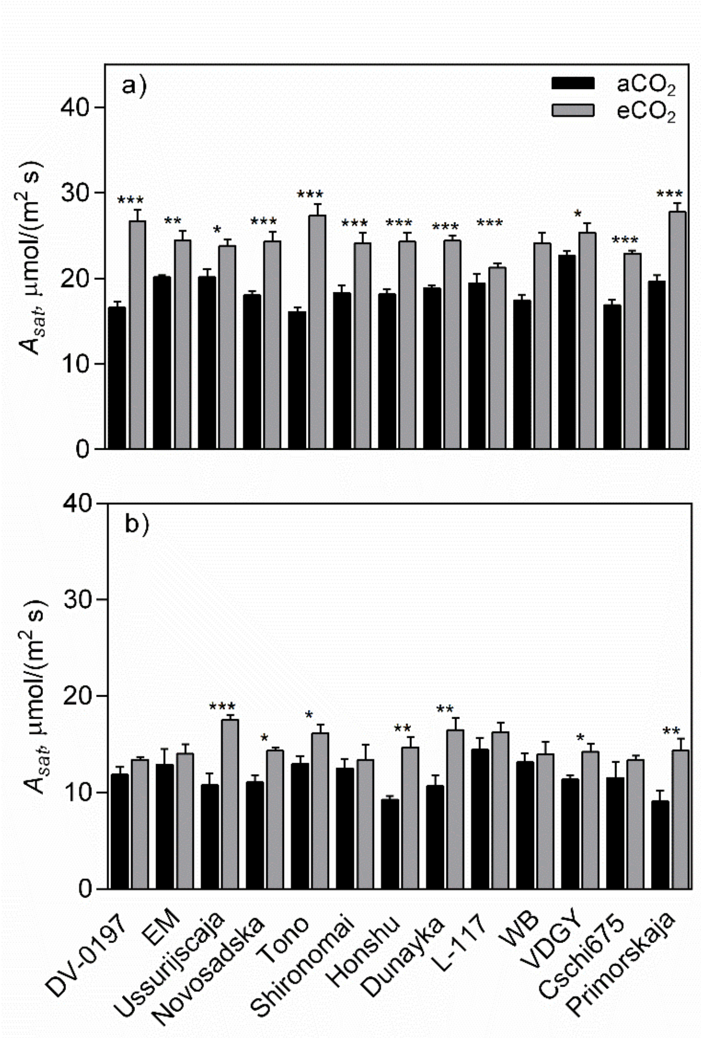

Figure 3.2 – Photosynthetic CO_2 assimilation of 13 soybean genotypes grown at aCO_2 (400 ppm) and eCO_2 (600 ppm). Values are the mean value ± standard error of the measurements made at (a) vegetative and (b) pod filling stages. Three plants from each subplot were sampled to assess the photosynthetic assimilation. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

3.4.5. Grain nutritional analysis

Elevated CO_2 affected significantly mineral concentrations in soybean grains at maturity. Calcium concentration decreased by 22.9% ($p < 0.001$, Figure 3.3 and Table 3.5) across soybean genotypes, and the concentrations responded differently to eCO_2 among cultivars ($p < 0.05$), with a significant CO_2 x genotype interaction ($p < 0.001$). The decrease was significant ($p < 0.01$) in EM, Honshu, Tono, Primorskaja, Dunayka, Cschi675, and Ussuriscaja (Table 3.6). Phosporous (P) concentration was also reduced by 9.0% ($p < 0.001$), and changed significantly among genotypes ($p < 0.001$), with a significant CO_2 x genotype interaction ($p < 0.001$). The concentration decreased by 15%, 26.3%, 20%, and 17.5% ($p < 0.01$) in Primorskaja, Cschi675, Novosadska, and WB, respectively. Potassium concentration was reduced by 11.4%, 10.7%, and 9.5% in Cschi675, Novosadska, and WB, respectively. A reduction of 10.1% ($p < 0.001$) was observed in magnesium (Mg) concentration among all soybean genotypes. Therefore, it was observed a significant decrease by 12.8%, 21.9%, 12.5%, 11.4%, and 12.6% ($p < 0.01$) in Honshu, Cschi 675, Novosadska, WB, and VDGY, respectively.

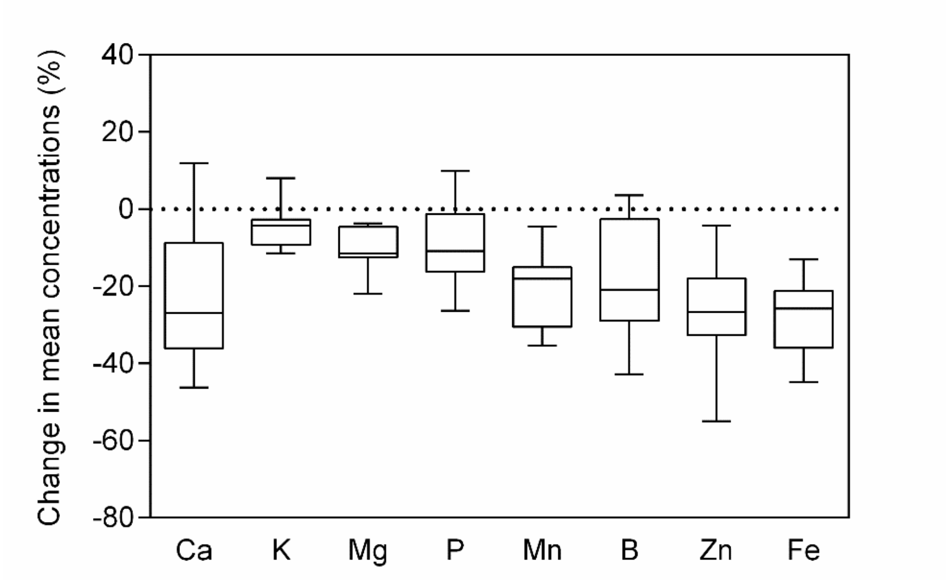

Figure 3.3 – Boxplot shows the response ratio of the grain mineral concentrations of 13 soybean genotypes. CO_2 response values are the mean value of each mineral at eCO_2/aCO_2. Ten seeds from independent plants from each subplot were pooled and used for mineral analysis.

Table 3.5 - Analysis of variance and significance levels of main effects and interactions of CO_2 and genotypes in mineral concentrations and phytochemical profiles from soybean genotypes exposed to aCO_2 (400 ppm) and eCO_2 (600 ppm).

Mineral	CO_2 effect	CO_2			G			CO_2 x G		
	(%)	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*	*df*
Ca, (mg/g)	-22.93	**85.72**	**<0.001**	1	**11.91**	**<0.001**	12	**3.54**	**<0.001**	12
P, (mg/g)	-9.02	**35.57**	**<0.001**	1	**4.84**	**<0.001**	12	**3.84**	**<0.001**	12
K, (mg/g)	-4.86	**24.81**	**<0.001**	1	1.83	0.06	12	**1.96**	**0.04**	12
Mg, (mg/g)	-10.11	**64.30**	**<0.001**	1	**5.63**	**<0.001**	12	1.59	0.11	12
Mn, (µg/g)	-21.29	**183.95**	**<0.001**	1	**11.72**	**<0.001**	12	**3.66**	**<0.001**	12
Fe, (µg/g)	-28.13	**79.51**	**<0.001**	1	**7.40**	**<0.001**	12	1.35	0.21	12
B, (µg/g)	-18.53	**113.37**	**<0.001**	1	**11.20**	**<0.001**	12	**5.74**	**<0.001**	12
Zn, (µg/g)	-25.90	**175.93**	**<0.001**	1	**4.05**	**<0.001**	12	**4.11**	**<0.001**	12
TPC, mg gallic acid/g	-5.39	2.62	0.11	1	**7.23**	**<0.001**	12	**4.84**	**<0.001**	12
ABTS, mmol Trolox/g	-36.87	**414.20**	**<0.001**	1	**12.48**	**<0.001**	12	**10.36**	**<0.001**	12
Sugar, %	9.07	**5.94**	**0.02**	1	**5.03**	**<0.001**	12	**1.96**	**0.04**	12
Starch, %	16.00	**6.74**	**0.02**	1	**12.17**	**<0.001**	12	0.41	0.95	12
Protein, %	-5.63	**37.44**	**<0.001**	1	**3.51**	**<0.001**	12	1.90	0.05	12
Phytate, %	8.10	**21.49**	**<0.001**	1	**2.17**	**0.015**	12	**3.01**	**<0.001**	12

Note: Results from the analysis of variance with degrees of freedom (d.f.), F ratios and probabilities (*p*) for some plant parameters. Significant effects are shown in boldface.

Table 3.6 – Effects of eCO_2 on soybean seed mineral concentrations from soybean genotypes exposed to aCO_2 (400 ppm) and eCO_2 (600 ppm).

	Ca (mg/g)		**P (mg/g)**		**K (mg/g)**		**Mg (mg/g)**		**Mn (µg/g)**		**Fe (µg/g)**		**B (µg/g)**		**Zn (µg/g)**	
Genotype	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
L-117	1.71	1.53	3.70	4.05	18.43	17.70	2.20	2.11	22.50	19.05	52.97	40.58	15.75	15.05	35.50	33.97
EM	**2.10**	**1.30**	3.57	3.92	18.14	17.38	2.23	2.08	**30.27**	**20.18**	**74.34**	**55.15**	21.86	22.64	38.94	32.79
DV-0197	1.25	1.17	4.16	3.75	17.07	16.76	2.21	1.96	19.48	18.60	50.19	40.80	**21.68**	**17.16**	34.84	27.86
Honshu	**1.97**	**1.29**	4.33	3.83	18.17	17.66	**2.43**	**2.12**	**25.82**	**16.83**	**67.01**	**36.94**	**21.07**	**14.97**	**39.67**	**29.08**
Tono	1.53	0.96	3.66	3.79	17.49	17.03	2.13	2.04	**22.91**	**16.56**	**51.97**	**34.48**	**19.73**	**11.29**	**38.97**	**27.86**
Primorskaja	1.77	1.29	**4.47**	**3.80**	18.59	17.62	2.33	2.13	**24.32**	**18.38**	**60.32**	**41.23**	**21.37**	**16.60**	**40.63**	**29.14**
Dunayka	**1.82**	**1.22**	3.65	3.31	18.52	17.31	1.96	1.89	**24.21**	**18.79**	41.48	32.02	20.46	17.13	**39.14**	**26.37**
Cschi675	**1.39**	**0.93**	**4.69**	**3.45**	**18.56**	**16.44**	**2.38**	**1.86**	**19.88**	**16.28**	**52.50**	**32.41**	**24.54**	**17.46**	**37.51**	**24.79**
Novosadska	1.34	1.25	**3.98**	**3.19**	**18.45**	**16.48**	**2.15**	**1.88**	**22.57**	**18.76**	42.61	32.90	**18.41**	**13.17**	**32.38**	**24.19**
Shironomai	0.85	0.95	3.54	3.32	18.62	16.95	1.98	1.88	17.87	14.75	36.49	31.72	20.08	19.94	**52.61**	**25.43**
WB	1.31	1.17	**3.98**	**3.29**	**18.30**	**16.56**	**2.10**	**1.86**	**22.07**	**19.51**	**41.11**	**33.12**	19.96	20.57	**32.63**	**25.44**
Ussurijscaja	**1.36**	**0.73**	3.67	3.16	17.14	18.52	2.06	1.72	20.64	13.34	**51.28**	**29.44**	**25.26**	**16.74**	36.04	23.30
VDGY	1.24	0.92	4.18	3.72	19.00	18.36	**2.15**	**1.88**	20.54	17.52	47.05	33.56	23.40	18.69	34.89	30.59

Note: Significant differences ($p < 0.05$) between aCO_2 and eCO_2 within a genotype are shown in boldface.

In terms of micronutrients, the reduction was greatest for Fe and Zn, decreasing by 28.1%, and 25.9% averaged among genotypes, respectively. Manganese (Mn) concentration was also significantly reduced by 33.3%, 34.8%, 27.7%, 24.4%, 22.4%, 18.1%, 16.7%, and 11.7% in EM, Honshu, Tono, Primorskaja, Dunayka, Cschi 675, Novosadska, and WB, respectively. Consistent decreases in boron (B) concentration among genotypes were also found under eCO_2, with a reduction of 20.9%, 29%, 42.8%, 22.3%, 28.9%, 28.5%, and 33.7% in DV-0197, Honshu, Tono, Primorskaja, Cschi 675, Novosadska, and Ussuriscaja, respectively ($p < 0.05$). The magnitude of variation in micronutrient concentrations varied significantly among genotypes, with a significant CO_2 x genotype interaction ($p < 0.001$), except for Fe. Genotypes with high mineral content at eCO_2 might be a crucial trait for breeding programs. Consequently, EM exhibited simultaneously the highest concentration of B (22.6 μg/g), Fe (55.2 μg/g), and Mn (20.2 μg/g), and L-117 exhibited the highest content of P (4.1 mg/g), Ca (1.5 mg/g), and Zn (34 μg/g).

Elevated CO_2 did not influence the TPC when compared with aCO_2 ($p > 0.05$; Figure 3.4 and Table 3.5), but a significant difference across genotypes was observed ($p < 0.001$), with a CO_2 x genotype interaction ($p < 0.001$). The ABTS values decreased significantly from 32.88 to 20.76 mmol Trolox/g ($p < 0.001$), with significant differences among genotypes ($p < 0.001$) and CO_2 x genotype interaction ($p < 0.001$). Soluble sugar and starch concentrations in soybean grains improved due to eCO_2 conditions by 9.1% and 16.0% ($p < 0.05$) averaged across soybean genotypes, respectively. We also evaluated phytate, a phosphate storage molecule that inhibits the absorption of some nutrients in humans. Phytate content increased significantly at eCO_2 ($p < 0.001$), and the extent of change varied between genotypes ($p < 0.05$), with CO_2 x genotype interaction ($p < 0.001$). Elevated CO_2 reduced grain protein concentration by 5.6% ($p < 0.001$). This decrease was significant in Tono, L-117, Cschi675, DV-0197, Primorskaja, and VGDY with a reduction of 13.3, 11.7, 9.0, 8.6, 7.2, and 6.4%, respectively (Figure 3.5).

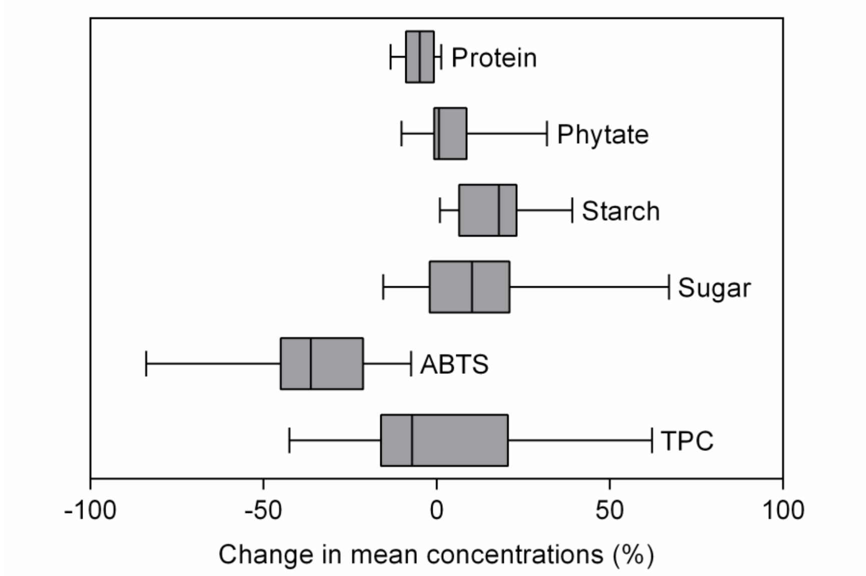

Figure 3.4 – Boxplot shows the response ratio of the grain phytochemical profiles of 13 soybean genotypes under eCO_2. CO_2 response values are the mean value of each variable at eCO_2/aCO_2. Ten seeds from independent plants from each subplot were pooled and used for phytochemical analysis. TPC, total phenolic content; ABTS, 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

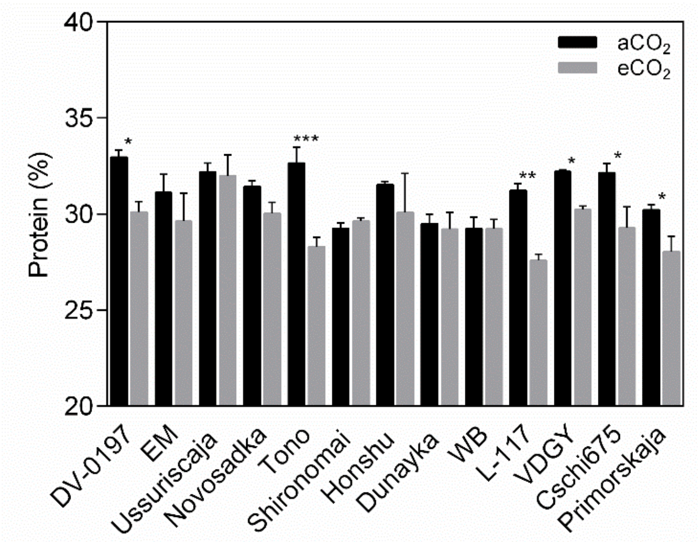

Figure 3.5 – Genotypic variation in grain protein response under eCO_2 conditions. Bars show the mean value ± standard error in 13 genotypes grown at the FACE facility in 2018. Ten seeds from independent plants from each subplot were pooled and used for protein analysis. * *$p < 0.05$*; ** *$p < 0.001$*; * *$p < 0.0001$*.**

3.4.6. Nutritional analysis association with soybean yield

The PCA was performed in order to associate the responses at eCO_2 of mineral concentrations and phytochemical profiles to that of grain yield. The results (Figure S3.2) shows the diversity of the samples, and also the identification of the variables responsible for that differentiation. The biplot revealed two principal components, together explaining 50.7% of the observed variability. The genotypes were mainly discriminated by PC1, with differentiation between genotypes growing at aCO_2 and eCO_2. The first principal component PC1 explained 38.1% of the variance showing a reduction in the grain nutritive value observed mainly through a decrease in mineral and protein concentrations, and also in the antioxidant activity. The genotypes positioned on the right-hand side of the PCA plot were those grown under aCO_2 conditions, and showed higher levels of the minerals, protein concentration, and antioxidant activity. The second principal component PC2 was responsible for 12.6% of the variation and positively correlated with starch and yield and negatively correlated with protein. Thus, PC2 showed the separation of the samples in the vertical direction, and the genotypes positioned in the higher half and on the left-hand side of the PCA scores plot contained higher grain yield and starch content. Therefore, genotypes such as WB, Primorskaja, and L-117 are probably good candidates for selection in future breeding programs mainly due to their high yield capacity and less affected in the grain quality. PCA shows that greater grain yield stimulation under eCO_2 was associated with a reduction in mineral concentrations, probably suggesting a yield dilution effect.

3.5. Discussion

Advances in soybean genetics, the discovery of new or improved genotypes, innovations in farming practices, and the increase in atmospheric CO_2, have greatly contributed to increase in soybean yield. However, the extent of yield enhancement is possibly insufficient to meet the future demands of a growing global population (Bishop et al., 2015). This study showed genotypic variation in soybean yield responses under FACE conditions ranging from no significant changes, to an increase in seed yield of almost 90%, and the averaged increase was 46.9% among all genotypes (Figure 3.1 and Table 3.2). DV-0197, EM, and Ussuriscaja did not increase seed yield under eCO_2, whereas Primorskaja was the most responsive genotype to eCO_2, followed by Cschi675, and VDGY. The best-adapted genotypes to aCO_2, were also the genotypes with the greatest seed yield at eCO_2 (viz. WB, L-117, and Shironomai) suggesting that the best-adapted genotypes to the current CO_2 might be useful in the upcoming CO_2 concentration. The genotypes investigated in the current study (Table 3.1) were previously grown in a controlled environment under hydroponic conditions at aCO_2 (400 ppm) and eCO_2 (800 ppm) conditions (Soares et al., 2019a). The range of soybean yield responses to eCO_2 was -23.8 to 39.6% with mean change of 7.1%. This contrasts with yield stimulation of 46.9% under FACE conditions corresponding to more than six times that of plants grown in hydroponic solutions. The reduction in seed yield increase was associated with the physical restriction in hydroponic root growth, since the volume for root growth was <2 L. Similarly, Ainsworth et al. (2002) also highlighted the effect of pot size in soybean growth and yield in a meta-analysis. The authors described that even large pots (>9 L) failed to predict the increase in yield seen in soybeans planted in the ground stimulation. Thus, seed yield increase in large pots was 12%, while yield stimulation of soybeans planted in the ground was 38% (Ainsworth et al., 2002). Therefore, there was little consistency between both studies. However, DV-0197 and Ussuriscaja did not increase yield at eCO_2 and were consistently unresponsive genotypes. Furthermore, WB and Shironomai showed a significant seed yield and biomass response to eCO_2 under controlled environment and FACE conditions (Table 3.1). From our results, and others (Ainsworth et al., 2002, Bishop et al., 2015, Bunce, 2014, Kumagai et al., 2015, Soares et al., 2019a, Ziska et al., 2001) yield responses to CO_2 enrichment varied considerably between genotypes, ranging from –10% to 90% for soybean (Bishop et al., 2015, Kumagai et al., 2015, Ziska et al., 2001, Soares et al., 2019a). Yield performance at eCO_2 is essential for selecting CO_2-responsive genotypes. To our knowledge, only Bishop et al. (2015) described the genotypic variation in soybean responses under FACE conditions using more than two genotypes simultaneously. Furthermore, it was our purpose to understand which characteristics would best predict yield responses. We found that number of pods ($r = 0.67$, $p < 0.05$), and number of seeds per plant ($r = 0.87$, $p < 0.001$) were useful indicators of the yield responses at eCO_2 conditions (Table 3.2). Moreover, the harvest index, i.e. the proportion of biomass partitioned into seeds, was not significantly changed by eCO_2 ($p > 0.05$). Therefore, in such conditions of more carbohydrates provided by photosynthesis stimulation, this suggests that there were no sink limitation restricting the capacity to generate more seeds. Therefore, the effect of CO_2 was mainly an increase in biomass and, consequently, an increase in the number of pods that reached maturity with filled seeds. There was also a weak positive correlation between changes in harvest index ($r = 0.50$, $p = 0.084$, Table 3.2) and yield at eCO_2, such that genotypes with significant reduction in harvest index showed no seed yield stimulation (viz. DV-0197 and EM). We also analyzed photosynthetic parameters, and it was observed that yield prediction is not directly correlated from leaf photosynthesis due to the influence of other factors, such as respiration, leaf growth, partitioning of assimilates, flowering, and pod setting (Steduto et al., 1997). Our results demonstrated that eCO_2 decreased leaf chlorophyll content (Table 3.3) at the pod filling stage implying that chlorophyll turnover might occur at this stage. It is generally accepted that photosynthesis acclimation occurs when the sink capacity is reduced (Morgan et al., 2001). In this study, we found an increase in A_{sat} under eCO_2 at either vegetative and pod filling stages (Figure 3.2 and Table 3.3), as also a significant increase in pod formation to avoid sink limitation. Interestingly, Fq′/Fm′values decreased at the pod filling stage and might be related to the start of leaf senescence and carbon remobilization to the new sinks. This could be explained by the fact that fluorescence measurements were made a week later than the gas exchange measurements due to the weather conditions.

We also studied the effects of CO_2 concentration on the grain nutritional quality since CO_2 enrichment can lead to changes in nutrients accumulation and pose a potential challenge to human health (Li et al., 2018b). Data evaluation demonstrates that eCO_2 shifts total mineral content towards a reduced level compared to aCO_2; the mean change across all the minerals is -17.6%. Elevated CO_2 significantly reduced Ca by 22.9%, P by 9.0%, K by 4.9%, Mg by 10.1%, Mn by 21.3%, Fe by 28.1%, B by 18.5%, and Zn by 25.9% (Figure 3.3). The magnitude of variation across mineral concentrations differed among soybean genotypes ($p < 0.05$), except for K (Table 3.5). The reduction in mineral concentrations was exacerbated under FACE conditions in relation to the growth chamber study of Soares et al. (2019a). This evidence probably reflects the significance of the greater dilution effect caused by the increase in carbon allocation in the current study. Loladze (2014) also found a decline in P, K, Ca, Mg, and Zn concentrations in foliar and edible tissues under FACE conditions, including wheat, barley, and rice. A reduction in grain Fe concentration has been reported in rice, wheat, barley, pea, and soybean, and Mn in rice and pea at FACE conditions (Myers et al., 2014). Wu et al. (2004) also suggested that nutrient concentrations (N, P, K, Zn) in wheat grains decreased by eCO_2. This phenomenon increases the incidence of nutrient deficiency and other related diseases, and current plant breeding programs have been focused on higher yields instead of preserving grain nutritional quality (Fernando et al., 2014). Consequently, genotypes with high mineral content and high yield capacity under eCO_2 might be important traits from a breeding perspective. Thus, among the high-responsive genotypes, L-117 had simultaneouly the highest concentration of P, Ca, and Zn. The exact mechanisms for the decrease in grain mineral concentrations remain unclear. Some authors have proposed this phenomenon to the dilution effect caused by the increased biomass under eCO_2 (Parvin et al., 2019, Gifford et al., 2000, Li et al., 2019). However, inhibition of photorespiration and malate production, decreased mass flow due to reduced transpiration rate might also be relevant in explaining the reduced mineral levels under eCO_2 conditions (Bloom, 2015, Pleijel et al., 2000, Gifford et al., 2000). Legumes are a great source of phenolic compounds which play substantial roles in many physiological and metabolic processes, and are directly related to the antioxidant activity (Singh et al., 2017). Data obtained in this study showed that plants grown under eCO_2 have lower antioxidant activity by 36.9%, but no significant effect was found on the TPC (Figure 3.4 and Table 3.5). These findings are consistent with previous studies showing that eCO_2 could induce a decrease in antioxidant capacity in fruit vegetables (Dong et al., 2018b), rice (Goufo et al., 2014), and soybean leaves (Gillespie et al., 2012). Pérez-López et al. (2018) suggested that CO_2 enrichment can reduce photorespiration, decreasing the formation of oxygen radicals, showing no need to induce antioxidant synthesis. This eCO_2-induced decrease in antioxidants of soybean seeds might have a great influence on human diet and on the food industry that produces antioxidants from soybean grains (Zheng et al., 2020). In the currrent study, eCO_2 increased sugar, and starch in soybean grains by 9.1% ($p < 0.05$), and 16.0% ($p < 0.05$), respectively; whereas, mean values of seed protein was lowered by 5.6% ($p < 0.001$, Table 3.5). Besides, CO_2 enrichment increased the concentration of soluble sugars in potato, and starch in potato and wheat using open-top chambers as described by Högy and Fangmeier (2008) and Kumari and Agrawal (2014). Although soybean plants can symbiotically fix N, to alleviate N deficiency, shortcomings still occur under eCO_2 conditions. Many studies support that lower seed protein concentration at eCO_2 can be attributed to accumulation of non-structural carbohydrates (Gifford et al., 2000, Wu et al., 2004). This evidence was supported by the greater increase in plant biomass, and consequently a great reduction in protein content, under FACE conditions as opposed to the growth chamber experiment described by Soares et al. (2019a). However, other mechanisms than carbohydrate dilution alone, might all be relevant to explain this phenomenon (Soares et al., 2019a, Dietterich et al., 2015, Myers et al., 2014). Thus, lower levels of protein could have nutritional implications for humans that use these crops as a food source. We also report phytic acid, a molecule present in most plants that has the potential for binding to positively charged protein, amino acids, and minerals in foods reducing their absorption in the human gut (Weaver and Kannan, 2002). This molecule increased at eCO_2 by 8.10% ($p < 0.01$, Figure 3.4 and Table 3.5), and might intensify complications of nutrient deficiency. At eCO_2, an increase of 1.2% and 12.8% in phytic acid concentration was also found in rice and sorghum, respectively (Myers et al., 2014). Therefore, genotypes such as WB, Primorskaja, and L-117 are probably good candidates for selection in future breeding programs mainly beacause of their yield capacity and resilience to grain quality losses.

3.6. Conclusion

In conclusion, this study showed that there is a variation among soybean genotypes grown in field conditions under eCO_2 conditions and that genetic background has the potential to adapt to the upcoming atmospheric CO_2 concentrations. Exploiting this genetic diversity in crops can help to mitigate the negative impacts of climate change and improve crop yields in the future. Our results suggest that eCO_2 has positive effects on the soybean yield but decreases the grain content of protein, minerals, and antioxidant capacity. However, it does appear that yield increase was driven by responsiveness in number of pods, and increased number of seeds. Therefore, it is essential to design strategies with a focus on increasing yield responses and select genotypes with minor nutritional losses that may occur under eCO_2. Overall, WB, Primorskaja and L-117 genotypes appear to be particularly promising to breed soybean to the future atmospheric conditions.

CHAPTER 4 - Transcriptomic and physiological analyses under elevated CO_2 conditions in soybean plants

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4.1. Abstract

Carbon dioxide is a chemical compound required for plant growth and development that influences the molecular response mechanisms of the soybean genome under elevated CO_2 (eCO_2), which are still not fully understood. In this study, soybean plants were grown under hydroponic conditions at ambient CO_2 (aCO_2, 400 ppm) and eCO_2 (800 ppm) conditions for three weeks. Plant dry weight increased by 46.9%, and soluble carbohydrates from 12.1 to 26.2 mmol g FW^{-1} in roots and from 33.6 to 52.2 mmol g FW^{-1} in leaves under eCO_2. The photosynthetic rate did not change at eCO_2, while stomatal conductance and transpiration rates decreased by 64.2% and 52.9%, respectively.

Gene expression analysis showed that in root tissues of soybean plants grown under eCO_2, 828 genes were differentially expressed (544 upregulated and 284 downregulated genes). Similarly, in leaf tissues, 3083 genes were differentially expressed (1717 upregulated and 1366 downregulated genes). Further analysis of KEGG pathways showed that differentially expressed genes were enriched in photosynthesis,

biosynthesis of secondary compounds, nitrogen metabolism, fatty acid metabolism, and circadian rhythm-related genes in both leaves and roots. Besides, eCO_2 significantly changed the expression of 362 transcription factors presumably involved in adaptation to eCO_2 conditions, suggesting that eCO_2 induces signaling pathways in soybean. The larger transcription factor families include ERF, MYB, bHLH, bZIP, NAC, and WRKY. Therefore, it was evident that leaf and root tissues in soybean plants respond differentially to CO_2 enrichment revealing key players in CO_2-based responses.

4.2. Introduction

Earth's atmospheric CO_2 concentration has been gradually increasing (420 ppm by June 2022) and is projected to reach about 500-700 ppm by 2050 and 650-1000 ppm by 2100, contributing to a global temperature increase of 2 to 4 °C (Huang et al., 2019). Soybean (*Glycine max*) is an essential leguminous crop worldwide since it is an important protein source in animal feed and human food and a source of dietary cooking oil (Schmutz et al., 2010, Zeng et al., 2018). Besides, soybean is an essential source of carbohydrates, amino acids, and mineral elements, all of which have considerable nutritional value (Singh et al., 2016). The physiological responses of soybean to elevated CO_2 (eCO_2) have been characterized (Bunce, 2016, Ainsworth et al., 2002, Bishop et al., 2015, Kimball, 2016, Kumagai et al., 2015, Ziska et al., 2001) and it is assumed that eCO_2 increases carbon uptake, carbohydrate content, plant growth, and seed yield while decreasing stomatal conductance (Ainsworth et al., 2006). The seed yield increase in C_3 grasses and grain legumes was about 19% and 16% due to eCO_2, respectively, under sufficient N and H_2O for most FACE experiments (Kimball, 2016). Moreover, eCO_2 changes the mineral composition of crop plants by affecting the uptake and distribution throughout the plant (Soares et al., 2019b). C3 grains and legumes grown under field conditions at eCO_2 have lower zinc and iron concentrations, and C3 crops other than legumes also have lower protein concentrations (Myers et al., 2014). Molecular changes under eCO_2 have been already reported in poplar trees (Gupta et al., 2005, Taylor et al., 2005), *Arabidopsis* (LI et al., 2008b, Li et al., 2006), maize (Huang et al., 2019), rice (GESCH et al., 2003), wheat (Lin et al., 2016b), cucumber (Song et al., 2020), chickpea (Palit et al., 2020a), and soybean (Ainsworth et al., 2006), and often CO_2 enrichment has been studied in combination with other stresses (Gupta et al., 2005, Zhang et al., 2018b, Wang et al., 2009). The response of plants to eCO_2 is complex, involving many regulatory proteins, signaling pathways, and biomolecules (Song et al., 2020). Ainsworth et al. (2006) reported upregulation of genes encoding for ribosomal proteins, cell cycle, and cell wall loosening, required for cytoplasmic growth and cell proliferation in soybean-growing leaves under FACE conditions. Identification of 327 CO_2-responsive genes suggested that eCO_2 induces the respiratory breakdown of carbohydrates providing energy and biochemical precursors for leaf expansion and growth. Moreover, Lin et al. (2016b) showed that under eCO_2 conditions, some functional genes related to energy metabolism and photosynthesis were enriched in wheat leaves grown in field conditions. In another study, Song et al. (2020) also reported several differentially expressed genes (DEGs) involved in chlorophyll biosynthesis, chlorophyll-binding protein, and chloroplast development. The transcriptome of *Populus* indicated that during long-term adaptation to eCO_2, some of the most CO_2-responsive transcripts include important regulators of carbon metabolism and signaling molecules. Moreover, the study revealed that the leaf developmental stage was of greater significance than CO_2 concentration in determining the gene-expression response (Taylor et al., 2005). The CO_2-based responses of *Arabidopsis thaliana* under field conditions included a reduction in gene expression associated with chloroplast functions. Variation among ecotypes in gene regulation was also associated with carbohydrate biosynthesis and partitioning, N-allocation, amino acid metabolism, cell wall biosynthesis, and hormone responses (Li et al., 2006). Besides, changes in pathways involved in carbohydrate metabolism, chlorophyll, and secondary metabolism, deciphered the crosstalk operating in response to chickpea grown in open-top chambers under eCO_2 conditions (Palit et al., 2020a).

We analyzed the soybean responses under eCO_2 using transcriptome and physiological analysis. We hypothesized that plants had developed common and different metabolic pathways between the aboveground and underground tissues in adaptation to eCO_2 conditions. As far as we know, this is the first study that provides information about the coordinated response of the root and leaf of soybean under eCO_2 to understand the signaling mechanisms between different organs. Moreover, DEGs identification following exposure to eCO_2 conditions can deepen our understanding of the plant responses to coping with the upcoming atmospheric CO_2 concentration. Therefore, this study has the potential to reveal novel genes and pathways responsible for adaptation to eCO_2 in soybean plants.

4.3. Materials and methods

4.3.1. Plant material and growth conditions

A strong-CO2 responsive soybean (var. Winsconsin Black) (Soares et al., 2019a) was chosen as plant material, and the study was performed at the Biotechnology School from Catholica University (Portugal). Plants were grown under hydroponic conditions in a controlled environment with the following solution: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4.7H_2O$, 0.2 mM $NH_4H_2PO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 0.5 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.1 µM $NiSO_4$, and 20 µM Fe(III)-EDDHA. The solution was buffered with MES (1 mM, pH 5.5) and changed every 3 days. The growth chamber was controlled to maintain the temperature at 25 ºC (day period) and 20 ºC (dark period), relative humidity at 75%, and photosynthetic photon flux density at 325 µmol s^{-1} m^{-2} (daytime light). The experiment was performed at aCO_2 (400 ppm) and eCO_2 (800 ppm) for 3 weeks.

4.3.2. Determination of plant biomass

At the end of the experiment, plant material was dried at 70 °C until constant weight for determination of plant biomass.

4.3.3. Leaf gas exchange parameters

Leaf gas exchange measurements were measured on day 17 after CO_2 treatment. We randomly selected the first expanded trifoliate leaf from 3 plants (n = 9) using a portable photosynthesis system (LI-6400XT; LICOR, Inc.). The CO_2 in the leaf chamber was set to match the CO_2 treatment with a photosynthetic photon flux density of 500 µmol photon m^{-2} s^{-1} at 25 °C. Moreover, the transpiration rate and stomatal conductance were also determined.

4.3.4. Root and leaf carbohydrates

Root and leaf samples (n = 5) were evaluated for carbohydrate analysis at the end of the experiment. The extraction protocol was described by López-Millán et al. (2009). About 100 mg of plant material was grounded using liquid nitrogen, suspended in 5 mM H_2SO_4, vortexed for 30 s, and then boiled for 30 min. The samples were centrifuged at

2320 x g for 10 min, supernatant was filtered through a 0.45 mm PTFE filter, and the volume adjusted to 2 mL and stored at –80 °C until further analysis. The HPLC system consisted of an ion exchange amines HPX-87H Column (300 x 7.8 mm) (Bio-Rad, USA) maintained at 40 °C, and the mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}.

4.3.5. Transcriptome analysis

4.3.5.1. RNA extraction

Root (n =3) and leaf (n = 2) samples were harvested and immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The total RNA was isolated using the Plant RNeasy kit (Qiagen, CA) following the manufacturer's instructions. RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies, USA) and checked by agarose gel.

4.3.5.2. Library preparation and sequencing

Poly(A) enriched strand-specific libraries were generated with the TruSeq Stranded mRNA sample preparation kit (Illumina) from approximately 1 µg of high-quality total RNA from each sample. Sequencing was performed in Genoinseq (Cantanhede, Portugal) on a NextSeq 550 Illumina sequencer (Illumina) with the NextSeq 500/550 High Output v2.5 kit (Illumina). All procedures were carried out according to manufacturer's instructions.

4.3.5.3. Data processing

Raw reads were extracted from Illumina Nextseq® System in fastq format and quality-filtered with fastp version 0.20.0 (Chen et al., 2018) to trim bases with an average quality lower than Q25 in a window of 5 bases, to remove polyadenylated tails bigger than 10 bases, and remove reads with less than 30 bases. The rRNA content was assessed by sortMeRNA version 2.0 (Kopylova et al., 2012). High-quality reads were mapped against the *Glycine max* genome (version 2.1) extracted from Ensembl, using TopHat version 2.0.10 (Trapnell et al., 2009). Cufflinks version 2.2.1 (Trapnell et al.,

2012) was used to assemble the aligned sequence into transcripts, and construct a map of the transcriptome guided by the reference transcript annotation extracted from Ensembl. Downstream analyses of FPKM data were conducted using iDEP 0.95 (integrated Differential Expression and Pathway analysis) online tools (Ge et al., 2018). DEGs were identified by comparing the eCO_2-treated and aCO_2-treated samples using the R package limma. Genes with FPKM values >1 in at least one sample were selected. Genes with a fold change ≥ 2 and false discovery rate (FDR) < 0.05 were considered differentially expressed. Enrichment analysis based on Gene Ontology (GO) (Ashburner et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017) databases were applied to analyze DEGs using g:Profiler (Raudvere et al., 2019). Enrichment analysis was performed for annotated genes only. The g:SCS threshold was chosen as the significance threshold and set to $p < 0.05$, and results were filtered to display only terms sized between 5 and 500 genes. Enriched GO terms were visualized using the Enrichment Map plugin (Merico et al., 2010) in Cytoscape v.3.9 (Shannon et al., 2003) based on the protocol by Reimand et al. (2019). Gene annotation was retrieved from the Phytozome database and the transcription factors (TFs) from the PlantTFDB database (Zhang et al., 2011).

4.4. Results

4.4.1. Plant growth and physiology

Soybean seedlings were exposed to aCO_2 (400 ppm) and eCO_2 (800 ppm) for three weeks in a controlled environment. The results showed that plant dry weight increased by 46.9% in plants exposed to eCO_2 (Figure 4.1a). In roots, soluble sugar concentrations increased from 12.1 to 26.2 mmol g FW^{-1} and from 33.6 to 52.2 mmol g FW^{-1} in leaves (Figure 4.1b). The photosynthetic rate was not affected by eCO_2 (Figure 4.2a), while stomatal conductance (Figure 4.2b) and transpiration rate (Figure 4.2c) decreased by 64.2% and by 52.9%, respectively.

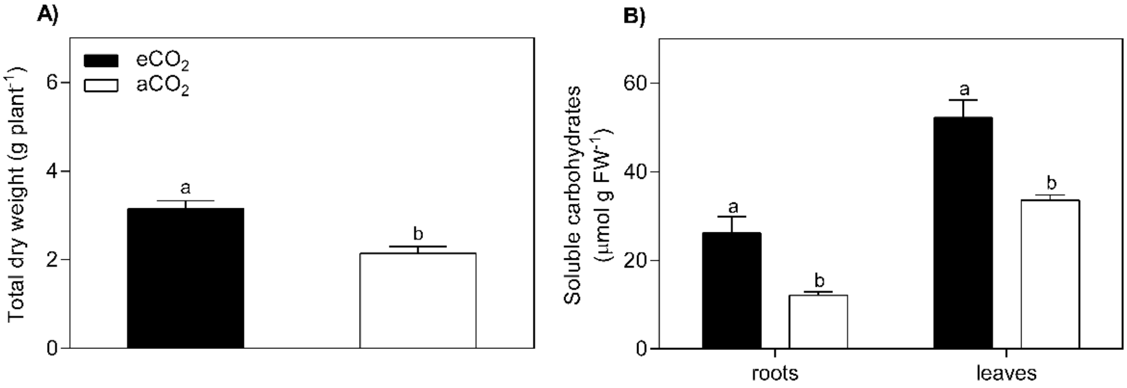


Figure 4.1 - Plant dry weight (a) and root and leaf level contents of soluble carbohydrates (sucrose, glucose, fructose; b) in soybean seedlings grown at different levels of CO_2 (aCO_2 = 400 ppm and eCO_2 = 800 ppm). Different letters indicate significant differences by Student′s t-test at $p < 0.05$ between aCO_2 and eCO_2. Values represent the means ± SE (n = 5).

4.4.2. Analysis of differentially expressed genes (DEGs)

The cluster analysis showed several genes differentially expressed in the roots and leaves of soybean plants grown under CO_2 enrichment (Figures S4.1 and S4.2). To determine the differences between gene expression at eCO_2 and aCO_2, a fold change ≥ 2 and *p*-value < 0.05 were set as the threshold. In roots, gene expression analysis revealed 828 transcripts differentially expressed under CO_2 enrichment (Figure 4.3a and Table S4.1). These include 544 upregulated and 284 downregulated genes.

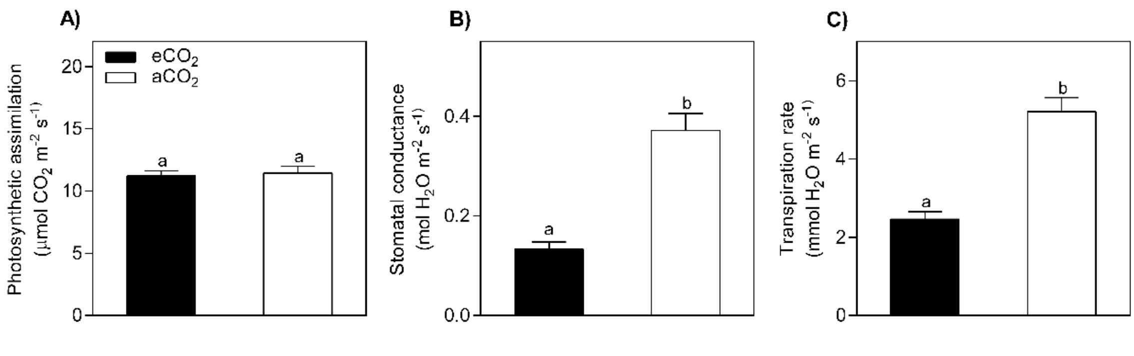


Figure 4.2 - Photosynthetic assimilation (a), stomatal conductance (b), and transpiration rate (c) of soybean seedlings grown at different levels of CO_2 (aCO_2 = 400 ppm and eCO_2 = 800 ppm). Different letters indicate significant differences by Student′s t-test at $p < 0.05$ between aCO_2 and eCO_2. Values represent the means ± SE (n = 5).

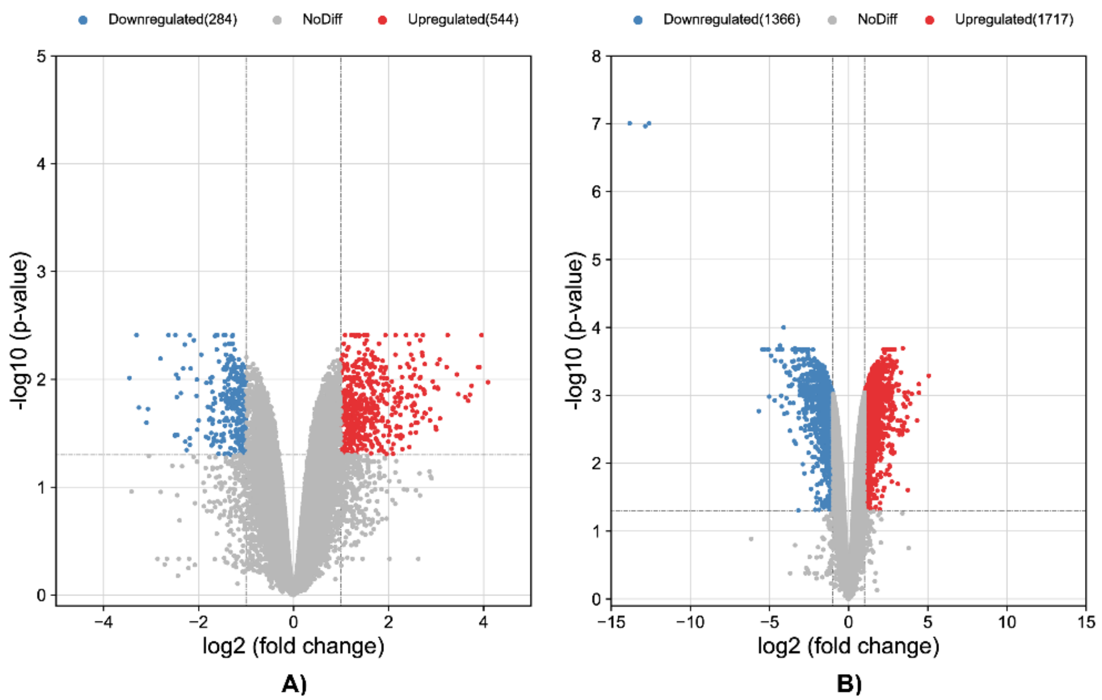
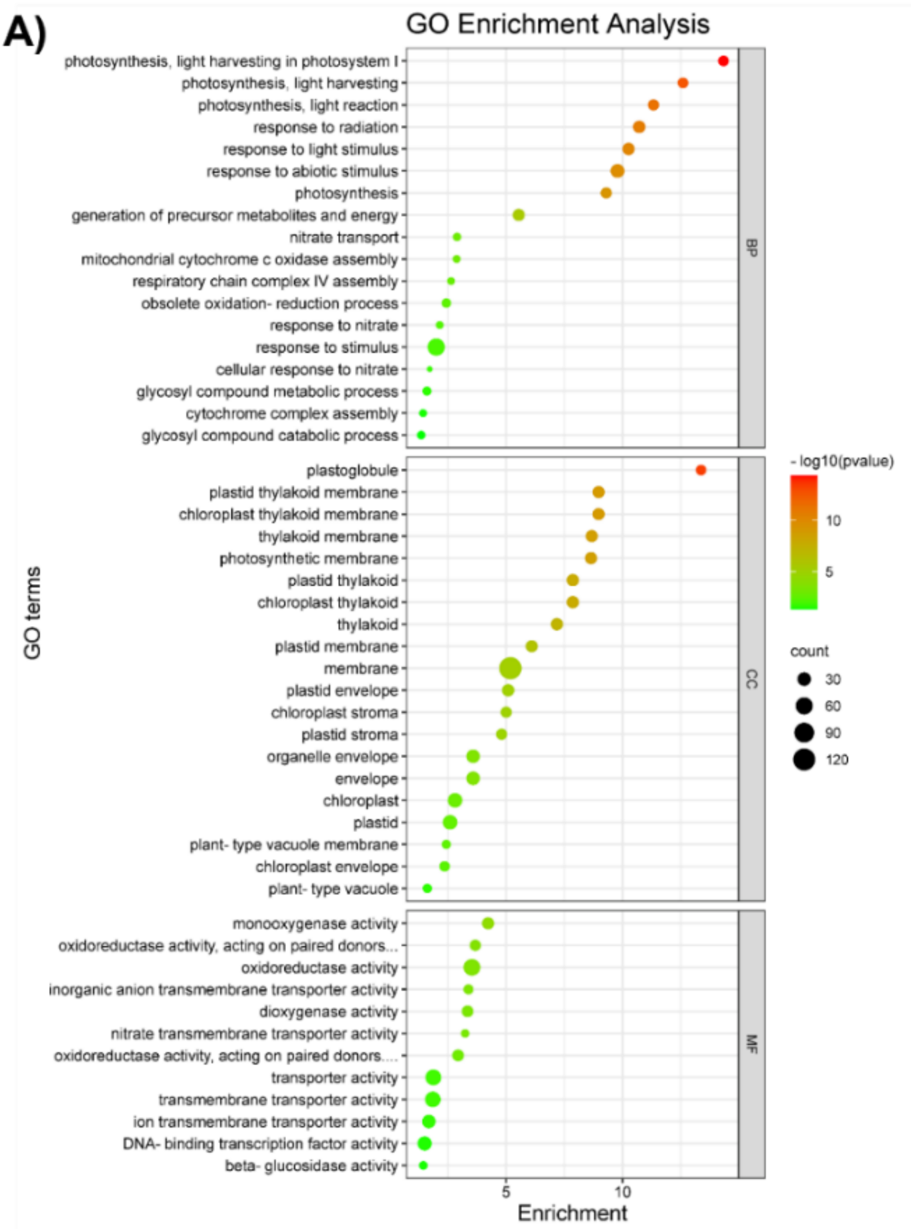


Figure 4.3 - Volcano plots of DEGs between the control (aCO_2) and eCO_2-treated soybean roots (a) and leaves (b). Red dots represent upregulated genes, blue dots represent downregulated genes, and grey dots indicate the non-significantly DEGs.

In leaves, gene expression analysis revealed that 3083 transcripts changed under eCO_2, including 1717 upregulated and 1366 downregulated transcripts (Figure 4.3b and Table S4.2). This evidence suggests that leaf tissues are more sensitive to eCO_2 conditions. Gene Ontology (GO) enrichment analysis was performed to understand the function of the DEGs (Figure 4.4a and b) and showed that eCO_2 significantly (adjusted p-value < 0.05) affected several GO terms in root and leaf tissues. Functional annotation showed that eCO_2 affected 18 biological processes, 21 cell component metabolic pathways, and 12 molecular functions in root tissues. Similarly, CO_2 enrichment significantly affected 53 biological processes, 29 molecular functions, and 13 cellular components in leaf tissues. Exposure to eCO_2 mainly affected the following biological processes in the roots of soybean: photosynthesis, response to radiation, light and abiotic stimulus, generation of precursor metabolites and energy, nitrate transport, and mitochondrial cytochrome c oxidase assembly; the affected cellular components include plastoglobule, plastid thylakoid membrane, photosynthetic membrane, and membrane; affected molecular functions include monooxygenase activity, oxidoreductase activity, inorganic anion transmembrane transporter activity, dioxygenase activity, and nitrate transmembrane transporter activity.


A)
GO Enrichment Analysis
photosynthesis, light harvesting in photosystem I
photosynthesis, light harvesting
photosynthesis, light reaction
response to radiation
response to light stimulus
response to abiotic stimulus
photosynthesis
generation of precursor metabolites and energy
nitrate transport
mitochondrial cytochrome c oxidase assembly
respiratory chain complex IV assembly
obsolete oxidation- reduction process
response to nitrate
response to stimulus
cellular response to nitrate
glycosyl compound metabolic process
cytochrome complex assembly
glycosyl compound catabolic process
BP
plastoglobule
plastid thylakoid membrane
chloroplast thylakoid membrane
thylakoid membrane
photosynthetic membrane
plastid thylakoid
chloroplast thylakoid
thylakoid
plastid membrane
membrane
plastid envelope
chloroplast stroma
plastid stroma
organelle envelope
envelope
chloroplast
plastid
plant- type vacuole membrane
chloroplast envelope
plant- type vacuole
CC
monooxygenase activity
oxidoreductase activity, acting on paired donors...
oxidoreductase activity
inorganic anion transmembrane transporter activity
dioxygenase activity
nitrate transmembrane transporter activity
oxidoreductase activity, acting on paired donors....
transporter activity
transmembrane transporter activity
ion transmembrane transporter activity
DNA- binding transcription factor activity
beta- glucosidase activity
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GO terms
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Enrichment
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count
30
60
90
120

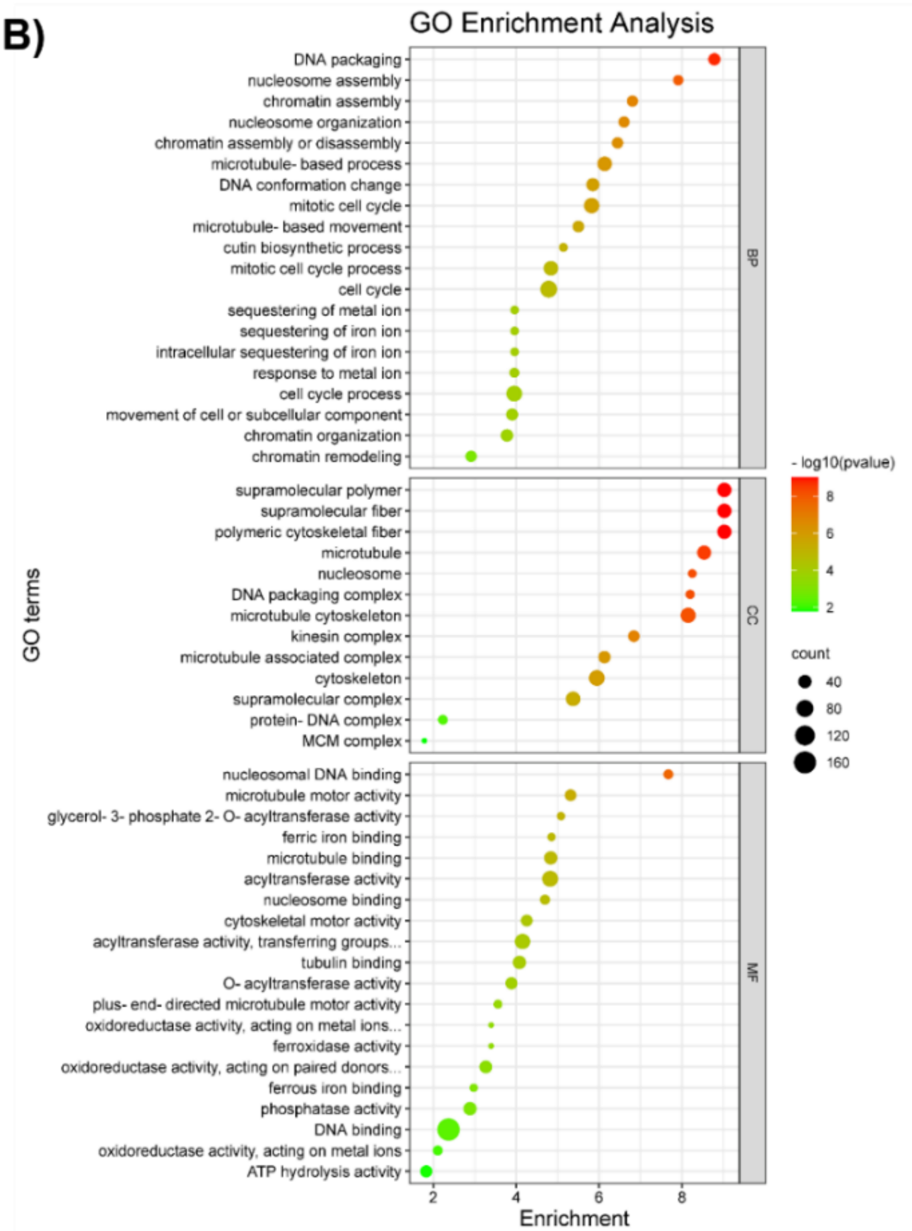

Figure 4.4 - GO enrichment analysis of DEGs between the control (aCO_2) and eCO_2-treated soybean roots (a) and leaves (b). The GO terms are provided in the vertical axis representing the top 20 GO terms, the enrichment factor in the horizontal axis, size of the point represent the number of DEGs, and the color represents the enrichment *p*-value of the GO term. BP, biological process; CC, cellular component; and MF, molecular function.

In leaves, under biological process, DEGs were enriched in terms of DNA packaging, nucleosome and chromatin assembly, nucleosome organization, microtubule-based process, DNA conformation change, mitotic cell cycle, microtubule-based movement, and cutin biosynthetic process. In the cellular component, genes were enriched in terms of polymeric cytoskeletal fiber, microtubule, nucleosome, DNA packaging complex, and microtubule cytoskeleton. Under molecular function, DEGs were significantly enriched in terms of nucleosome DNA binding, microtubule motor activity, glycerol 3-phosphate 2-O-acyltransferase activity, ferric iron binding, microtubule binding, acyltransferase activity, and nucleosome binding. Further analysis was performed on GO terms in downregulated and upregulated genes (Tables S4.3 and S4.4) and represented through the enrichment map in Cytoscape in Figures 4.5 and 4.6. In roots, enriched downregulated GO terms in the biological process include terms related to photosynthesis, nitrate metabolism, and response to the abiotic stimulus. The enriched upregulated GO terms included cytochrome c oxidase, oxidation-reduction process, hormone biosynthesis, and sterols metabolism.

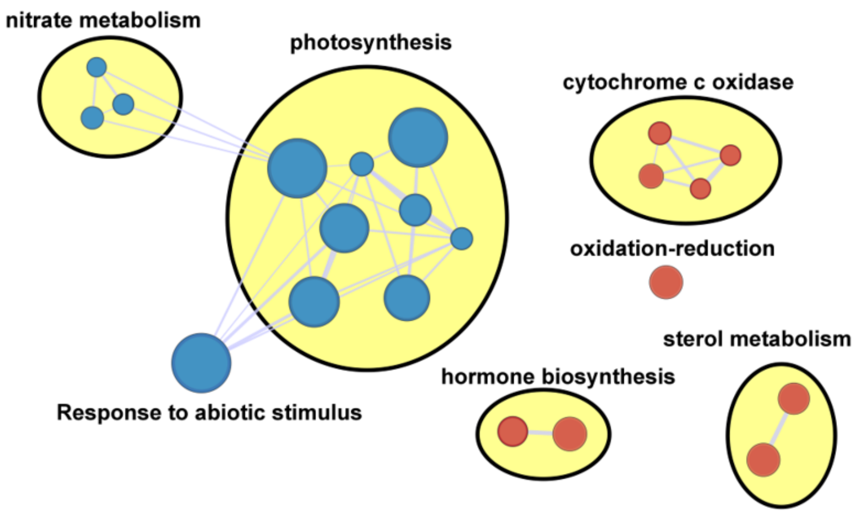

Figure 4.5 - Biological process analysis of DEGs in root tissues. GO modules enriched with upregulated (red circles) and downregulated genes (blue circles) were visualized by Enrichment Map in Cytoscape. Circle size represents the number of genes in the GO term, and connections among circles represent overlapping gene sets of each GO term.

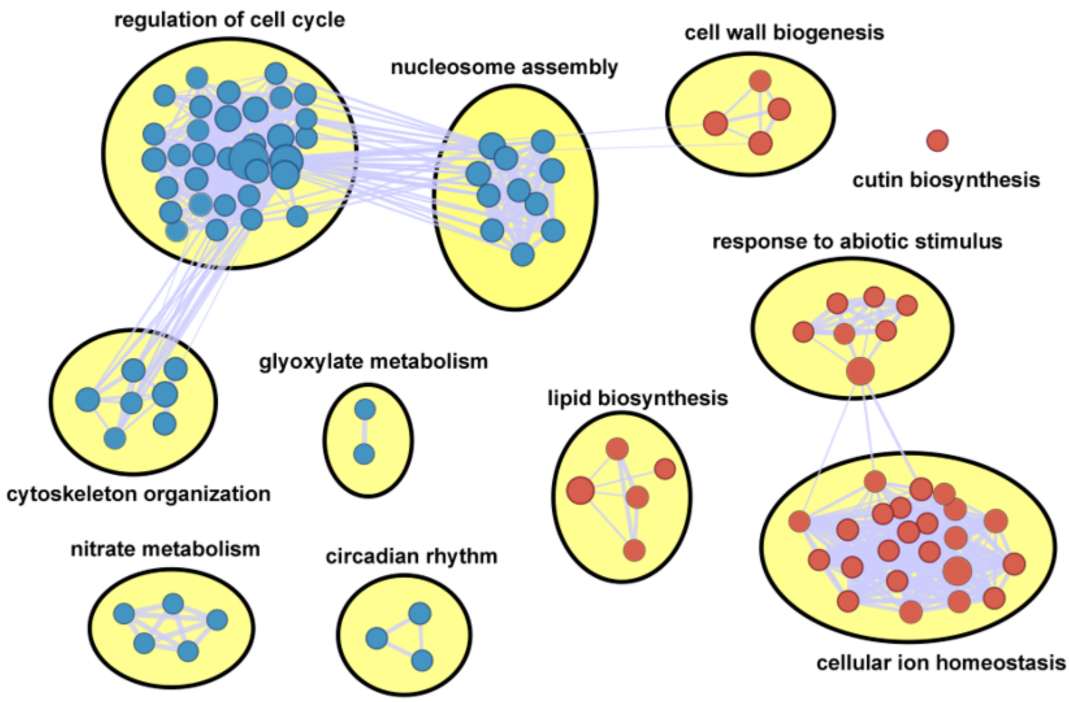

Figure 4.6 - Biological process analysis of DEGs in leaf tissues. GO modules enriched with upregulated (red circles) and downregulated genes (blue circles) were visualized by Enrichment Map in Cytoscape. Circle size represents number of genes in the GO term, and connections among circles represent overlapping gene sets of each GO term.

Downregulated GO terms in leaf tissues included those involved with cell cycle regulation, nitrate metabolism, circadian rhythm, and glyoxylate metabolism. Enriched upregulated GO terms include terms related to the cell wall, response to abiotic stimulus, lipid biosynthesis, and cellular ion homeostasis.

Kyoto encyclopedia of genes and genomes (KEGG) pathways enrichment analysis of DEGs are demonstrated in Figure 4.7. KEGG analysis in root tissues showed that photosynthesis, metabolic pathways, nitrogen metabolism, and biosynthesis of secondary compounds were the most enriched represented pathways. Furthermore, a higher number of genes were included in metabolic pathways and biosynthesis of secondary metabolites in root and leaf tissues (Figures 4.7a and b). In leaves, biosynthesis of secondary metabolites, fatty acid elongation, metabolic pathways, glycerolipid metabolism, and circadian rhythm were the most enriched pathways.

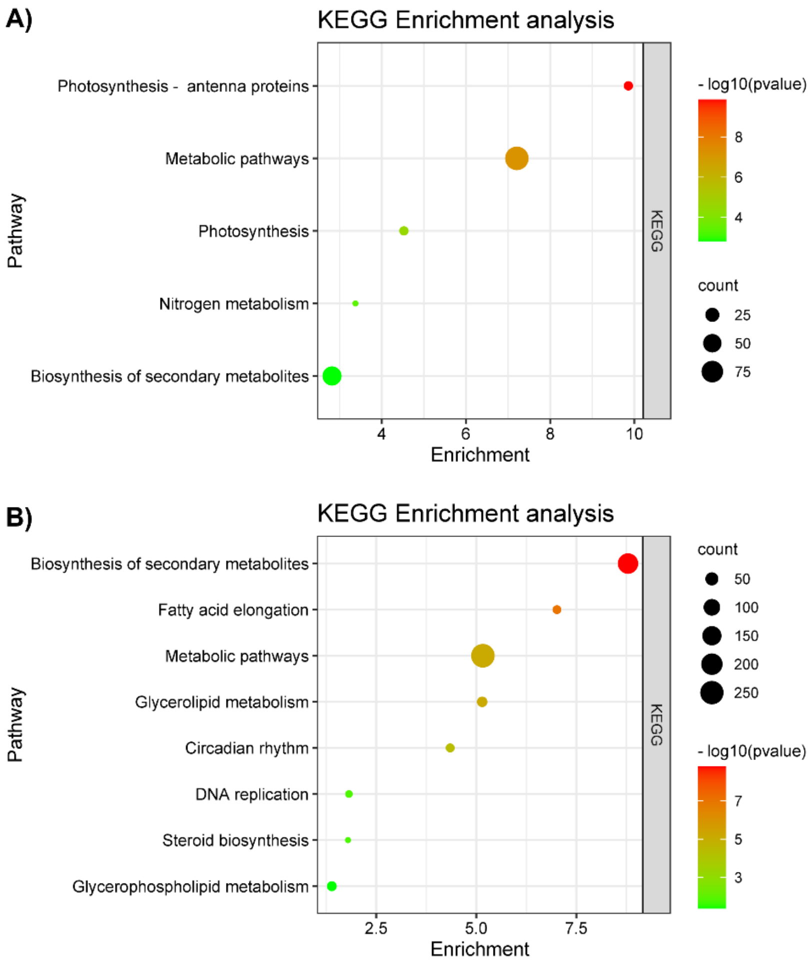

Figure 4.7 - KEGG pathway enrichment analysis of DEGs between aCO_2 and eCO_2 treatments identified in soybean roots (a) and leaves (b). The pathway names are provided on the vertical axis, the enrichment factor on the horizontal axis, the bubble size represents the number of DEGs, and the color represents the enrichment *p*-value of the corresponding pathway.

4.4.3. Expression of major TFs

Due to the importance of TFs in the regulation of stress-responsive genes, DEGs were studied using the Plant Transcription Factor Database and the TF families listed in Tables S4.5 and S4.6. We identified 97 and 265 TFs encoding genes in root and leaf tissues, respectively (Figures 4.8a and b). Of the TFs identified, 20 were downregulated, and 77 were upregulated in root tissues. In leaf tissues, we found 109 TFs downregulated and 156 upregulated. The CO_2-responsive genes were included in 37 families in both tissues analyzed. The prevalent TF families expressed in this study include ERF (48 genes), MYB (43 genes), bHLH (35 genes), bZIP (18 genes), NAC (17 genes), and WRKY (17 genes).

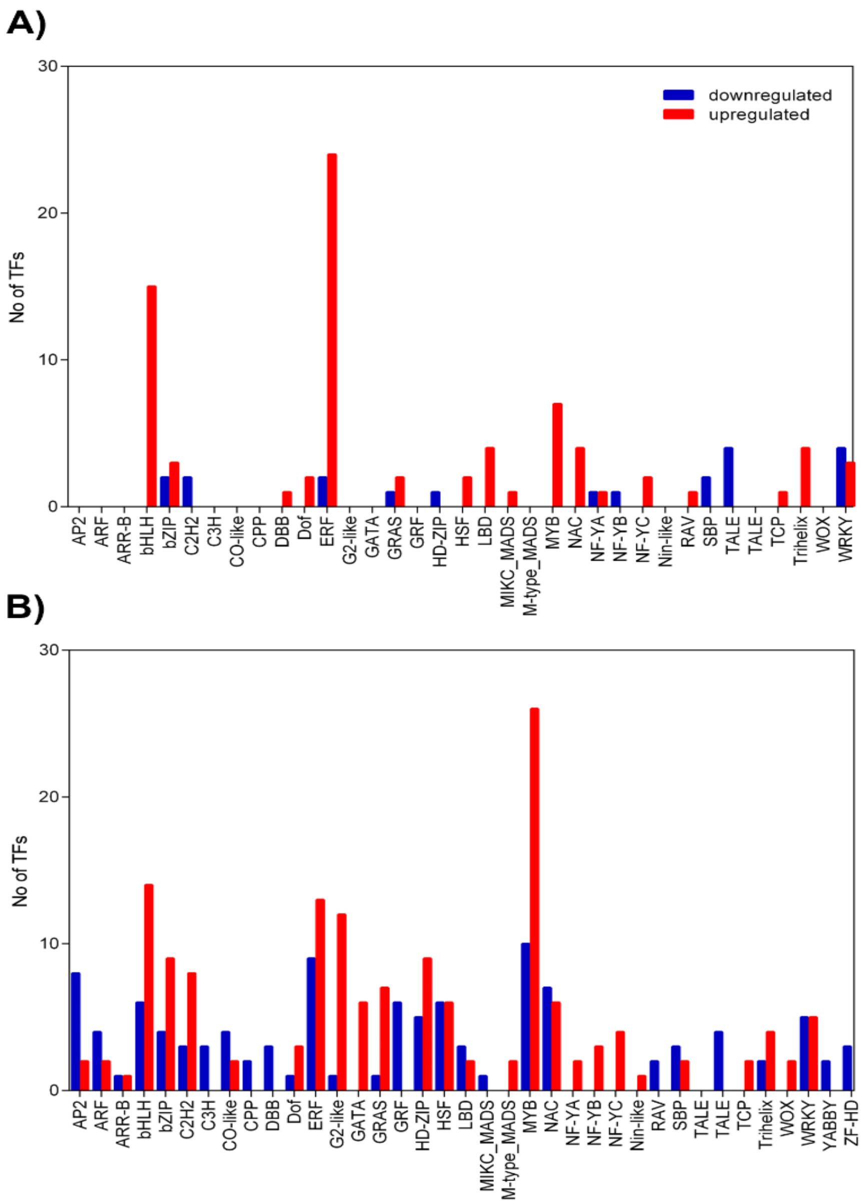

Figure 4.8 - Transcription factors identified in DEGs between aCO_2 and eCO_2 treatments in soybean roots (a) and leaves (b). The number of genes is provided on the vertical axis and the TF family on the horizontal axis.

4.5. Discussion

4.5.1. Plant growth response under eCO_2

A strong CO_2-responsive soybean genotype (*Glycine max* var. Wisconsin Black) (Soares et al., 2019a) was used in this study. RNA-seq has been used to understand the transcriptomic changes that occur under eCO_2 conditions in several crops such as maize (Huang et al., 2019), wheat (Lin et al., 2016b), chickpea (Palit et al., 2020a), and barley

(Córdoba et al., 2017). Carbon dioxide is a chemical compound crucial for plant growth and development, and the molecular bases of the adaptation to eCO_2 conditions have not been fully addressed in soybean, and only a few reports are available (Ainsworth et al., 2006, Bencke-Malato et al., 2019). However, the effects of eCO_2 on soybean physiology, growth, and metabolism have been well documented (Soares et al., 2019a, Jin et al., 2019, Huang et al., 2019, Dong et al., 2018b, Ainsworth et al., 2006, Ainsworth et al., 2002). Besides, eCO_2 generally stimulates plant biomass and crop yield through the CO_2 fertilization effect (Soares et al., 2019a, Ziska et al., 2001, Ainsworth et al., 2002, Bencke-Malato et al., 2019, Zheng et al., 2020). In the present study, plant dry weight increased by 46.9% (Figure 4.1a). These results were in agreement with previous studies on the impact of eCO_2 on the physiology of crop plants (Jin et al., 2009, Masuya et al., 2021, Asadi and Eshghizadeh, 2021). Moreover, several authors reported that photosynthesis usually increases by about 20–30% under eCO_2 (Ainsworth et al., 2002, Long et al., 2004, Leakey et al., 2009, Bencke-Malato et al., 2019). The photosynthetic rate did not change due to eCO_2 conditions (Figure 4.2a). Downregulation of photosynthesis might be associated with limiting factors such as lower Rubisco concentration, reduced stomatal conductance, or excessive carbohydrate accumulation in leaves (Zheng et al., 2019). Under eCO_2, plants might surpass what they are using or distributing to sinks, increasing the carbohydrate content and possibly leading to feedback inhibition of photosynthesis (Thompson et al., 2017). Herein, we found an increase in soluble sugars in leaves (Figure 4.1b) and a decline in stomatal conductance (Figure 4.2b) probably attributed to a decrease in stomatal density and stomatal openness (Singh et al., 2013), which could explain photosynthetic downregulation.

4.5.2. The key roles of DEGs under eCO_2 conditions

The 828 CO_2-responsive genes identified in the root tissues of soybean include 65.7% and 34.3% up and downregulated genes, respectively (Figure 4.3a). Leaves are the first organ in direct contact with CO_2 and possibly would differentially express a higher number of genes when compared to roots. Therefore, DEGs analysis showed 3083 genes differentially expressed, and 55.7% and 44.3% of the genes were up and downregulated, respectively (Figure 4.3b). GO enrichment analysis showed that most DEGs were enriched in the category of biological process in both tissues. DEGs involved in photosynthesis, response to abiotic stimulus, nitrate metabolism, and cytochrome c oxidase assembly were enriched in root tissues. However, genes associated with DNA packaging, nucleosome assembly, microtubule-based process, cell cycle genes, and cutin biosynthetic process were enriched in leaves (Figures 4.4a and b). Several studies have suggested that genes involved in such processes might play essential roles in plant adaptation to eCO_2 conditions. For example, evidence has shown that a large number of transcripts for ribosomal proteins, cell cycle, cell wall loosening, and photosynthesis were differentially expressed in soybean under eCO_2 conditions (Ainsworth et al., 2006). In maize, eCO_2 significantly altered gene expression of photosynthesis and carbohydrate biosynthesis pathways (Huang et al., 2019). Elevated CO_2 also resulted in significant differences in gene expression associated with carbohydrate and nitrogen metabolism in barley (Córdoba et al., 2017). Some proteins with regulation properties in energy and metabolism processes are activated during adaptation to environmental changes (Wang et al., 2019). In another study, upregulated proteins induced by eCO_2 at sufficient N conditions, were enriched in metal binding, carbohydrate binding, small molecule binding, and lyase activity in a perennial grass species (Yu et al., 2019). Our study demonstrated that genes related to oxidoreductase, monooxygenase activity, and dioxygenase activity in roots (Table S4.3) and ferric iron binding, acyltransferase, and phosphate activity in leaves (Table S4.4) had high expression levels under eCO_2 conditions. This evidence may provide the necessary material and energy for soybean adaptation to eCO_2.

4.5.3. Pathways involved under eCO_2 conditions

KEGG analysis showed that DEGs were mainly involved in photosynthesis, metabolic pathways, nitrogen metabolism, biosynthesis of secondary metabolites, lipid metabolism, and circadian rhythm in soybean seedlings under eCO_2 conditions (Figures 4.7a and b). These pathways were mainly related to energy and lipid metabolism, probably leading to soybean adaptation to a high-CO_2 environment. The following discussion focuses on metabolic pathways involved in CO_2-based responses in soybean plants.

4.5.3.1. Photosynthesis

Photosynthesis is a crucial process that provides the energy source for plant metabolism (Wang et al., 2019). Surprisingly, in this study, photosynthesis-related genes were identified in the roots of soybean seedlings under CO_2 enrichment (Figure 4.5). The downregulated genes included chlorophyll a-b binding proteins, ferredoxin, oxygen-evolving complex protein, and photosystem I and II reaction center subunits involved in energy metabolism (Table S4.7). Similarly, many chlorophyll a-b binding proteins-coding genes were downregulated in the roots of jatropha after 7 days of drought and salt stress (Zhao et al., 2020b). Kang et al. (2014) also observed photosynthesis-related genes in rice roots under low-phosphorus conditions. The decreased expression of photosynthesis-related genes might be associated with saving energy for other processes (Li et al., 2010) and may play an active role in regulating the early responses to eCO_2, but this evidence needs further elucidation. Besides, leaf sugar accumulation in plants generally leads to the downregulation of photosynthesis-related genes (Stitt et al., 2010) and may also be the case in roots. A reduction in the expression level of phosphoenolpyruvate carboxylase and Rubisco enzymes, included in the top downregulated genes, were found in leaves (Table S4.2). In addition, to cope with the excess of assimilates, starch and sucrose metabolism-related genes were also decreased (Table S4.8). Some authors also reported photosynthesis feedback regulation by sugars in sugarcane leaves involving the decrease of carbon fixation enzymes, such as Rubisco and phosphoenolpyruvate carboxylase (Lobo et al., 2015, Marquardt et al., 2021).

4.5.3.2. Nitrogen metabolism

Our results showed that eCO_2 affected the efficiency of nitrogen metabolism in roots (Figure 4.7), inhibiting the expression of nitrate transporter, glutamate dehydrogenase, and glutamine synthase (Table S4.7). Dong et al. (2017) explained that inhibition of nitrogen assimilation possibly decreased sink strength, thus limiting the plant responses to eCO_2 conditions. The results with cucumber plants revealed sugar accumulation in leaves after nitrogen limitation. Therefore, eCO_2 is more likely to inhibit N assimilation first, and after that, restricts the plant capacity to transport photosynthates from leaves. Since eCO_2 inhibits photorespiration and several pathways of nitrogen assimilation, it might be possible that the signals from nitrogen assimilation, such as nitrate, play a role in limiting carbohydrate synthesis and transportation from leaves. Similarly, eCO_2 inhibits the expression of genes involved in nitrogen metabolism in Masson pine, including glutamate synthase, nitrite reductase, glutamine synthase, nitrate reductase, and glutamate dehydrogenase (Wu et al., 2020). In another study, eCO_2 decreased nitrate uptake from the soil in *Medicago Trunculata* by downregulating nitrate reductase and nitrate transporter Guo et al. (2013).

4.5.3.3. Biosynthesis of secondary metabolites

KEGG analysis showed that genes associated with the biosynthesis of secondary metabolites were affected by eCO_2 in root and leaf tissues (Figure 4.7). The phenylpropanoid pathway generates several metabolites in plants required for the lignin biosynthesis and production of other compounds (Fraser and Chapple, 2011). This evidence is an adaptation that allows plants to survive under stress conditions producing compounds with essential functions, such as lignin for structural support, flavones and flavonols for UV protection, anthocyanins, chalcones, and aurones as pigments for the attraction of pollinators, and isoflavonoids and furanocoumarins as phytoalexins for pathogen defense (Jia et al., 2019, Novaes et al., 2010). Our results showed that most proteins involved in lignin synthesis were downregulated and included 4-coumarate-CoA ligase, aldehyde dehydrogenase, O-methyltransferase, omega-hydroxypalmitate O-feruloyl transferase, spermidine hydroxycinnamoyl transferase, and several peroxidases (Tables S4.7 and S4.8). Downregulation of lignin biosynthesis might be a defense mechanism against abiotic stress (Gallego-Giraldo et al., 2011). Moreover, Novaes et al. (2010) showed that phenylpropanoid synthesis represents a significant pathway for carbon and energy flow during lignin biosynthesis. Therefore, reducing the phenylpropanoid biosynthesis might conserve carbon and energy for other processes (Jia et al., 2019).

4.5.3.4. Lipid metabolism

Lipid metabolism is considered an essential factor for environmental adaptation, and under stress conditions, modifications in lipid metabolism are possibly providing defensive mechanisms (Zhang et al., 2018b). In this study, fatty acid elongation and glycerolipid metabolism pathways were upregulated at eCO_2 conditions in the leaves (Figure 4.7). Therefore, nine genes encoding a 3-ketoacyl-CoA synthase involved in long-chain fatty acids synthesis essential for wax biosynthesis were upregulated (Table

S4.8). Cuticular waxes are considered a barrier to avoiding unnecessary water loss and a defense mechanism from several types of damage, such as UV irradiation and pathogen attack (Yang et al., 2021). In addition, we found upregulation of the glycerolipid metabolism, including the glycerol-3-phosphate acyltransferase, which participates in the synthesis of cutin or suberin associated with the plant cell wall (Yang et al., 2010). Zhang et al. (2018b) showed that enzymes involved in lipid biosynthesis had higher expression levels at eCO_2 in wheat plants. Differences in the balance of glycerolipid-related compounds under temperature stress have also been reported in various plant species (Chen et al., 2006, Johnson and Williams, 1989). Our results suggest that adjustments in the lipid metabolism pathway may be an adaptive strategy of plants to cope with eCO_2 conditions.

4.5.3.5. Circadian rhythm

The presence of several DEGs involved in circadian rhythm further confirmed the changes under eCO_2 conditions (Figure 4.7). The transcript levels of response regulators 1 and 12, E3 ubiquitin-protein ligase, protein gigantea, acyl-CoA-binding domain, protein early flowering, protein kinase, DNA photolyase, and flowering locus protein T decreased after exposure to eCO_2 in leaves (Table S4.8). These genes are involved in light signaling pathways and stomatal movements and might be associated with stress response regulation, such as drought, salinity, heat, and high radiation (D'Amico-Damião and Carvalho, 2018). Although the underlying mechanism is currently undiscovered, these circadian rhythm-related genes might be involved in eCO_2 responses (Higuchi-Takeuchi et al., 2020).

4.5.3.6. Genes associated with cell cycle and leaf development

Our results showed that the expression levels of genes related to the cell cycle, nucleosome assembly, DNA replication, microtubule-based process, and motor activity decreased (Figures 4.6 and 4.7 and Table S4.8). Cell cycle genes that regulate cytoplasm growth include cyclins, cyclin-dependent kinases, mitotic cyclins, and tubulins. Besides, eCO_2 also influenced the expression of genes associated with cell wall loosening and leaf expansion. Cell wall proteins are crucial in the control of cell wall extensibility, which mediates cell enlargement and expansion (Le Gall et al., 2015). Herein, we found cellulose synthase, FASCICLIN-like arabinogalactan-protein,

protein COBRA-like, and alpha-1,4-glucan-protein synthase proteins which were most upregulated. Our results suggest that cell expansion rather than cell division could be more important in determining early responses to eCO_2 in soybean leaves. Previous studies have shown that eCO_2 concentration could increase cell expansion rather than enhance cell division (Ferris et al., 2001). Taylor et al. (1994) also reported stimulation in leaf growth, which is usually associated with increased leaf cell expansion rather than increased leaf cell production, following exposure to eCO_2 in beans and two hybrid poplar clones. This evidence may be a plant strategy to increase leaf growth and development in response to eCO_2. Shoot elongation was stimulated by eCO_2 in arrowhead and associated with high increased transcript levels of genes encoding xyloglucan endotransglucosylase/hydrolase genes (Ookawara et al., 2005). Moreover, long-term exposure to eCO_2 in *Arabidopsis* also induces cellulose synthase genes, indicating an alteration of plant cell wall biosynthesis (May et al., 2013).

4.5.4. TFs responding to eCO_2

Elevated CO_2 promotes extensive changes in genes encoding TFs. Similarly, Ainsworth et al. (2006) showed that most genes with high expression levels were TFs in soybean leaves at eCO_2 under fully open-air conditions. Under eCO_2, 9687 TFs were identified in two chickpea cultivars grown in open-top chambers at different stages of plant growth (Palit et al., 2020b). In our study, we identified 362 TFs belonging to 37 TFs families. The predominant TFs families identified in this study include ERF (48), MYB (43), bHLH (35), bZIP (18), NAC (17), and WRKY (17) (Figure 4.8a and b). These TF families play a critical role in gene expression regulation in most biological processes (Shiu et al., 2005).

The APetala 2/Ethylene-Responsive Factor (AP2/ERF) superfamily is a large group of plant-specific transcription factors that contains four subfamilies: the AP2, RAV (Related to Abscisic Acid Insensitive 3/Viviparous 1), ERF and DREB (Dehydration-Responsive Element-Binding) protein subfamilies (Mizoi et al., 2012). Many AP2/ERF TFs from various plant species have been involved in abiotic stress responses and play a crucial role in several developmental processes (Xu et al., 2008). Zhao et al. (2019) identified 160 soybean ERF genes after exposure to several abiotic stresses. The authors characterized the ABA-induced ERF TF gene GmERF75 as essential in enhancing osmotic stress tolerance in Arabidopsis and soybean. A variety of ERFs was also identified in Arabidopsis, tobacco, tomato, rice, and hot pepper and reviewed by Magnani et al. (2004). Furthermore, overexpression of the soybean gene GmERF3 increased tolerance to salt, drought, and disease in tobacco (Zhang et al., 2009). In this study, 48 ERF TFs were affected by exposure to eCO_2, with 24 upregulated and two downregulated in the root tissues. Similarly, in leaves 13 and nine ERF TFs were upregulated and downregulated, respectively (Figures 4.8a and b). Upregulation of most of these TFs suggests that this TF family could trigger the CO_2-adaptation response in soybean plants.

The MYB TFs widely distributed in the plant kingdom act as regulators for plant development, metabolism, differentiation, and responses to biotic and abiotic stresses (Dubos et al., 2010, Ambawat et al., 2013). MYB proteins have been identified in several crop plants, such as 157 R2R3-MYB encoding genes in maize (Du et al., 2012a), five 3R-MYB genes, 192 R2R3-MYB genes in Populus (Wilkins et al., 2009), 252 MYB genes in soybean (Du et al., 2012b), and 329 MYB genes in chickpea (Palit et al., 2020a). In our study, 43 MYB TFs were affected by eCO_2 in soybean plants. In roots, we found upregulation of seven MYB genes, whereas, in leaves, 26 and 10 genes were up and downregulated, respectively. Overexpression of MYB genes increases the tolerance to drought and salt stresses in transgenic plants (Ramalingam et al., 2015, Wu et al., 2019). Furthermore, an MYB gene plays a role in positively regulating the phenylpropanoid metabolism in *Arabidopsis thaliana* by increasing the production of compounds to increase the plant's ability to stand for pathogen infection (Borevitz et al., 2000).

The basic helix-loop-helix (bHLH) family is also another family of TFs that regulates a variety of plant's developmental and metabolic processes, such as secondary metabolite biosynthesis, biotic or abiotic stresses responses, such as salinity (Kim and Kim, 2006), drought (Zhao et al., 2020a, Dong et al., 2014), low temperature (Xu et al., 2014), and nutrient deficiencies (Guo et al., 2021, Tissot et al., 2019). The bHLH TFs were already identified in eggplant (Xu et al., 2020) and chickpea (Palit et al., 2020a) under eCO_2 conditions, suggesting that this TF family may be involved in CO_2-based responses. Herein, the expression of 15 bHLH TFs was upregulated in root tissues, while six and 14 bHLH TFs were down and upregulated, respectively, in leaf tissues of soybean. The upregulation of bHLH might reduce photo-oxidation damage by increasing the content of anthocyanins in leaves (Nawaz et al., 2018).

The basic leucine zipper (bZIP) family is one of the larger TF families found in plants, mainly involved in pathogen defense, abiotic stress, hormone signaling pathways, plant developmental processes, and senescence, as reviewed by Tolosa and Zhang (2020). Palit et al. (2020a) identified 332 bZIP TFs in chickpea under eCO_2 concentrations. In this study, 18 bZIP TFs were affected in the leaf and root tissues of soybean, suggesting a possible role in adaptation to eCO_2.

In plants, NAC (NAM, ATAF, and CUC) proteins are responsible for stress tolerance and growth promotion (Tolosa and Zhang, 2020). Besides, this TF family is involved in signal transduction, innate immune system, defense, and acquired resistance in several plant species (Nuruzzaman et al., 2013). Thus, it is probable that most of the defense and stress pathways are induced by NAC genes. NAC proteins were also involved in the response of two sea buckthorn cultivars under different CO_2 concentrations (Zhang et al., 2018a). In our study, 17 NAC genes were affected by eCO_2 conditions. In the roots, we found upregulation of four NAC genes, and in the leaves, seven and six NAC genes were down and upregulated under $eCO_{2,}$ respectively.

The WRKY gene family has been studied in different plants with crucial roles in biotic and abiotic stress responses (Tolosa and Zhang, 2020). By regulating several signal transduction pathways, these TFs also participate in several biological processes involving nutrient deficiency, embryogenesis, seed and trichome development, senescence, and other developmental and hormone-regulated processes (Wani et al., 2021, Tolosa and Zhang, 2020). WRKY genes were identified in different plant species such as *Glycine max*, *Oryza sativa*, Populus, *Arabidopsis thaliana*, *Sorghum bicolor*, and *Hordeum vulgare*, among others, as reviewed by Wani et al. (2021). In the root, four and three WRKY TFs were down and upregulated, respectively. Nevertheless, we found downregulation in five WRKY genes and upregulation in five genes in leaf tissues. Considering the differential expression of most TFs under eCO_2, further recognized their general implication on plant stress signaling and possible crosstalk between stress pathways and CO_2-based responses.

4.6. Conclusion

In conclusion, we conducted a comparative analysis of the physiological and transcriptomic responses in soybean seedlings under eCO_2, highlighting the most significant pathways changed after exposure to eCO_2. Several DEGs were found in leaf (3083 genes) and root (828 genes) tissues, suggesting that CO_2-induced responses in plants are mediated mainly by leaves.

The number of pathways regulated in soybean shows its significant metabolic activity under CO_2 enrichment. Therefore, DEGs are enriched in pathways associated with energy and metabolic processes, implying a possible role in adaptation to eCO_2 conditions. Moreover, several TFs were identified and might be also involved in soybean adaptation to eCO_2 conditions. Our results highlight the molecular mechanisms in soybean under eCO_2, which will provide a valuable resource for further research on soybean response to CO_2 enrichment.

PART III - Interaction between CO_2 enrichment and Fe-limitation in soybean plants

CHAPTER 5 - Short-term exposure to eCO_2 stimulates growth and metabolic responses that alleviate early-stage Fe deficiency symptoms in soybean

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5.1. Abstract

Elevated CO_2 (eCO_2) increase plant biomass and might lead to nutritional losses. The results showed that eCO_2 under Fe deficiency stimulates root dry weight from 0.37–0.80 g plant^{-1} and shoot dry weight from 0.82–2.10 g plant^{-1}, without compromising root/shoot ratio. Besides, eCO_2 improved the chlorophyll content of Fe-deficient plants. However, downregulation of photosynthesis, reduction in stomatal conductance and transpiration rate, and an increase in water-use efficiency was observed due to eCO_2. Moreover, under Fe deficiency, eCO_2 decreased K and Mg in roots, and Mg in leaves, whereas increased P and Zn in roots and leaves. In Fe-sufficient plants, eCO_2 increased K, P, Mn, Zn and Fe in leaves and did not change the concentration in roots. The Fe deficiency-induced responses in roots, including the increase in FCR activity, and the expression of Fe-uptake genes were stimulated by eCO_2, but were not sufficient to increase Fe concentration in Fe-deficient plants.

5.2. Introduction

The global atmospheric carbon dioxide concentration increased by 48% since the beginning of the industrial era from 280 ppm to the current level of about 420 ppm (https://gml.noaa.gov/ccgg/trends/), and is predicted to reach 700–1000 ppm by the end of this century (IPCC, 2014). This steady increase in atmospheric CO_2 is responsible for global climate change affecting crops worldwide (Bencke-Malato et al., 2019). Therefore, one of the great questions for agriculture in the upcoming future is to improve biomass production and plant product quality, and atmospheric CO_2 concentration and regulation of mineral concentrations are major actors in this scenario (Briat et al., 2015). Soybean is a consistent source of nutrients as consumption of soybean-based products is on the rise due to its high content of proteins, fatty acids, natural antioxidants, vitamins, and minerals. (Zheng et al., 2020). Previous findings have demonstrated that elevated CO_2 (eCO_2) promotes photosynthetic CO_2 assimilation rate in C3 plants, increasing biomass and yield (Haase et al., 2008, Kimball, 2016, Bishop et al., 2015, Leakey et al., 2009, Bencke-Malato et al., 2019). For example, Kimball (2016) showed that biomass and seed yield increased in C3 species under FACE conditions. Yields of C3 grain crops increased by an average of 19%. In another study, eCO_2 stimulated aboveground biomass by 22% and seed yield by 9% among 18 soybean genotypes grown under field conditions (Bishop et al., 2015). Plants exposed to eCO_2 changed their root size and activity to increase nutrient uptake and translocation for a given nutrient (Guo et al., 2015, Pérez-López et al., 2014). The results from Guo et al. (2015) in rice suggest that eCO_2 might support the translocation of calcium (Ca), magnesium (Mg), iron (Fe), zinc (Zn), and manganese (Mn) from soil to stem and panicle. A great deal of attention has been dedicated to the effects of eCO_2 on the nutrient quality of crop plants and expanding this knowledge to Fe status is of particular interest. Fe is a crucial element of biomass production and plant quality due to its essential role in the structure and function of the photosynthetic electron transfer chain and chlorophyll synthesis (Briat et al., 2015). Besides, Fe intake for a significant proportion of the worldwide population is delivered by plant consumption (Smith et al., 2017). Even though the Fe concentration in soils regularly surpasses plant requirements, its bioavailability is often limited, predominantly in calcareous soils representing 30% of cultivated soils (Jin et al., 2009). Furthermore, Fe is also the most commonly deficient micronutrient in the human diet, impacting roughly an estimated 2 billion people (Briat et al., 2015, Smith et al., 2017). Recent studies revealed that C3 plants, legumes, and maize have lower Fe concentrations ranging from 4–10% when grown at eCO_2 (550 ppm) under field conditions (Myers et al., 2014). Similar findings were obtained by Loladze (2014) in C3 plants, reflecting foliar and edible tissues, FACE, and non-FACE studies. Consequently, Fe metabolism in higher plants is likely to be affected by the atmospheric CO_2 concentration, and it is crucial to address the impact of eCO_2 on plant Fe nutrition. Jin et al. (2009) reported a relative increase in tomato biomass and the root/shoot ratio at eCO_2 under Fe-limited and Fe-sufficient conditions. Under eCO_2, the Fe deficiency-induced responses in roots, including ferric chelate reductase (FCR) activity, proton secretion, subapical root hair development, and the expression of FER, FRO1, and IRT genes, were higher than in plants grown at aCO_2. Also, eCO_2 has a positive effect on biomass when Fe is limited in barley (Haase et al., 2008). Thus, understanding the Fe-uptake mechanisms and Fe-metabolism under eCO_2 conditions is crucial for selecting nutrient-rich and more tolerant genotypes to Fe deficiency (Morrissey and Guerinot, 2009). Moreover, little is known about the interaction of eCO_2 and Fe deficiency in soybean plants, and the underlying mechanisms responsible for such changes. In plants, the Fe-uptake mechanism is divided into two different approaches: strategy I occur in nongraminaceous plants, and Fe^{3+} is reduced via a membrane-bound reductase to make it accessible for uptake by a Fe^{2+} transporter, and strategy II occurs in grasses which secrete phytosiderophores that readily bind Fe^{3+}, and these complexes are then transported back into the roots (Morrissey and Guerinot, 2009, Jin et al., 2009). In the present study, soybean plants were grown in a hydroponic culture system to report the effects of the interaction of eCO_2 and Fe deficiency in the morphological, physiological, and molecular responses under controlled conditions. It was previously demonstrated in tomato (Jin et al., 2009) and barley (Haase et al., 2008) that eCO_2 has a positive impact on plant biomass when Fe is limited. Here, we aimed to understand if eCO_2 may have a positive effect on soybean growth under Fe deficiency without impairing mineral accumulation, since Fe homeostasis and eCO_2 could be linked to the interplay occurring among nutrient uptake and translocation.

5.3. Materials and Methods

5.3.1. Plant culture

Soybean (*Glycine max* cv 'Winsconsin Black') seeds were surface sterilized with 75% (v/v) ethanol, rinsed four times with ultra-pure water, and germinated at 25 °C in the dark for 5 days. Then, plants were transplanted to black plastic pots (5 L) filled with an aerated, full-strength nutrient solution with the following composition: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4.7H_2O$, 0.2 mM $NH_4H_2PO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 0.5 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.1 µM $NiSO_4$, and 20 µM Fe(III)-EDDHA. The solution was buffered with the addition of 1mM MES (pH 5.5) and changed every 3 days. Plants were grown in the controlled environment at 75% humidity with a daily cycle of 16 hours at 25 ºC (day) and 8 hours at 20 ºC (night). The daytime light intensity was 325 µmol s^{-1} m^{-2} of the photosynthetic photon flux density at the plant level. After 7 days of pre-treatment in the full-strength nutrient solution, plants were transferred to a nutrient solution with Fe(III)-EDDHA at 0.5 µM (Fe deficiency) or 20 µM (Fe-sufficiency) for 12 days. The plants were grown at 400 ± 10 ppm (aCO_2) or 800 ± 10 ppm (eCO_2). The CO_2 concentration was continuously monitored and maintained by an automated CO_2 control system, which measured and adjusted the CO_2 concentration from soybean planting to the end of the experiment.

5.3.2. Physiological parameters

At the end of the experiment, the chlorophyll content was evaluated using the first expanded trifoliate leaf with a chlorophyll meter (SPAD-502; Minolta). Then, plants were measured for length and separated into shoots and roots, and the material was dried at 70 ºC until constant weight and stored for ICP-OES analysis. Each analysis was performed from 5 plants and mean values calculated.

5.3.3. Determination of FCR activity

Root FCR activity was measured as described by Vasconcelos et al. (2006). The assays were performed by the spectrophotometric determination of Fe^{2+} chelated to BPDS (bathophenanthroline disulfonic acid). Plant roots were submerged in the assay solution containing: 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 3.75 mM $NH_4H_2PO_4$, 0.25 mM $MgSO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM H_2MoO_4, 0.1 µM $NiSO_4$, 100 µM Fe(III)-EDTA, 100 µM BPDS, and buffered with 1 mM MES at pH5.5. The assays were conducted under dark conditions at room temperature for 60 min. Absorbance values were obtained spectrophotometrically at 535 nm, and the solution with no roots was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 $mM^{-1}cm^{-1}$. The assays were performed from 5 samples and mean values calculated.

5.3.4. Mineral analysis by ICP-OES

About 200 mg of the roots and leaves dried samples were mixed with 5 mL of 65% HNO_3 (v/v) and 1 mL of H_2O_2 30% (v/v) in a Teflon reaction vessel and heated in a Speedwave TMMWS- 3+ (Berghof, Germany) microwave system. The digestion procedure was described in detail by Santos et al. (2015), and the resulting solutions were diluted to 50 ml with ultra-pure water for further analysis. Determination of mineral concentrations was performed using the ICP-OES Optima7000 DV (Perkin Elmer, USA) with a radial configuration from five independent digestions of each plant organ from all the treatments. The assays were performed from 5 samples and mean values calculated.

5.3.5. Leaf gas exchange parameters

Gas exchange parameters were performed in the last fully expanded leaves, ten days after the Fe-treatment, using a portable photosynthesis system (LI-6400XT; LICOR, Inc.). The CO_2 in the leaf chamber was set to match the CO_2 treatment with a PPFD of 500 µmol photon m^{-2} s^{-1} at 25 °C. A/Ci curves were also measured, and data were analyzed using the PS-FIT software (http://www.life.illinois.edu/bernacchi/links.html). This software uses the leaf model of photosynthesis (Farquhar et al., 1980) to calculate the maximum rates of electron transport (J_{max}) and maximum velocity of carboxylation by Rubisco ($V_{c,max}$). Moreover, the transpiration rate (T_r), stomatal conductance (g_s), and water use efficiency (WUE = A/T_r) were determined. The assays were performed from 3 plants and mean values calculated.

5.3.6. Gene expression analysis

For gene expression analysis, root samples were frozen in liquid nitrogen and stored at –80 ºC. About 100 mg of tissue was grounded in liquid nitrogen, and total RNA was extracted using a Qiagen RNeasy Plant Mini Kit (Qiagen, USA). Therefore, single-stranded cDNA was synthesized with the first-strand cDNA synthesis kit (Fermentas, USA) in a Thermal cycler (VWR, Doppio, Belgium), according to the manufacturer's instructions. The mRNA levels were detected by the SYBR Green Supermix (Bio-Rad) with the following pairs of gene-specific primers: *FER* fw, 5'-GAACAAACGTGGTGGAAAA G-3'; rev, 5'- AACTGCACGTCACCATTCTT-3'; *FRO2* fw, 5'-TGCTTGGACTCACACCA GAG-3'; rev, 5'-AGAGGTAGAAACCGGG GAGA-3'; *Ferritin* fw, 5'-CCCCTTATGCCTCTTTCCTC -3'; rev, 5'-GCTTTTCAGC GTGCTCTCTT-3'; *IRT1* fw, 5'-GATTGCACCTGTGACA CAAA-3'; rev, 5'-CAGCA AAGGCCTTAACCATA-3'; *DTM1* fw, 5'-GCCGCAAGAAACAGCTTATG-3'; rev, 5'-AGCTTCTTCCACGAGA ATCG-3'. The respective housekeeping genes were used as reference genes: *Actin* fw, 5'- ATCTTGACTGAGCGTGGTTATTCC-3'; rev, 5'-GCTGGTCCTGGCTGTCTCC-3'; and *ELF1B* fw, 5'-GTTGAAAAGCCAGGGGACA -3'; rev, 5'-TCTTACCCCTTGAGCGTGG-3'. *FER and DMT1 gene* sequences were searched in the NCBI database, and primers were designed using the Primer-Blast tool from NCBI with an expected PCR product of 100–200 bp. *FRO2*, *ferritin*, and *IRT1* primer pairs were described by Santos et al. (2015), and *Actin* and *ELF1B* by Wan et al. (2017). qPCR reactions were performed on a Chromo4 thermocycler (Bio-Rad, Hercules, CA, USA) with the following reaction conditions: 10 min at 95 ºC and 40 cycles with 15 s at 95 ºC, 15s at 58 ºC, and 15 s at 68 ºC. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used for the relative quantification of gene expression analysis. All the assays were performed with three biological and two technical replicates.

5.3.7. Organic acids and sugar analysis

The extraction protocol was described in detail by López-Millán et al. (2009). About 100 mg of plant material was grounded using liquid nitrogen and suspended in 5 mM H_2SO_4, vortexed for 30 seconds, and then boiled for 30 minutes. The samples were centrifuged at 2320 x g for 10 minutes, the supernatant filtered through a 0.45 mm PTFE filter, and the volume adjusted to 2 ml and stored at –80 ºC until further analysis. The HPLC system consisted of an ion exchange aminex HPX-87H Column (300 x 7.8 mm) (Bio-Rad, USA) maintained at an oven temperature of 40 °C and two detectors in series (Refractive Index and UV 210 nm; K-2301 and K-2501, Knauer, Germany). The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 mL/min. The assays were performed from 5 samples, and mean values were calculated.

5.3.8. Statistical analysis

The data for each dependent variable was subjected to the two-way ANOVA at $p < 0.05$ in all cases. All statistical analyses were performed with SPSS software (SPSS version 26.0).

5.4. Results

5.4.1. Effects of eCO_2 and Fe deficiency on chlorophyll synthesis, plant growth, and Fe-uptake

After 12 days of plant growth at aCO_2 and under Fe deficiency (0.5 µM Fe-EDTA), the soybean leaves were chlorotic (Figure 5.1) with an average SPAD reading of 12.7 (Figure 5.2a). However, leaves of plants grown in the same growth conditions but at eCO_2 had SPAD readings of 19.9 indicating that eCO_2 conditions ($p < 0.05$; Table 5.1) significantly improved the chlorophyll synthesis of plants grown in Fe-deficient conditions.

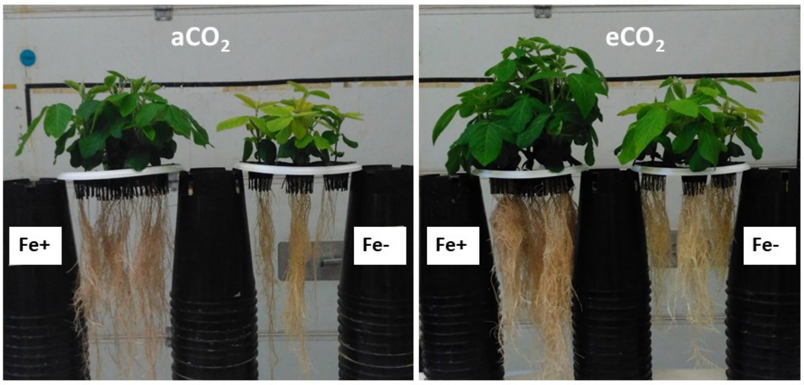

Figure 5.1 - Soybean plants grown in nutrient solution at aCO_2 (400 ppm) and eCO_2 (800 ppm). After 7 days of pre-treatment in the complete nutrient solution, plants were transferred to Fe-sufficient (20 µM Fe-EDDHA) and Fe-deficient (0.5 µM Fe-EDDHA) conditions for 12 days.

In Fe-sufficient plants (20 µM Fe-EDTA) the chlorophyll content was also higher under eCO_2 in comparison to aCO_2 conditions ($p < 0.05$; Figure 5.2a). The effects of CO_2 and Fe-supply were statistically significant on SPAD readings ($p < 0.001$), with a significant CO_2 x Fe interaction ($p < 0.05$, Table 5.1). Moreover, in Fe-sufficient plants, the height was higher compared to Fe-deficient plants, and this effect was exacerbated in response to eCO_2 (Figure 5.2b). In hydroponic growth, root dry weight was increased at eCO_2 by 66.4% in Fe-sufficient ($p < 0.01$) and by 119.7% in Fe-deficient ($p < 0.001$) plants, as demonstrated in Figure 5.3a. Shoot dry weight (Figure 5.3b) was also increased under eCO_2 by 40.1% in Fe-sufficient ($p < 0.001$) and by 157.6% in Fe-deficient plants ($p < 0.001$). Furthermore, the root/shoot ratio (Figure 5.3c) was not affected in Fe-deficient plants ($p > 0.05$) but increased in Fe-sufficient plants ($p < 0.05$) by 20.1% under eCO_2 conditions. The effects of CO_2 levels and Fe-supply were statistically significant on FCR activity ($p < 0.001$), with a significant CO_2 x Fe interaction ($p < 0.001$, Table 5.1). Therefore, FCR activity was induced in plants grown in Fe deficiency, and this induction was higher when combined with aCO_2 (Figure 5.4). Contrastingly, FCR activity was higher under eCO_2 conditions in Fe-sufficient conditions.

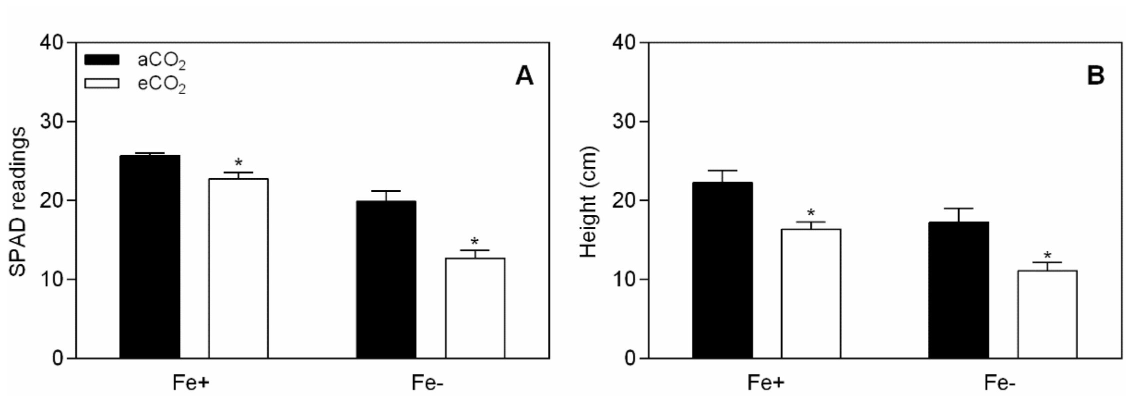

Figure 5.2 - SPAD readings (a) and height (b) of soybean plants grown in the nutrient solution, depending on Fe-supply (0.5 and 20 µM Fe-EDDHA) and atmospheric CO_2 concentration (400 and 800 ppm). Data are mean ± SE (n = 5). *, Significant differences ($p < 0.05$) between aCO_2 and eCO_2 treatments.

Table 5.1 - Two-factorial ANOVA results for the effect of eCO_2 and Fe-supply on leaf chlorophyll content (SPAD values), plant height (cm), plant biomass (root and shoot dry weight, g plant^{-1}), root/shoot ratio, FCR activity (µmol Fe g^{-1} FW h^{-1}), A (µmol m^{-2} s^{-1}), g_s (mol m^{-2}s^{-1}), T_r (mol m^{-2} s^{-1}), WUE (µmol mol^{-1}), $V_{c,max}$ (µmol m^{-2} s^{-1}), and J_{max} (µmol m^{-2} s^{-1}).

Parameters	d.f	CO_2	Fe	CO_2 x Fe
SPAD	1	**31.434;<0.001**	**77.547;<0.001**	**5.573;0.025**
Height	1	**19.286;<0.001**	**14.147;0.002**	0.009;0.924
Root dry weight	1	**64.970;<0.001**	**13.163;0.002**	0.280;0.604
Shoot dry weight	1	**89.608;<0.001**	**16.628;<0.001**	**10.753;0.005**
Root/shoot ratio	1	0.031;0.863	0.276;0.606	**9.405;0.007**
FCR activity	1	**2.439;<0.001**	**82.105;<0.001**	**23.406;<0.001**
A	1	0.006;0.939	**15.180;<0.001**	0.208;0.654
g_s	1	**84.401;<0.001**	4.021;0.059	1.477;0.239
T_r	1	**68.916;<0.001**	**5.217;0.035**	2.765;0.114
WUE	1	**120.798;<0.001**	1.296;0.270	0.597;0.450
$V_{c,max}$	1	**41.212;<0.001**	4.656;0.063	**9.83;0.014**
J_{max}	1	**20.418;0.002**	0.606;0.459	**6.943;0.030**

A, Photosynthetic carbon assimilation rate; g_s, stomatal conductance; T_r, transpiration rate; WUE, water-use efficiency; $V_{c,max}$, maximum velocity of carboxylation by Rubisco; J_{max}, maximum rate of electron transport. Results from the analysis of variance with degrees of freedom (d.f.), F ratios and probabilities (p) for some parameters. Significant effects are shown in boldface.

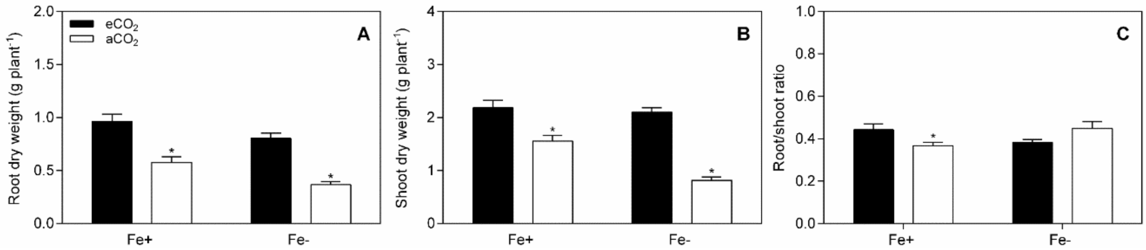

Figure 5.3 - Root biomass (a), shoot biomass (b) and root/shoot ratio (c) of soybean plants grown in the nutrient solution, depending on Fe-supply (0.5 and 20 µM Fe-EDDHA) and atmospheric CO_2 concentration (400 and 800 ppm). Data are mean ± SE (n = 5). ∗, Significant differences ($p < 0.05$) between aCO_2 and eCO_2 treatments.

5.4.2. Effects of eCO_2 and Fe deficiency on the photosynthetic parameters

Leaf photosynthetic assimilation rate (A) decreased in Fe-deficient conditions ($p < 0.05$; Table 5.2) when compared to Fe-sufficient conditions. However, in Fe-sufficient and Fe-deficient plants the response of A was not affected by exposure to eCO_2 ($p > 0.05$), with

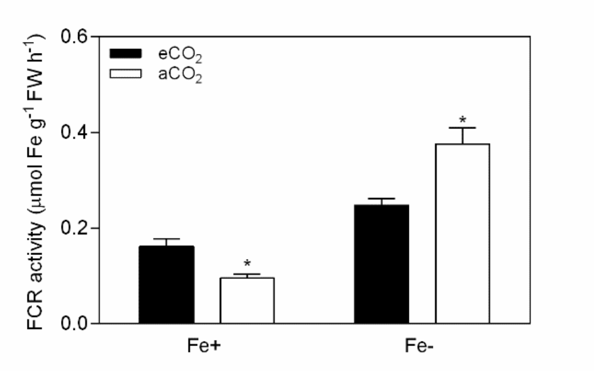

Figure 5.4 - Effect of eCO_2 on FCR activity in soybean plants grown in Fe-sufficient (20 µM Fe-EDDHA) and Fe-deficient (0.5 µM Fe-EDDHA) conditions. Data are mean ± SE (n = 5). *, Significant differences ($p < 0.05$) between aCO_2 and eCO_2 treatments.

no significant CO_2 x Fe interaction ($p > 0.05$, Table 5.1). Also, eCO_2 reduced stomatal conductance (g_s) from 0.37 to 0.13 mol H_2O m^{-2} s^{-1} in Fe-sufficient ($p < 0.05$) and from 0.30 to 0.12 mol H_2O m^{-2} s^{-1} in Fe-deficient ($p < 0.05$) plants. The transpiration rate (T_r) also decreased by eCO_2, in both Fe-supplies. The water-use efficiency (WUE) increased significantly from 2.23 to 4.76 mmol mol^{-1} ($p < 0.01$) in Fe-sufficient plants, and from 2.17 to 3.97 mmol mol^{-1} ($p < 0.01$) in Fe-limited plants due to exposure to eCO_2. The A/Ci response curves showed a reduction in the $V_{c,max}$ and in J_{max} under eCO_2 in Fe-sufficient and Fe-deficient plants ($p < 0.05$, Table 5.2). However, the response of $V_{c,max}$ and J_{max} were not affected by Fe-supply ($p > 0.05$) but a significant CO_2 x Fe interaction was found ($p < 0.05$, Table 5.1).

5.4.3. Effects of eCO_2 and Fe deficiency on the sugar and organic acid concentrations

In hydroponic culture, elevation of atmospheric CO_2 did not affect sucrose concentration ($p > 0.05$) in roots and leaves of Fe-sufficient and Fe-deficient plants (Table 5.3). Moreover, eCO_2 increased glucose concentration in roots of Fe-deficient plants ($p < 0.05$) and in leaves of Fe-sufficient plants ($p < 0.05$). Besides, fructose concentration increased in Fe-deficient and Fe-sufficient plants ($p < 0.05$) in both tissues analyzed due to CO_2 enrichment. The effect of Fe-supply was not statistically significant on the carbohydrate responses ($p > 0.05$), as well as the CO_2 x Fe interaction ($p > 0.05$, Table 5.4).

Table 5.2 - Effect of eCO_2 on photosynthetic parameters of soybean plants grown in Fe-sufficient (20 µM Fe-EDDHA) and Fe-deficient (0.5 µM Fe-EDDHA) conditions.

Fe treatment	CO_2	A	g_s	T_r	WUE	Vc,max	Jmax
Fe-sufficient	Ambient	11.43 ± 0.55	0.37 ± 0.03	5.22 ± 0.36	2.23 ± 0,39	57.63 ± 2.00	125.95 ± 6.26
	Elevated	11.21 ± 0.43	0.13 ± 0.02 *	2.46 ± 0.19 *	4.76 ± 0,95 *	29.56 ± 3.79 *	70.22 ± 11.46 *
Fe-deficient	Ambient	8.90 ± 0.80	0.30 ± 0.02	4.12 ± 0.29	2.17 ± 0,33	42.08 ± 2.71	99.35 ± 4.95
	Elevated	9.21 ± 0.40	0.12 ± 0.01 *	2.28 ± 0.16 *	3.97 ± 0,31 *	32.43 ± 2.95 *	84.68 ± 1.96 *

A, Photosynthetic carbon assimilation rate (µmol m^{-2} s^{-1}); g_s, stomatal conductance (mol $m^{-2}s^{-1}$); T_r, transpiration rate (mol m^{-2} s^{-1}); WUE, water-use efficiency (µmol mol^{-1}); $V_{c,max}$, maximum velocity of carboxylation by Rubisco (µmol m^{-2} s^{-1}); J_{max}, maximum rate of electron transport (µmol m^{-2} s^{-1}). Data are mean ± SE (n = 3). *, Significant differences ($p < 0.05$) between aCO_2 and eCO_2 treatments.

Table 5.3 - Effect of eCO_2 on sugars and organics acids content (µmol gFW^{-1}) of soybean plants grown in Fe-sufficient (20 µM Fe-EDDHA) and Fe-deficient (0.5 µM Fe-EDDHA) conditions.

	Roots				Leaves			
	Fe-sufficient		Fe-deficient		Fe-sufficient		Fe-deficient	
	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2
Suc	4.41 ± 0.53	3.31 ± 0.21	3.08 ± 0.74	3.96 ± 0.42	16.36 ± 1.40	13.38 ± 0.86	15.29 ± 1.42	14.81 ± 1.54
Gluc	4.91 ± 0.87	3.78 ± 0.68	5.02 ± 0.90	2.67 ± 0.28 *	10.87 ± 1.08	7.31 ± 0.69 *	9.05 ± 1.12	8.71 ± 0.99
Frut	16.85 ± 2.47	5.11 ± 0.75 *	13.33 ± 0.66	2.11 ± 0.28 *	23.94 ± 2.29	12.93 ± 1.23 *	22.16 ± 2.22	15.14 ± 1.85 *
CA	17.40 ± 1.03	13.64 ± 0.97 *	12.55 ± 0.49	8.07 ± 1.06 *	57.88 ±3.88	56.84 ± 5.73	69.51± 2.23	72.85 ± 2.88
MA	41.33 ± 5.69	25.57 ± 2.63 *	33.13 ± 4.93	11.85 ± 0.97 *	60.54 ± 7.10	40.11 ± 2.82 *	63.86 ± 8.64	62.26 ± 8.38

Suc, sucrose; Gluc, glucose; Frut, fructose; CA, citric acid; MA, malic acid. Data are mean ± SE (n = 5). *, Significant differences ($p < 0.05$) between aCO_2 and eCO_2 treatments.

Table 5.4 - Analysis of variance for the effect of eCO_2 and Fe-supply on sugar (µmol g FW^{-1}), organic acid (µmol g FW^{-1}), and mineral concentrations (µg g^{-1}) in roots and leaves of soybean plants.

Parameters	Tissue	d.f	CO_2	Fe	CO_2 x Fe
Sucrose	Roots	1	0.056; 0.817	0.504;0.493	4.311;0.062
	Leaves	1	1.835;0.209	0.020;0.890	0.958;0.353
Glucose	Roots	1	**7.533;0.019**	0.635;0.443	0.920;0.358
	Leaves	1	3.903;0.072	0.047;0.833	2.677;0.128
Fructose	Roots	1	**56.533;<0.001**	4.549;0.059	0.030;0,866
	Leaves	1	**23.209;<0.001**	0.013;0.910	1.137;0.311
Citric acid	Roots	1	**19.240;0.001**	**30.839;<0.001**	0.144;0.711
	Leaves	1	0.078;0.785	**11.331;0.006**	0.283;0.605
Malic Acid	Roots	1	**22.169;<0.001**	**7.762;0.018**	0.491;0.498
	Leaves	1	4.033;0.068	**5.395;0.039**	2.947;0.112
Calcium	Roots	1	**3.734;0.075**	**319.693;<0.001**	0.084;0.776
	Leaves	1	0.058;0.814	**81.918;<0.001**	0.266;0.615
Potassium	Roots	1	0.881;0.364	**21.721;<0.001**	**6.980; 0.019**
	Leaves	1	**10.694;0.006**	**47.637;<0.001**	3.944;0.067
Phosphorous	Roots	1	**19.763;<0.001**	**5.220;0.038**	3.704;0.075
	Leaves	1	**48.860;<0.001**	1.190;0.295	**12.069;0.004**
Magnesium	Roots	1	0.645;0.437	**49.296;<0.001**	**7.957;0.014**
	Leaves	1	0.819;0.383	**90.837;<0.001**	**14.331;0.003**
Manganese	Roots	1	2.336;0.149	**26.449;<0.001**	1.354;0.264
	Leaves	1	**20.476;<0.001**	**42.025;<0.001**	**18.519;<0.001**
Zinc	Roots	1	**20.406;<0.001**	**18.340;0.001**	**10.278;0.008**
	Leaves	1	**36.354;<0.001**	**5.300;0.038**	1.057;0.323
Iron	Roots	1	1.058;0.319	**63.952;<0.001**	0.074;0.789
	Leaves	1	**26.087;<0.001**	**111.620;<0.001**	**23.688;<0.001**

Results from the analysis of variance with degrees of freedom (d.f.), F ratios and probabilities (p) for some parameters. Significant effects are shown in boldface.

The citric acid concentration increased by the exposure to eCO_2 from 13.64 to 17.40, and from 8.07 to 12.55 µmol g FW^{-1} in roots of Fe-sufficient and Fe-deficient plants, respectively. In leaves, citric acid was not affected under eCO_2 ($p > 0.05$, Table 5.3). At the end of the growth period, eCO_2 increased malic acid concentration in roots from 25.57 to 41.33 in Fe-sufficient and from 11.85 to 22.13 µmol g FW^{-1} in Fe-deficient plants. By comparison, leaves of soybean plants grown in the same nutrient conditions, eCO_2 only increased malic acid in Fe-sufficient plants from 40.11 to 60.54 µmol g FW^{-1}. Moreover, the effect of Fe-supply was significant on citric and malic acid concentrations ($p < 0.05$, Table 5.4).

5.4.4. Effects of eCO_2 and Fe deficiency on the mineral concentrations

The mineral-based responses under different Fe-supplies in soybean plants were also influenced by eCO_2, as demonstrated in Figures 5.5 and 5.6. Elevation of atmospheric CO_2 concentration did not affect Ca concentration in root and leaf tissues, but it was significantly reduced in Fe-deficient plants ($p < 0.05$, Table 5.4).

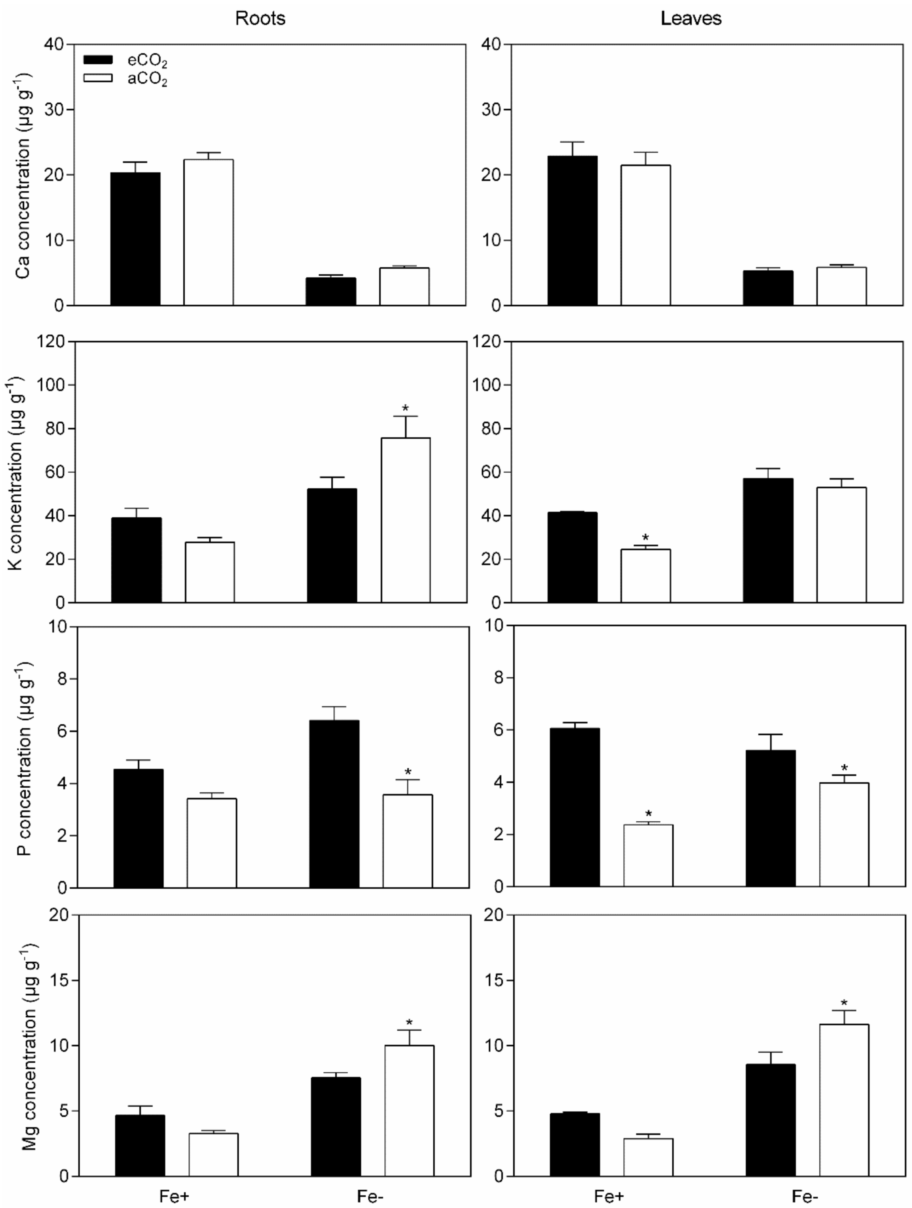

Figure 5.5 - Effect of eCO_2 on the macronutrient concentrations from soybean plants grown in Fe-sufficient (20 µM Fe-EDDHA) and Fe-deficient (0.5 µM Fe-EDDHA) conditions. Data are mean ± SE (n = 5). *, Significant differences (*p* < 0.05) between aCO_2 and eCO_2 treatments.

Elevated CO_2 increased K concentration ($p < 0.05$) from 24.6 to 41.4 µg g^{-1} in leaves of Fe-sufficient plants, and decreased K concentration ($p < 0.05$) from 75.7 to 52.3 µg g^{-1} in roots of Fe deficiency plants. The concentration of P was generally higher under eCO_2 conditions ($p < 0.05$), except in roots of Fe-sufficient plants. Furthermore, eCO_2 did not affect Mg concentration ($p > 0.05$) in Fe-sufficient plants, but a significant decrease was achieved in Fe-deficient plants ($p < 0.05$). Regarding to micronutrients, Mn concentration was not changed by CO_2 treatment in Fe-deficient and Fe-sufficient plants ($p > 0.05$), apart from the increase in Mn concentration occurring in leaves of Fe-sufficient plants ($p < 0.05$) from 56.7 to 155.8 µg g^{-1}. CO_2 enrichment led to a significant increase in Zn concentration, except in roots of Fe-sufficient plants ($p > 0.05$). The concentration of Fe was not changed by eCO_2, excluding the leaves of Fe-sufficient plants, which were significantly higher compared to aCO_2 conditions.

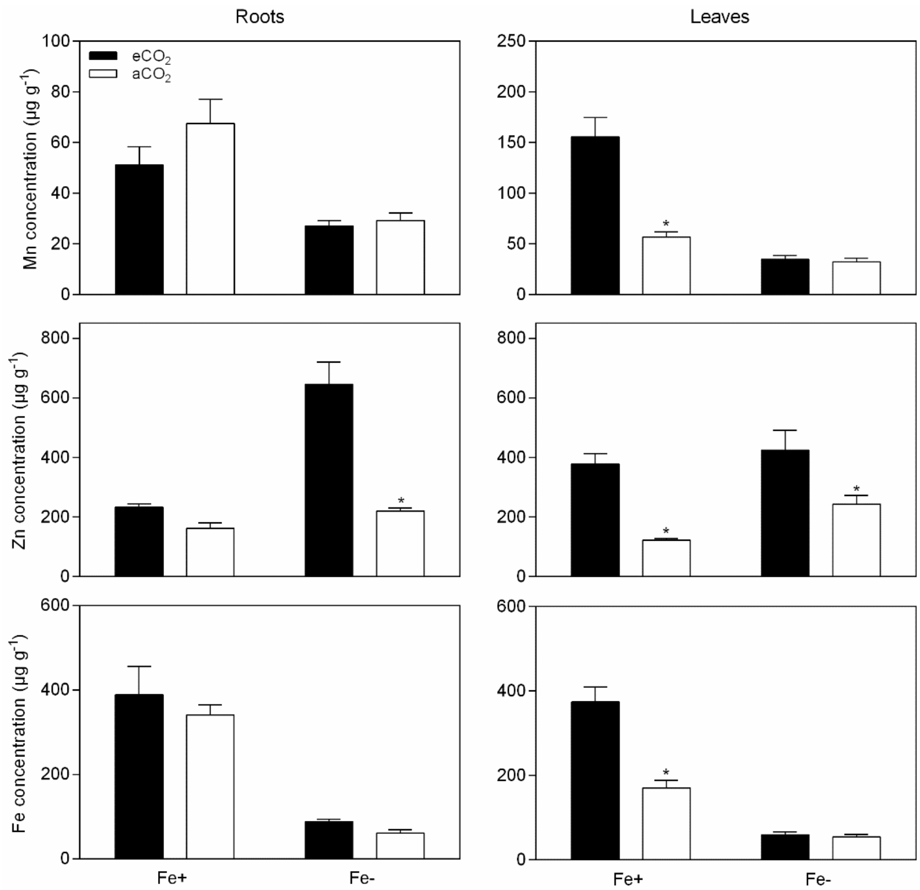

Figure 5.6 - Effect of eCO_2 on the micronutrient concentrations from soybean plants grown in Fe-sufficient (20 µM Fe-EDDHA) and Fe-deficient (0.5 µM Fe-EDDHA) conditions. Data are mean ± SE (n = 5). *, Significant differences ($p < 0.05$) between aCO_2 and eCO_2 treatments.

5.4.5. Effects of eCO_2 and Fe deficiency on the expression of genes involved in Fe-uptake mechanisms

The effects of eCO_2 and Fe deficiency on genes related to the Fe-uptake mechanisms – *DMT1*, *FER*, *FRO2*, *IRT1*, and *ferritin* – were analyzed in soybean roots using qPCR. Overall, higher gene expression levels were associated with Fe deficiency responses as demonstrated in Figure 5.7. In the case of the *FRO2* gene, the expression differed between treatments, with higher expression levels observed in Fe deficiency plants, and this change was higher under eCO_2 conditions. Similar findings were obtained in *DMT1* and *FER* gene expression levels. Moreover, *IRT1* gene expression was higher in Fe-deficient plants, but eCO_2 did not affect the expression levels. Contrastingly, the *ferritin* gene expression was detected only in Fe-sufficient plants, and this change was higher under eCO_2 conditions.

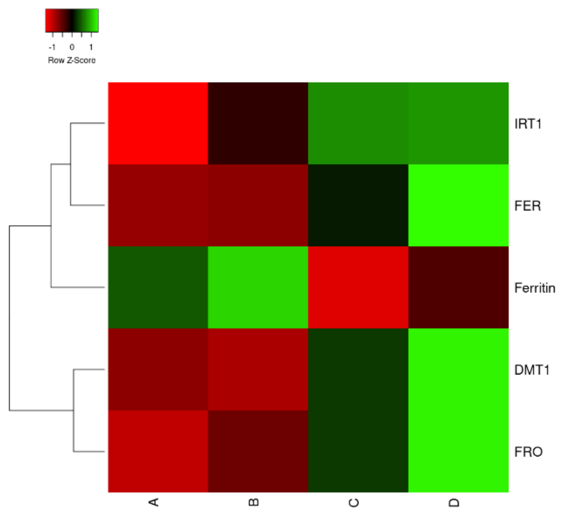

Figure 5.7 - Heatmap of the expression profiles of *DMT1*, *FRO2*, *ferritin*, *FER* and *IRT1* genes in roots of soybeans plants grown at Fe-sufficient and aCO_2 (A), Fe-sufficient and eCO_2 (B), Fe-deficient and aCO_2 (C), Fe-deficient and eCO_2 (D) from three independent replicates.

5.5. Discussion

The beneficial effects of eCO_2 on stimulating plant growth and biomass accumulation of several crops have been widely studied (Ainsworth et al., 2002, Long et al., 2004, Bishop et al., 2015, Ziska and Bunce, 2007, Bunce, 2008, Bunce, 2015, Bunce, 2016, Kimball, 2016, Santos et al., 2015, Vasconcelos et al., 2014). However, there is a lack of information about the influence of eCO_2 on the changing demands for Fe-supply and is crucial to consider how eCO_2 affects and might alleviate the Fe deficiency responses. In this sudy, we treated soybean plants with limited (0.5 μM) and sufficient (20 μM) Fe-supply under eCO_2 conditions. Supplying soybean plants with 0.5 μM Fe is sufficiently low to induce FCR activity. Furthermore, soybean plants revealed clear Fe chlorosis symptoms in young leaves at aCO_2 but not at eCO_2 conditions, which affected biomass accumulation and growth (Figure 5.1). An increase from 0.5−20 μM Fe in hydroponic solution alleviated the chlorosis symptoms in soybean plants. As reported for other species (Bunce, 2016, Wu et al., 2004, Bourgault et al., 2016, Bourgault et al., 2017), soybean growth increased at eCO_2 conditions, as well as the requirement for nutrients and water, to sustain such CO_2-based stimulation. Consequently, more nutrients will need to be applied, or plants will have to become more effective at obtaining those nutrients from the soil (Jin et al., 2009). In this study, eCO_2 led to an increase in biomass, even at reduced Fe-supply, similar to plants grown at Fe-sufficient conditions (Figure 5.3). Therefore, in Fe-sufficient plants, root and shoot dry weight increased by 66.4% and 40.1%, respectively, whereas in Fe-deficient plants increased by 119.7% and 157.4% due to eCO_2 conditions. Similar findings were reported in tomato (Jin et al., 2009) and barley (Haase et al., 2008) grown at eCO_2 with or without Fe-supply. The root/shoot ratio increased in Fe-sufficient plants due to eCO_2 and was not affected in Fe-deficient plants, suggesting that the biomass accumulation changed with Fe-supply. The reason proposed might be that under eCO_2 and Fe-supply, upregulation of the root over the shoot growth was observed appearing in a higher root/shoot ratio. Also, eCO_2 alleviated the Fe deficiency chlorosis symptoms (Figure 5.1), as demonstrated by the increase in chlorophyll concentration (Figure 5.2). The driving force for increased soybean growth at eCO_2 is a higher A (Ainsworth et al., 2002). We noticed that the A was not changed due to CO_2 enrichment and could not predict the magnitude of biomass increase. However, the photosynthetic capacity was affected by Fe-supply ($p < 0.001$, Table 5.1), with a concomitant decrease in Fe-deficient plants of about 20% (Table 5.2). Therefore, under eCO_2 photosynthetic acclimation was observed and attributed to a decrease in $V_{c,max}$, and J_{max}. Nevertheless, despite acclimation of photosynthetic capacity, biomass production is greater in plants exposed to eCO_2. It is generally accepted that Fe deficiency decreased photosynthesis in higher plants (Jiang et al., 2007, Pestana et al., 2001), since the Fe-status is a crucial parameter that affects metabolic changes, photosynthesis, and consequently plant growth at eCO_2 (Briat et al., 2015). eCO_2 reduces the transpiration-driven mass flow due to the stomatal closure (Leakey et al., 2009, Prior et al., 2010). In our study, eCO_2 decreased the g_s by 60−64.9% and T_r by 44.7−52.9% compared to aCO_2 conditions (Table 5.2). The decrease in g_s at eCO_2 was associated with higher WUE and consistent with previous studies (Prior et al., 2010, Zheng et al., 2020). Our results suggested that soybean plants may have increased drought tolerance under eCO_2 conditions. The decrease in the T_r might be a modification to maximize the carbon fixation under eCO_2 conditions (Jauregui et al., 2015). The carbohydrate accumulation, mainly glucose and fructose, which are assimilates from photosynthesis, exhibited significant variation with eCO_2 in leaf and root tissues resulting in differential carbon allocation, as demonstrated in Table 5.3. Therefore, eCO_2 increased carbohydrate accumulation independently of Fe-supply, while an increase of photosynthesis capacity was not confirmed. A possible reason for this phenomenon, at eCO_2, is that plants exceed what they are capable of using or distributing to sinks, increasing the carbohydrates levels, and leading to feedback inhibition of photosynthesis (Thompson et al., 2017).

Furthermore, the results showed a differential variation of sugars in plant tissues, but this variation was independent of Fe-supply ($p > 0.05$, Table 5.4). Therefore, variation in nutrient uptake mechanisms should be less or partially influenced by sugars under Fe deficiency. However, there are new insights on sugar sensing and signaling pathways of how plants are influenced by the higher sugars content produced under eCO_2 conditions (Thompson et al., 2017). Sugars are recognized to crosstalk with hormones (e.g. auxin, nitric oxide and ethylene) and act on gene regulation and therefore modify nutrient uptake and transport, among other functions (Thompson et al., 2017). Hindt and Guerinot (2012) proposed a model of the Fe deficiency response, where auxin, ethylene, and nitric oxide were involved as positive regulators of the Fe acquisition genes FIT, FRO2, and IRT1. A recent study of Lin et al. (2016a) proposed sucrose as the upstream signaling molecule of auxins, causing an increase in auxin and a subsequent increase in nitric oxide, ultimately causing FIT-mediated transcriptional regulation of FRO2 and IRT1 genes and inducing Fe-uptake. In this study, soybean plants allocate greater amounts of sugars (mainly glucose and frutose) to root and leaf tissues during exposure to eCO_2 conditions, which might cause an imbalance in carbon and nitrogen metabolism. Therefore, Fe deficiency signals with sugar variation induced by eCO_2 conditions might act in concert and affect Fe-uptake mechnisms. Besides, if these genes are regulated by the increase of sugars, then it stands to reason that an increase in sugar content in roots mediated by exposure to eCO_2 might also induce Fe deficiency induced responses. The levels of organic acids increased at eCO_2 in roots, and remained almost unchanged in leaves. The increased levels of carbohydrates and organic acids in plants exposed to eCO_2 indicates a higher energy condition capable of maintaining the greater carbon allocation (Jauregui et al., 2015). Moreover, Pavlovic et al. (2013) demonstrated that increased concentration of citrate and malate in the root tissues of cucumber was also important in alleviating Fe deficient responses. Since eCO_2 and sugars increase plant root growth, it was expected greater uptake of nutrients, thus alleviating nutrient deficiencies, however, there are other mechanisms that are affected by eCO_2 that lead to nutrient deficiencies (Thompson et al., 2017). The influence of eCO_2 and Fe deficiency, on plant mineral concentrations, were also addressed. The roots and leaves were differentially affected by eCO_2 and Fe-supply, as described in Figures 5.5 and 5.6. Overall, eCO_2 increased K, P, Mn, Zn, and Fe concentrations in leaves of Fe-sufficient plants, and P and Zn in Fe-deficient plants but did not affect the other minerals. In roots of Fe-sufficient plants, the mineral concentrations were not affected by eCO_2, whereas in Fe-deficient plants, eCO_2 increased P and Zn, and Ca, Mn, and Fe remained unchanged. Consequently, our results suggested that eCO_2 can induce the accumulation of minerals particularly in soybean leaves and at Fe-sufficient conditions probably indicating that the mineral uptake and upward transport by soybean roots was not restricted. As also reported by others (Haase et al., 2008, Jin et al., 2009, Guo et al., 2015) CO_2 enrichment could considerably increase the mineral uptake by the roots promoting the transport from soil to leaves. Also, it is recognized that increase in biomass at eCO_2 changes the demands for nutrients (Seneweera, 2011). In this study, with a consistent supply of nutrients under flooded conditions, the bioavailability was relatively higher to maintain that nutrient supply under eCO_2. Thus, the increase in the root/shoot ratio, mainly in Fe-sufficient plants, under eCO_2 can promote nutrient uptake and possibly lead to higher nutrient concentrations under eCO_2 conditions. Also, we found a decrease in K (roots) and Mg (roots and leaves) in Fe-deficient plants. This reduction could be attributed to the unbalanced mineral translocation from the roots to the leaves or by differential mineral uptake.

The adaptation of soybean plants to Fe deficiency under eCO_2 conditions was also demonstrated by evaluating the expression of genes involved in Fe-uptake and the activity of root FCR. It was reported in tomato and Arabidopsis, that the *FER* gene is upregulated by Fe-deficiency and encodes a root-specific bHLH transcription factor regulating the expression of some Fe-responsive genes, such as the ferric chelate reductase *FRO2* and the transporter *IRT1* (Paolacci et al., 2014, Jin et al., 2009, Connolly et al., 2003). In the present study, we found typical Fe-deficiency responses in soybean plants grown with different Fe-supplies at eCO_2 under controlled conditions. In agreement with previous findings (Jin et al., 2009), we found that FCR activity increased in response to Fe deficiency. Moreover, *DMT1*, *FER*, *FRO2*, and *IRT1* gene expression levels were also upregulated by Fe deficiency, and this phenomenon usually increased by exposure to eCO_2 (Figure 5.7). Graziano and Lamattina (2007) argued that these are crucial Fe deficiency responses, but they are not sufficient to significantly increase Fe concentration in Fe-deficient plants (Figure 5.6). Furthermore, expression of the *ferritin* protein gene increased in Fe-sufficient plants, particularly at eCO_2 conditions. Iron homeostasis is strongly dependent on *ferritin* expression levels produced in response to Fe as previously demonstrated (Arnaud et al., 2006, Wei and Theil, 2000, Briat et al., 2010). Fe homeostasis has to be precisely controlled, to prevent starvation that impairs the metabolism, and to avoid excess that may lead to cell death (Arnaud et al., 2006). Therefore, at eCO_2 conditions Fe-sufficient plants have more Fe content in their roots (Figure 5.6), and this could be a strategy (i.e. increasing *ferritin* expression) that plants use to control the Fe excess.

5.6. Conclusion

We have demonstrated that changes in CO_2 concentration and Fe-supply can induce a set of morphological, physiological, and molecular responses in soybean plants. CO_2 enrichment has a decisive influence on the adaptation to Fe deficiency in soybean plants. According to our results, biomass accumulation was significantly increased by eCO_2 irrespective of Fe-supply. Therefore, our results suggest that eCO_2 alleviated symptoms of Fe deficiency and might support biomass increase in plants grown in calcareous soils, at least during plant's earlier developmental stages, which is usually the most sensitive to Fe deficiency. However, it would be interesting to replicate the responses under eCO_2 of soybean plants grown on alkaline and calcareous soils which can induce Fe deficiency chlorosis. Improved acquisition of several minerals under sufficient Fe-supply may suggest that eCO_2 can support the unbalanced translocation of minerals to the leaves. eCO_2 also mitigates the Fe deficiency-induced chlorosis and enhances Fe-acquisition mechanisms but they are not sufficient to increase the Fe-status of plants grown in Fe-deficient conditions. It will be meaningful to understand if these observed effects will be sustained under a longer-term exposure and what will be the reported influence of eCO_2 on nutritional value of soybean grains.

CHAPTER 6 - Effect of the interaction between elevated carbon dioxide and iron limitation on proteomic profiling of soybean

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6.1. Abstract

Elevated CO_2 (eCO_2) and iron (Fe) availability are key factors affecting plant growth that may impact the proteomic profile of crop plants. The impact of each abiotic factor is well studied, but the interaction is complex, and the underlying mechanisms of plant response are poorly understood. In the present study, soybean plants treated with Fe(III)-EDDHA at 0.5 µM (Fe-limitation) and Fe(III)-EDDHA at 20 µM (Fe-sufficiency) were grown for 12 days at ambient (400 µmol mol^{-1}) and eCO_2 (800 µmol mol^{-1}) in environment-controlled growth chambers. Elevated CO_2 increased biomass from 2.14 to 3.14 g $plant^{-1}$ and from 1.18 to 2.91 g $plant^{-1}$ under Fe-sufficient and Fe-limited conditions, respectively, but did not affect leaf photosynthesis. Besides, sugar

concentration in roots increased from 10.92 to 26.17 μmol g FW^{-1} in Fe-sufficient plants and from 8.75 to 19.89 μmol g FW^{-1} in Fe-limited plants after exposure to CO_2 enrichment. In leaves, sugar concentration increased from 33.62 to 52.22 μmol g FW^{-1} and from 34.80 to 46.70 μmol g FW^{-1} in Fe-sufficient and Fe-limited conditions, respectively, under eCO_2 conditions. However, Fe-limitation decreased photosynthesis from 11.43 to 8.90 μmol m^{-2} s^{-1} at aCO_2 and from 11.21 to 9.21 μmol m^{-2} s^{-1} at eCO_2. Moreover, Fe-limitation decreased plant biomass from 2.14 to 1.18 g $plant^{-1}$ but only under aCO_2 conditions. Pathway enrichment analysis showed that cell wall organization, glutathione metabolism, photosynthesis, stress-related proteins, and biosynthesis of secondary compounds changed in root tissues to cope with Fe-stress. Plant growth, with or without Fe-limitation, occurred at eCO_2 associated with the increased abundance of proteins involved in glycolysis, starch and sucrose metabolism, biosynthesis of plant hormones gibberellins, and decreased levels of protein biosynthesis. Our results revealed that proteins and metabolic pathways related to Fe-limitation changed the effects of eCO_2 and negatively impacted soybean production, which may have important implications for soybean production in the future.

6.2. Introduction

Atmospheric carbon dioxide (CO_2) concentrations are estimated to increase to at least 700-1000 μmol mol^{-1} at the end of this century (Meehl et al., 2006), rising from the current levels of 420 ppm (https://gml.noaa.gov/ccgg/trends/). The trend in crop responses under elevated CO_2 (eCO_2) is supposed to have a noticeable influence on the global food chain and may threaten human nutrition (Yu et al., 2019). The impact of eCO_2 varies among different crop species, but eCO_2 generally improves the photosynthesis of the C3 plants (Zheng et al., 2020) by repressing ribulose-1,5-bisphosphate oxygenase activity and by improving carbon assimilation used for plant growth (Briat et al., 2015). Proteomic analysis in creeping bentgrass showed that eCO_2 affected various metabolic processes and pathways, such as photosynthetic carbon fixation, respiratory metabolism, cellular growth, and stress defense (Burgess and Huang, 2016, Burgess and Huang, 2014). Yu et al. (2017) also demonstrated that eCO_2 improved heat tolerance in bermudagrass attributed to metabolic pathways during which proteins and metabolites were upregulated, including light reaction and carbon fixation of photosynthesis, glycolysis and TCA cycle, and amino acid metabolism. Nevertheless, plant-based responses to eCO_2 are affected by nutrient bioavailability, including iron (Fe) (Jin et al., 2009). Iron is an essential micronutrient that impacts plant productivity, and its bioavailability to plants is often limited in calcareous soils (Robin et al., 2008), which account for ~30% of the world's agricultural soil. The mineral element is of particular significance because of its role in photosynthetic CO_2 fixation, which utilizes Fe as a crucial element to ensure photosynthetic efficiency (Briat et al., 2015). Anyway, Fe is the most frequently deficient micronutrient in the human diet, affecting an estimated 2 billion people (Myers et al., 2014). While eCO_2 promotes plant growth, the interaction with Fe-limitation is not fully understood. Due to the increase in plant growth, the plant requirement for nutrients also increases, and the restriction of macro and micronutrient content at eCO_2 generally inhibits the increase in plant biomass (Briat et al., 2015). Accordingly, low nitrate supply limits shoot growth and hormonal responses to eCO_2 (Yu et al., 2019), involving alterations in protein synthesis and metabolic pathways associated with eCO_2 and nitrate. Furthermore, the proteomic analysis demonstrated an increase in the expression of many proteins due to eCO_2 under adequate nitrate levels involved with cell cycle and proliferation, transcription and translation, photosynthesis, amino acids synthesis, sucrose, and starch metabolism, and ABA signaling pathways. In tomato plants grown in Fe-limited and Fe-sufficient conditions, eCO_2 increased plant biomass and root-to-shoot ratio compared to plants grown in aCO_2 conditions. The percentage increase in biomass was higher in Fe-limited plants compared to Fe-sufficient plants. Therefore, shoot fresh weight increased under eCO2 conditions by 22% and 44% in Fe-sufficient and Fe-limited conditions, respectively, and fresh root weight by 43% and 97% (Jin et al., 2009). Likewise, eCO_2 induced biomass production in barley grown at Fe-sufficient and Fe-deficient conditions and increased shoot Fe concentration and Fe-acquisition mechanisms in Fe-deficient plants indicating an improved internal Fe utilization (Haase et al., 2008). The proteomic characterization of Fe deficiency responses in cucumber roots revealed that most of the increased proteins belong to glycolysis and nitrogen metabolism, and proteins with low expression levels were related to the metabolism of sucrose, structural carbohydrates, and proteins (Donnini et al., 2010). In addition, proteins associated with stress adaptation, reactive oxygen species-related proteins, and mitochondrial proteins were affected under long-term Fe-restriction on the roots of different pea cultivars (Meisrimler et al., 2016). López-Millán et al. (2013) revealed some common elements in proteome under Fe-limitation involving several plant species. Oxidative stress and defense-related proteins, C and N metabolism-related proteins, cell wall proteins, secondary metabolism-associated proteins, and energy-related proteins have been identified as differentially accumulated proteins among plant species.

Therefore, we consider that it is relevant to discover the complex response mechanisms of soybean plants to future climatic conditions, such as eCO_2 and Fe-limitation. This study aimed to understand how soybean plants respond to eCO_2, Fe-stress, and their combination at the level of plant growth, carbohydrate content, photosynthesis, and the expression profile of the leaf and root proteome. At the same time, it was our purpose to investigate if eCO_2 can mitigate the adverse effects of Fe-limitation in soybean plants and provide several insights into improving stress tolerance in the future environment.

6.3. Materials and Methods

6.3.1. Plant material and growth conditions

A previously identified highly-CO_2 responsive soybean variety Wisconsin Black (Soares et al., 2019a), was used as plant material. This study was performed at the Biotechnology School from Catholica University (Portugal). Plants were grown under hydroponic conditions in black plastic pots (5 L) filled with an aerated, full-strength nutrient solution with the following composition: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.3 mM $MgSO_4.7H_2O$, 0.2 mM $NH_4H_2PO_4$, 25 µM $CaCl_2$, 25 µM H_3BO_3, 0.5 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.1 µM $NiSO_4$, and 20 µM Fe(III)-EDDHA. The solution was buffered by MES (1mM, pH 5.5) addition and changed every 3 d. The growth chamber was controlled to maintain the temperature at 25 ºC (day period) and 20 ºC (dark period), relative humidity at 75 %, and photosynthetic photon flux density at 325 µmol s^{-1} m^{-2} (daytime light). After 7 days of pre-treatment in the complete nutrient solution under aCO_2, plants were transferred to a nutrient solution with Fe(III)-EDDHA at 0.5 µM (Fe-limited) or 20 µM (Fe-sufficient). Then, half of the plants were grown at 400 ppm (aCO_2), and the other half grown at 800 ppm (eCO_2) in independent growth chambers for 12 days. The CO_2 concentration was continuously monitored and maintained by an automated CO_2 control system, which measured and adjusted the CO_2 concentration from soybean planting to the end of the experiment.

6.3.2. Evaluation of plant biomass

At the end of the experiment, plants were dried in an oven at 70 ºC until constant weight for total plant biomass determination.

6.3.3. Leaf gas exchange parameters

Leaf gas exchange measurements were performed on day 17 after the beginning of the experiment. We randomly selected the first expanded trifoliate leaf from 3 plants (n = 9), and the photosynthetic rate was measured using a portable photosynthesis system (LI-6400XT; LICOR, Inc.). The CO_2 in the leaf chamber was set to match the CO_2 treatment, with a photosynthetic photon flux density of 500 µmol photon m^{-2} s^{-1} at 25 °C. Moreover, the transpiration rate and stomatal conductance were determined.

6.3.4. Root and leaf carbohydrates

Root and leaf samples (n = 5) were evaluated for carbohydrate analysis at the end of the experiment. The extraction protocol was described by López-Millán et al. (2009). About 100 mg of plant material was grounded using liquid nitrogen, suspended in 5 mM H_2SO_4, vortexed for 30 s, and then boiled for 30 min. Samples were centrifuged at 2320 x g for 10 min, supernatant was filtered through a 0.45 mm PTFE filter, and the volume adjusted to 2 ml and stored at –80 ºC until further analysis. The HPLC system consisted of an ion exchange aminex HPX-87H Column (Bio-Rad, USA) maintained at 40 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}.

6.3.5. Protein Extraction and LC−MS/MS Analysis

The protein extraction was based on the protocol developed by Wu et al. (2014). We used three biological replicates to perform the proteome analysis. From each sample, 250 mg of frozen plant tissue was ground in liquid nitrogen. The powdered tissue was dissolved in cold acetone (−20 °C) with TCA (10% wt/vol), homogenized with acid-washed sand, and centrifuged at 15,000g for 5 min at 4° C to collect the precipitated proteins. The pellet was resuspended in cold TCA/acetone, vortexed, and centrifuged at 15,000g for 5 min at 4° C to collect proteins and repeated until the pellet turned white. Then, the pellet was resuspended twice in 1.5 ml of cold acetone, centrifuged at 15,000g for 5 min at 4°C, and the supernatant discarded. After that, the pellet was air-dried in a fume hood and resuspended in an SDS extraction buffer for 1.5 h. Then, centrifuged at 15,000 g for 10 min, the supernatant was collected into new Eppendorf tubes, mixed with an equal volume of Tris-buffered phenol, and vortexed for 3 min. The mixture was centrifuged at 15,000g for 5 min, and the lower phenol phase was collected into a new Eppendorf tube. An equal volume of washing buffer I was added to the phenol phase, vortexed by 3 min, and centrifuged at 15,000g for 5 min at room temperature. The upper phase (phenol phase) was collected into a new Eppendorf tube, and 0.1 M ammonium acetate in methanol was added to a final volume of 2.0 ml. The mixture was vortexed for 30 sec and stored at − 20 °C for 2 hr to precipitate the phenol-extracted proteins. The solution was centrifuged at 15,000g for 10 min at 4 °C, and the supernatant was discarded. The protein pellets were resuspended with 0.1 M ammonium acetate in methanol and centrifuged at 15,000g for 5 min at 4 °C, and the supernatant was rejected. Therefore, the pellets were resuspended in 80% (vol/vol) acetone, centrifuged at 15,000g for 5 min at 4 °C, and the supernatant was removed. The protein pellets were air-dried at room temperature and resuspended in 100 µl of rehydration buffer (50 mM ammonium carbonate, 8 M urea). The concentrations of the protein extracts were determined by the Bradford assay (Bradford, 1976).

Each sample was processed for proteomic analysis following the solid-phase-enhanced sample-preparation (SP3) protocol and enzymatically digested with Trypsin/LysC as previously described (Osório et al., 2021). Protein identification and quantitation were performed by nanoLC-MS/MS following an already published procedure (Casanova et al., 2021) using a nanoLC flow rate of 300 nL/min. For protein identification and quantification, the UniProt database was considered for the *Glycine max* Proteome (2020_01). The proteomics data analysis was performed with the Proteome Discoverer 2.4.0.305 software (Thermo Scientific).

6.3.6. Database Search and Protein Quantification

Only high-confidence peptides and proteins with at least two unique peptides detected in all three replicates were used in quantification. For all quantified proteins, only those showing a fold change of above 1.2 or below 1/1.2 (for the mean of the three replicates) in the quantitative ratios and $p < 0.05$ were considered as differentially expressed proteins (DEPs). Protein sequences were submitted to the online Mercator 4 annotation tool (Schwacke et al., 2019) for proteome annotation. Protein functions were categorized using Mapman bin codes, and the protein abundance ratio was visualized through MapMan software (Thimm et al., 2004). Pathway enrichment analysis of DEPs was performed using Fisher's exact test. Information about the subcellular location was accomplished from the location available from SUBA4 (Hooper et al., 2017).

6.3.7. Statistical Analysis

Data were analyzed using SPSS software (SPSS version 26.0). Analysis of variance (2way ANOVA) was used to determine differences among different treatments after data normality and equal variance analysis. The means ± SE were calculated for each parameter.

6.4. Results

6.4.1. Interactive effects of eCO_2 and Fe-limitation on plant biomass

Both eCO_2 and Fe-limitation showed significant effects ($p < 0.05$) on plant biomass with a significant CO_2 x Fe interaction (Table 6.1). Elevated CO_2 increased plant dry weight from 2.14 to 3.14 g $plant^{-1}$ under Fe-sufficient conditions and from 1.18 to 2.91 g $plant^{-1}$ under Fe-limited conditions. Furthermore, Fe-limitation significantly decreased plant biomass at aCO_2 ($p < 0.05$) but not at eCO_2 ($p > 0.05$).

6.4.2. Interactive effects of eCO_2 and Fe-limitation on photosynthesis

Fe-limitation showed a significant effect on Pn ($p < 0.05$), whereas at eCO_2 ($p > 0.05$, Table 6.1) a significant effect was not detected. The decrease in Pn due to Fe-limitation was -21.7% and -28.4% under eCO_2 and aCO_2 conditions, respectively. Elevated CO_2 significantly decreased gs under both Fe supplies ($p < 0.05$). Moreover, under Fe-limitation, gs was reduced at aCO_2 ($p < 0.05$) and at eCO_2 conditions ($p > 0.05$). The transpiration rate (Tr) was decreased by eCO_2 ($p < 0.05$) and by Fe-stress at aCO_2 conditions ($p < 0.05$).

Table 6.1 - Effects of eCO_2 and Fe-limitation on biomass, sugar content, and gas exchange parameters in soybean plants.

	Treatments				Two-way ANOVA			
Measurements	Fe+ELE	Fe+AMB	Fe-ELE	Fe-AMB	df	CO_2	Fe	CO_2 x Fe
Total biomass (g plant $^{-1}$)	3.14 ± 0.19^a	2.14 ± 0.15^b	2.91 ± 0.12^a	1.18 ± 0.08^c	**1**	**94.29; <0.001**	**18.04; <0.001**	**6.59; 0.021**
Pn (µmol m^{-2} s^{-1})	11.21 ± 0.43^a	11.43 ± 0.55^a	9.21 ± 0.40^b	8.90 ± 0.80^b	**1**	0.006; 0.939	**15.17; <0.001**	0.21; 0.654
gs (mol m^{-2} s^{-1})	0.13 ± 0.01^a	0.37 ± 0.03^b	0.12 ± 0.01^a	0.30 ± 0.02^c	**1**	**85.74; <0.001**	**6.568; 0.021**	**5.42; 0.033**
Tr (mol m^{-2} s^{-1})	2.46 ± 0.19^a	5.22 ± 0.36^b	2.28 ± 0.16^a	4.13 ± 0.29^d	**1**	**54.19; <0.001**	3.84; 0.068	4.44; 0.051
Sugar (roots, µmol g FW^{-1})	26.17 ± 3.72^a	10.92 ± 1.12^b	19.89 ± 2.36^c	8.75 ± 0.92^b	**1**	**9.90; 0.009**	4.82; 0.051	1.85; 0.201
Sugar (leaves, µmol g FW^{-1})	52.22 ± 4.03^a	33.62 ± 1.21^b	46.70 ± 2.54^a	34.80 ± 2.44^b	**1**	**37.71; <0.001**	0.761; 0.406	1.82; 0.210

Note: Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2; Data are mean ± SE (n = 5). Different lowercase superscript letters in the same row indicate the significant difference at $p < 0.05$ level. CO_2, Fe, and CO_2 × Fe indicate CO_2 treatment, Fe treatment, and the interaction of CO_2 by Fe treatment, respectively. Results from the analysis of variance with degrees of freedom (d.f.), F ratios and probabilities (p) for some parameters. Significant effects are shown in boldface.

6.4.3. Interactive effects of eCO₂ and Fe-limitation on sugar content

Elevated CO_2 showed a significant effect on sugar concentration in the root ($p < 0.05$, Table 6.1) and leaf tissues ($p < 0.05$) but was not affected by Fe-limitation ($p > 0.05$). Sugar concentration increased from 10.92 to 26.17 µmol g FW^{-1} in the roots of Fe-sufficient plants and from 8.75 to 19.89 µmol g FW^{-1} in the roots of Fe-limited plants after exposure to eCO_2. In leaves, sugar concentration increased from 33.62 to 52.22 µmol g FW^{-1} in Fe-sufficient conditions and from 34.80 to 46.70 µmol g FW^{-1} in Fe-limited conditions.

6.4.4. Functional categories of differentially expressed proteins

Tables S6.1 and S6.2 go into depth about the entire list of proteins that were found and measured. Proteins with a *p*-value below 0.05 and a fold change ratio above 1.2 or below 1/1.2 were considered as DEPs (Figure 6.1 and Tables S6.3 and S6.4).

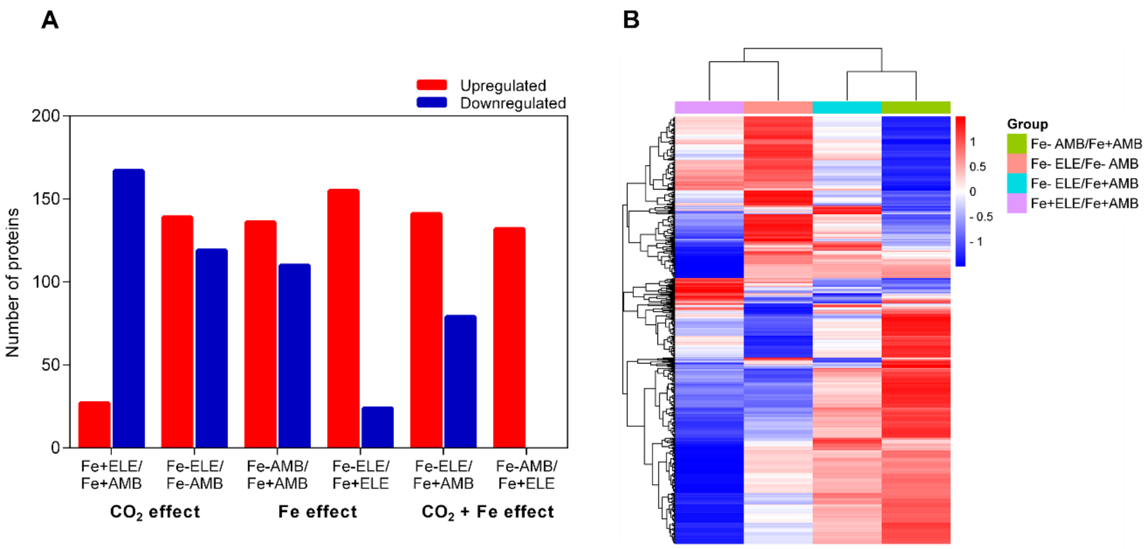
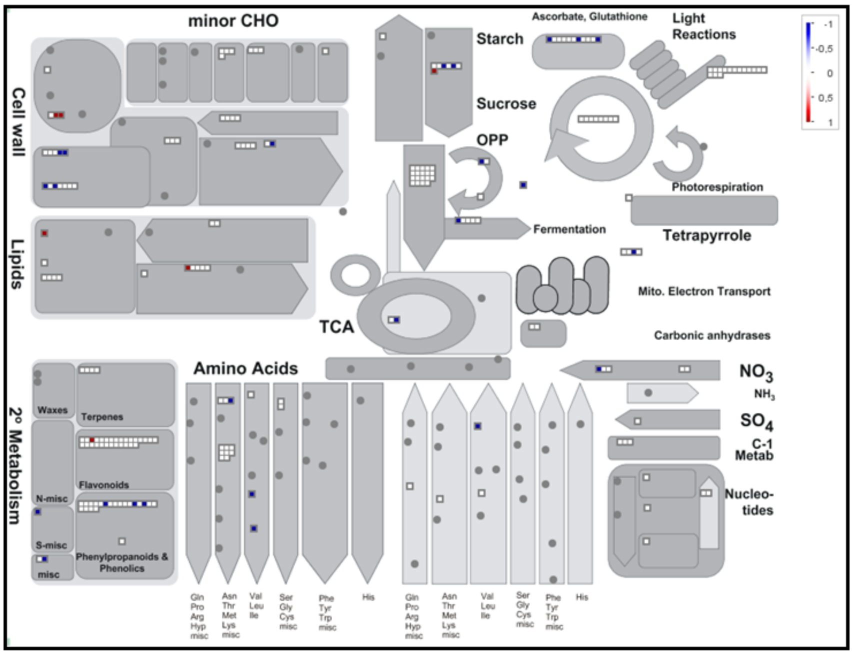


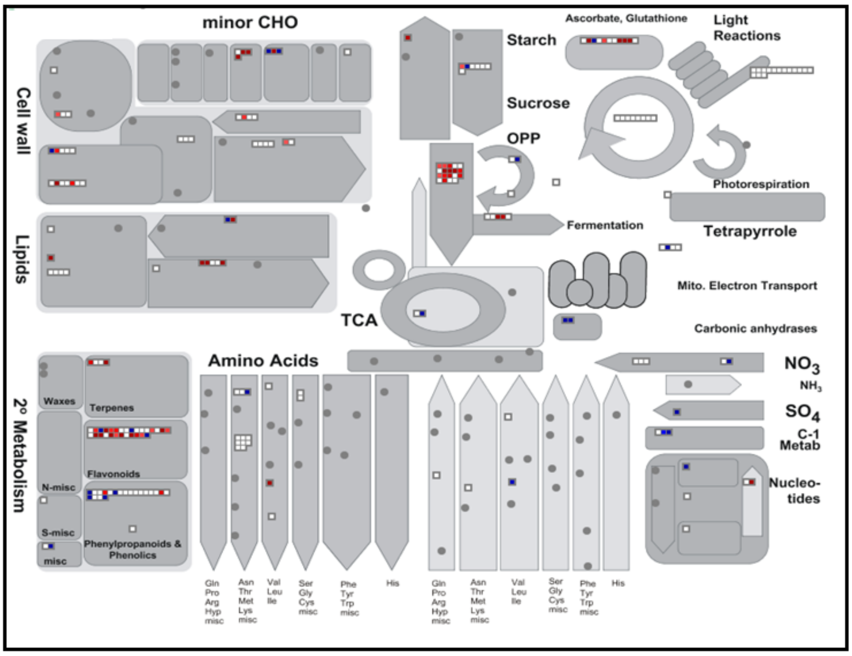
Figure 6.1 – Soybean root proteome profiles under eCO_2 and Fe-limitation. (A) The number of upregulated and downregulated proteins in soybean root in response to eCO_2 and Fe-limitation; (B) Cluster analysis of all differently regulated proteins among different treatments. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2; Fe+ELE/Fe+AMB and Fe-ELE/Fe-AMB – CO_2 effect; Fe-AMB/Fe+AMB – Fe effect; Fe-ELE/Fe+AMB – interaction of CO_2 and Fe limitation.

Among the identified proteins, 705 were differentially expressed in root tissues considering a fold change ratio > 1.2 or < 1/1.2 ($p < 0.05$, Figure 6.1a). We found 27 and 136 proteins that were upregulated under eCO_2 (Fe+ELE/Fe+AMB) and Fe-limitation (Fe-AMB/Fe+AMB), respectively, with an overlap of one protein (Fe2OG dioxygenase domain-containing protein, Figure S6.1). Furthermore, 167 and 110 proteins were downregulated by eCO_2 and Fe-limitation, respectively, with an overlap of two HMA domain-containing proteins, one superoxide dismutase and one 2-isopropylmalate synthase. Compared to control plants, 141 proteins were up and 79 downregulated by the interaction of eCO_2 with Fe-limitation (Fe-ELE/Fe+AMB). We also found 139 up and 119 downregulated proteins in Fe-ELE/Fe-AMB conditions. Protein expression profiles could be correctly distinguished between treatments using the heat map of DEPs (Figure 6.1b). The metabolism maps showed changes at the protein level obtained using the MapMan software. Proteins associated with redox regulation and cell wall metabolism decreased the expression levels under eCO_2, and photosynthetic light reactions proteins were upregulated by Fe-limitation, as shown in Figure 6.2. In addition, proteins related to photosynthetic light reactions, glycolysis, and redox regulation were upregulated in Fe-ELE/Fe+AMB conditions. Enzymes associated with secondary metabolism, glycolysis, and redox regulation were upregulated in Fe-ELE/Fe-AMB conditions. The number of differentially regulated proteins, according to functional categories and cellular localization, is summarized in Figure 6.3. Most enzymes in roots with assigned functions were "protein-related," "stress-related," "secondary metabolism-related," and "RNA-related," and were mostly downregulated in response to eCO_2. Protein synthesis, photosynthesis-related, and redox-related enzymes were mainly upregulated under Fe-limited conditions. In Fe-ELE/Fe+AMB conditions, most proteins were associated with photosynthesis, hormone metabolism, redox regulation, signaling, and glycolysis and upregulated. In addition, most enzymes related to protein synthesis were downregulated, and enzymes associated with secondary metabolism, hormone metabolism, stress, and glycolysis were upregulated in the Fe-ELE treatment compared with Fe-AMB. According to their cellular location, most proteins were found in the cytosol, followed by the extracellular space and nucleus. In leaves, a total of 589 proteins were differentially expressed (Figure 6.4). The gene expression profile between treatments could be distinguished and included 72 and 101 proteins that were up and downregulated, respectively, under eCO_2. Iron limitation resulted in 146 up and 104 downregulated proteins.

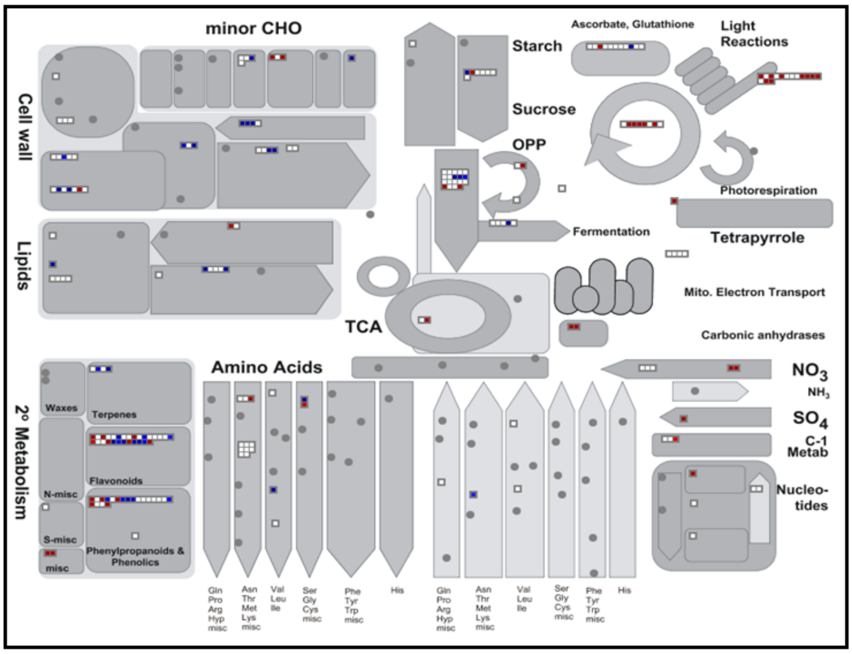
Fe+ELE/Fe+AMB



Fe-ELE/Fe-AMB



Fe-AMB/Fe+AMB



Fe-ELE/Fe+AMB

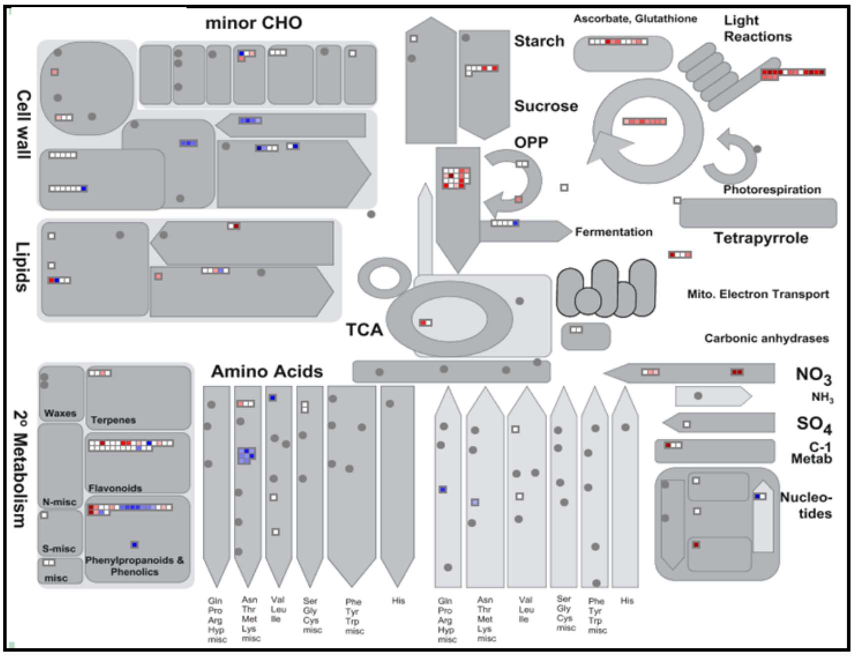


Figure 6.2 - MapMan metabolism overview maps showing changes in DEPs in root tissues under eCO_2 and Fe-limitation. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2. Boxes represent log2 expression values, genes in red are upregulated and those in blue are repressed.

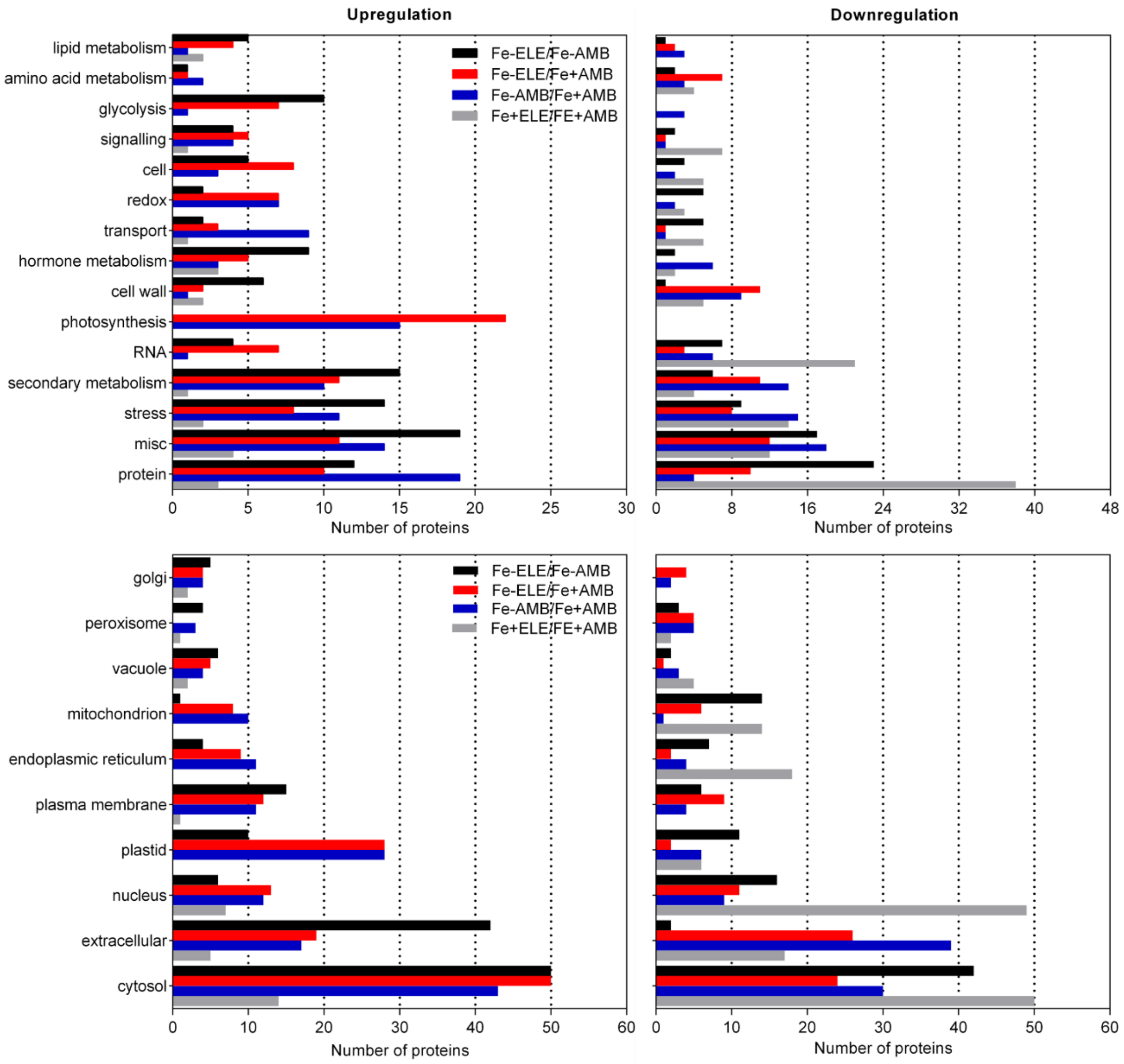


Figure 6.3 - Numbers of DEPs identified from soybean roots at different CO_2 levels under sufficient and limited Fe-supply according to functional categories and subcellular compartments by MapMan. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2.

In upregulated, an overlap of 14 proteins (Figure S6.2), whereas, in downregulated proteins, an overlap of 27 proteins was observed between eCO_2 and Fe-limitation. The interaction of Fe-limitation and eCO_2 resulted in 83 and 76 proteins being upregulated and downregulated, respectively. From the upregulated proteins, the interaction induced an overlap of 28 proteins with Fe-limitation or 27 proteins with eCO_2. In downregulated proteins, plants grown under Fe-limitation and eCO_2 had an overlap of 41 proteins with Fe-stress and 36 proteins with eCO_2. Furthermore, 74 and 136 proteins were upregulated and downregulated in the Fe-ELE/Fe-AMB conditions. The metabolism overview maps (Figure 6.5) in the leaves showed that eCO_2 increased the expression of enzymes involved in glycolysis, photosynthesis, and redox homeostasis, while Fe-limitation decreased proteins involved in photosynthetic light processes.

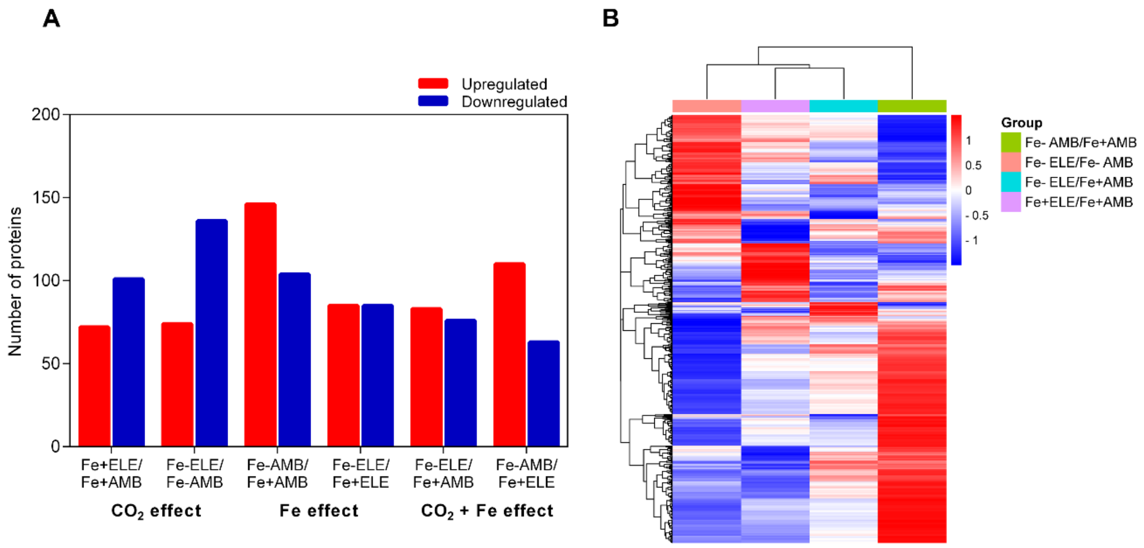
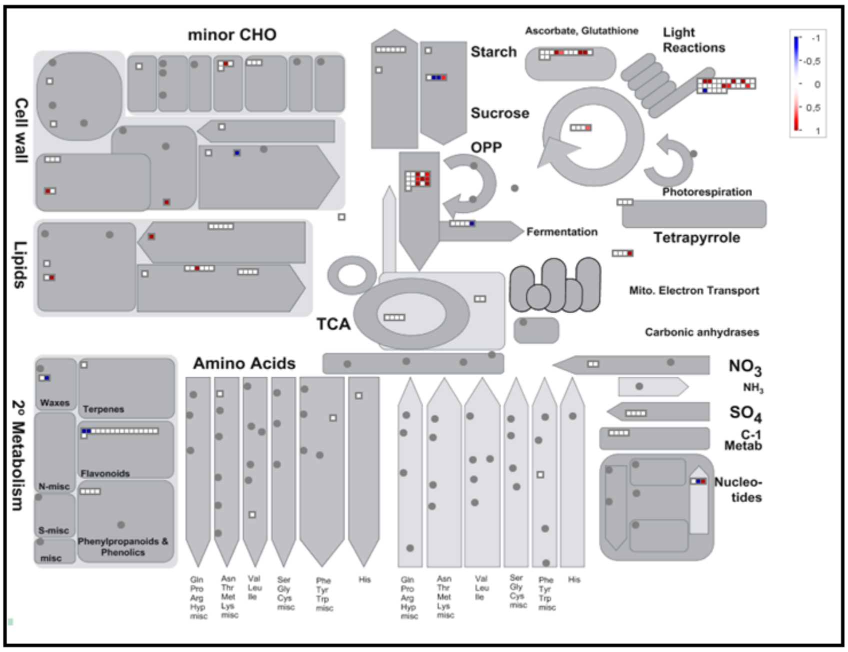


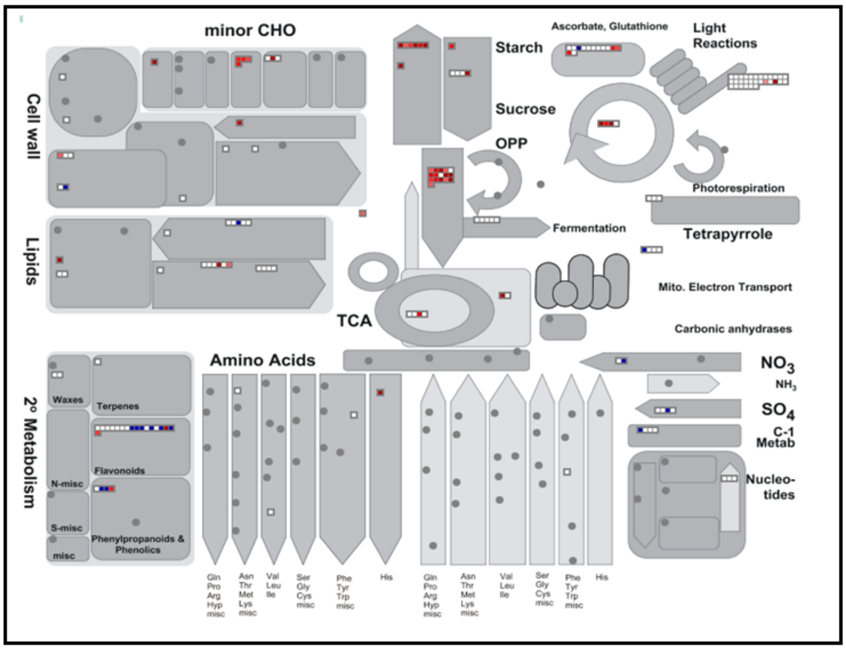
Figure 6.4 – Soybean leaf proteome profiles under eCO_2 and Fe-limitation. (A) The number of upregulated and downregulated proteins in soybean leaf in response to eCO_2 and Fe-limitation; (B) Cluster analysis of all differently regulated proteins among different treatments. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2; Fe+ELE/Fe+AMB and Fe-ELE/Fe-AMB – CO_2 effect; Fe-AMB/Fe+AMB – Fe effect; Fe-ELE/Fe+AMB – interaction of CO_2 and Fe-limitation.

In Fe-ELE/Fe+AMB, the expression of proteins involved in glycolysis and redox homeostasis increased. However, in Fe-ELE/Fe-AMB, enzymes related to glycolysis and carbohydrate metabolism were increased. The number of DEPs, according to functional categories and cellular compartments, is described in Figure 6.6. Elevated CO_2 increases the proteins implicated in stress, photosynthesis, and glycolysis while downregulating the enzymes involved in signaling and protein synthesis. Furthermore, protein synthesis and secondary metabolism proteins exhibited high expression levels, while photosynthesis-related proteins had low levels at Fe-limitation. Most glycolysis-related proteins were downregulated in Fe-ELE/Fe+AMB. In addition, most proteins associated with carbohydrate metabolism, photosynthesis, stress, and glycolysis were upregulated, and with protein synthesis were downregulated in Fe-ELE compared with Fe-AMB. Concerning their cellular location, most of the proteins identified were in the cytosol, followed by the plastid and extracellular space (Figure 6.6).

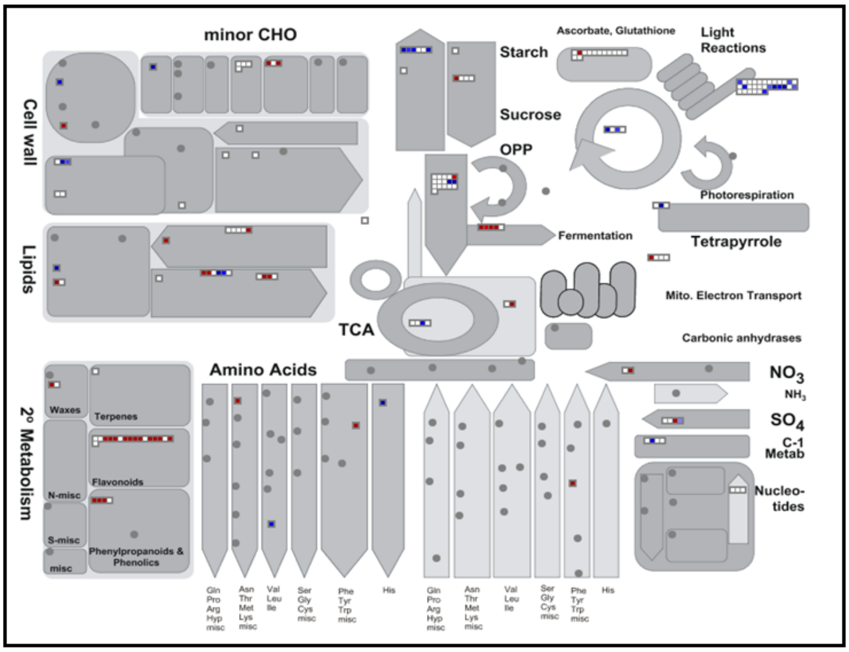
Fe+ELE/Fe+AMB



Fe-ELE/Fe-AMB



Fe-AMB/Fe+AMB



Fe-ELE/Fe+AMB

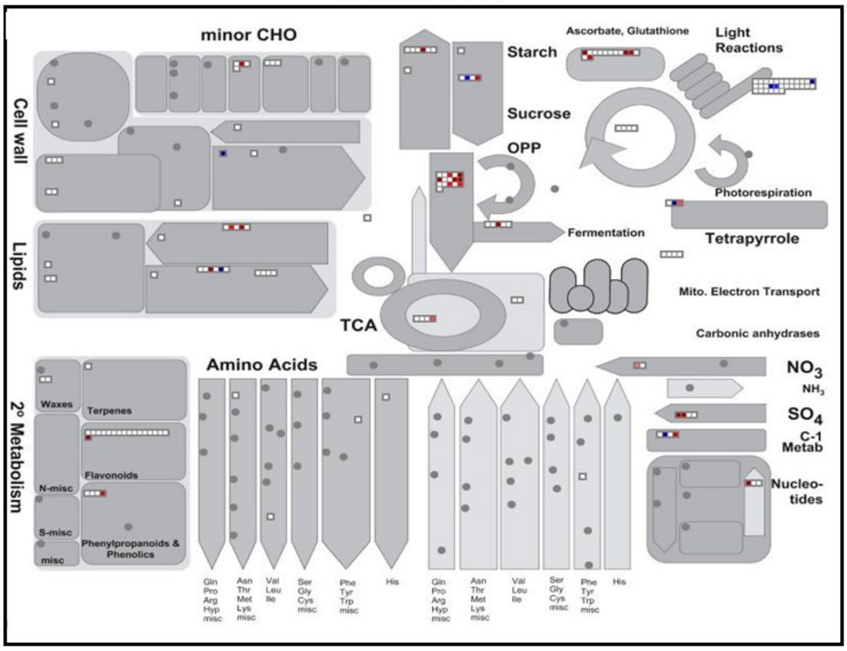


Figure 6.5 - MapMan metabolism overview maps showing changes in DEPs in leaf tissues under eCO_2 and Fe-limitation. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2. Squares represent log2 expression values, genes in red are upregulated and those in blue are repressed.

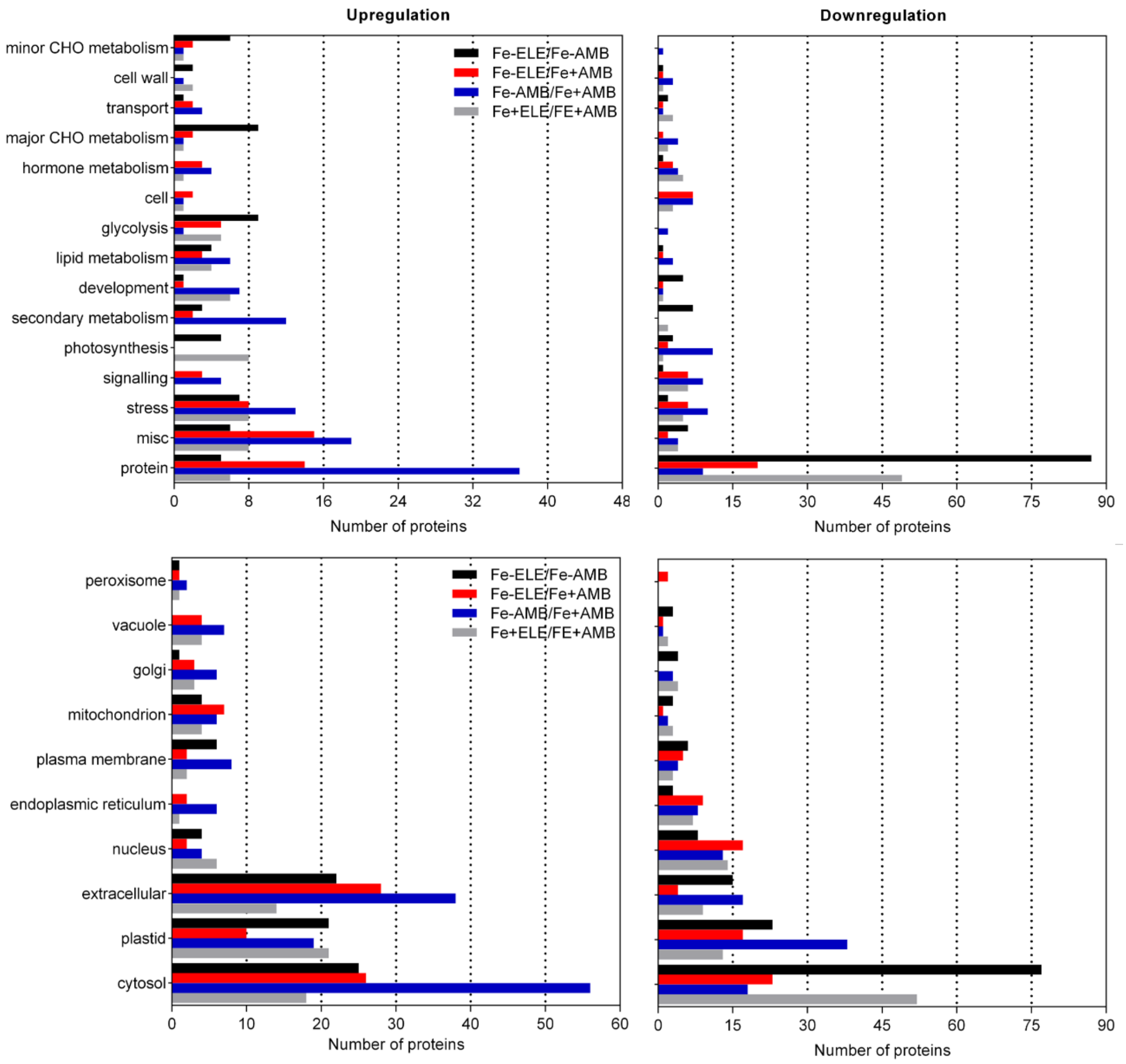


Figure 6.6 - Numbers of DEPs identified from soybean leaves at different CO_2 levels under sufficient and limited Fe-supply according to functional categories and subcellular compartments by MapMan. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2.

6.4.5. Metabolic pathways related to the interaction of eCO_2 and Fe-limitation

To gain a better understanding of the DEPs between different treatments, pathway enrichment analysis was conducted and shown in Tables 6.2 and 6.3. The photosynthesis-related pathways (bin 1) were upregulated in the Fe-AMB vs Fe+AMB and Fe-ELE vs Fe+AMB treatments in the root tissues. Glycolysis (bin 4.1) was not affected by eCO_2 and Fe-limitation but was upregulated under Fe-ELE treatment compared to Fe+AMB and Fe-AMB.

Cell wall organization (bin 10) and metal binding (bin 15.2) were not affected by eCO_2 but downregulated by Fe-limitation and the interaction of eCO_2 and Fe-limitation. The secondary metabolism (bin 16) was negatively downregulated by Fe-limitation and upregulated by eCO_2 under Fe-limitation (Fe-ELE vs Fe-AMB). Enzymes involved in hormone metabolism (bin 17.6) had a higher level of expression at eCO_2 under Fe-sufficient and Fe-limited conditions. Proteins associated with stress responses (bins 20.1 and 20.2.1) had low expression under Fe-stress. Moreover, an increase in ascorbate and glutathione metabolism (bin 21.2.1.3), which is an effective mechanism of plant detoxification, was induced by Fe-ELE compared with Fe+AMB conditions. Peroxidases (bin 26.12), phosphatases (bin 26.13), and oxygenases (bin 26.14) were not affected by CO_2 enrichment but downregulated by Fe-limitation. The expression of glutathione S-transferase proteins (bin 26.9) was higher in Fe-limited plants. Inhibition of gene expression in RNA (bin 27) and protein synthesis (bin 29) pathways occurred under eCO_2. The expression levels of transport proteins (bin 34) involved in the translocation of solutes across membranes were not affected by eCO_2.

Regarding leaf tissues, listed photosynthesis (bin 1) associated pathways were downregulated by Fe-stress. Upregulation of sucrose and starch biosynthesis (bin 2.1) genes occurred at eCO_2 under Fe-limited conditions, while downregulation was noticeable in low-Fe supply. Furthermore, glycolysis (bin 4.1) involved in the breakdown of sugars was upregulated by eCO_2 under Fe-limited and Fe-sufficient conditions and not affected by Fe-stress. Stimulation of fatty acid degradation was apparent by eCO_2 under Fe-limited conditions. Flavonoid biosynthesis (bin 16.8) was induced by Fe-stress and persisted unchanged under eCO_2 conditions. Proteins related to stress response (bin 20.1) were upregulated by eCO_2, Fe-limitation, and interaction of eCO_2 with Fe-limitation. Furthermore, several heat-shock proteins (bin 20.2.1) were downregulated by Fe-limitation. Glutathione S-transferases (bin 26.9) and peroxidases (bin 26.12) were upregulated by Fe-limitation and not affected by eCO_2. In addition, several enzymes involved in protein synthesis (bin 29) had lower expression levels at eCO_2 under Fe-sufficient and Fe-limited conditions. Cysteine protease degradation (bin 29.5.3) was upregulated by Fe-limitation and by the interaction of eCO_2 and Fe-limitation.

Table 6.2 – Pathway enrichment analysis of DEPS in roots of soybean plants under eCO_2 and Fe-limitation.

Bin code	Bin name	Fe+ELE vs Fe+AMB	*p*-value	Fe-AMB vs Fe+AMB	*p*-value	Fe-ELE vs Fe+AMB	*p*-value	Fe-ELE vs Fe-AMB	*p*-value
1.1.1	PS. Light reaction. Photosystem II	–	–	UP	$2.9E^{-05}$	UP	$1.7E^{-05}$	–	–
1.1.2	PS. Light reaction. Photosystem I	–	–	UP	$1.5E^{-02}$	UP	$3.8E^{-05}$	–	–
1.3	PS. Calvin cycle	–	–	–	–	UP	$4.4E^{-04}$	–	–
4.1	Glycolysis. Cytosolic branch	–	–	–	–	UP	$1.5E^{-03}$	UP	$1.7E^{-02}$
10	Cell wall	–	–	DOWN	$5.6E^{-07}$	DOWN	$1.6E^{-03}$	–	–
13.1.3.4	Amino acid metabolism. Synthesis. Aspartate family. Methionine	–	–	–	–	DOWN	$6.9E^{-03}$	–	–
15.2	Metal handling. Binding, chelation, and storage	–	–	DOWN	$3.3E^{-03}$	DOWN	$4.3E^{-07}$	–	–
16.1	Secondary metabolism. Isoprenoids	–	–	DOWN	$8.3E^{-03}$			UP	$1.6E^{-04}$
16.2.1	Secondary metabolism. Phenylpropanoids. Lignin biosynthesis	–	–	DOWN	$1.8E^{-03}$	DOWN	$2.6E^{-02}$	–	–
16.8	Secondary metabolism. Flavonoids	–	–	DOWN	$1.4E^{-02}$	–	–	UP	$3.1E^{-04}$
17.6	Hormone metabolism. Gibberellin	UP	$1.4E^{-04}$	DOWN	$8.3E^{-03}$	–	–	UP	$3.6E^{-04}$
20.1	Stress. Biotic	–	–	DOWN	$5.6E^{-03}$	–	–	UP	$2.1E^{-03}$
20.2.1	Stress. Abiotic. Heat	–	–	DOWN	$4.6E^{-02}$	–	–	–	–
21.2.1.3	Redox. Ascorbate and glutathione. Ascorbate. L-galactose-1-phosphate phosphatase	–	–	–	–	UP	$2.1E^{-02}$	–	–
26.9	Misc. Glutathione S-transferases	–	–	UP	$1.5E^{-02}$	–	–	DOWN	$1.3E^{-03}$
26.12	Misc. Peroxidases	–	–	DOWN	$2.7E^{-03}$	–	–	UP	$1.3E^{-03}$
26.13	Misc. Acid and other phosphatases	–	–	DOWN	$6.1E^{-03}$	–	–	UP	$3.6E^{-04}$
26.14	Misc. Oxygenase	–	–	DOWN	$4.2E^{-02}$	–	–	–	–
27.3	RNA. Regulation of transcription	DOWN	$9.4E^{-03}$	–	–	–	–	–	–
27.4	RNA.RNA binding	DOWN	$4.4E^{-02}$	–	–	–	–	–	–
29.2.1	Protein. Synthesis. Ribosomal protein	DOWN	$4.2E^{-02}$	UP	$2.9E^{-05}$	–	–	DOWN	$1.0E^{-14}$
34	Transport	–	–	UP	$3.1E^{-02}$	–	–	DOWN	$3.7E^{-02}$

Table 6.3 – Pathway enrichment analysis in leaves of soybean plants under eCO_2 and Fe-limitation.

Bin code	Bin name	Fe+ELE vs Fe+AMB	*p*-value	Fe-AMB vs Fe+AMB	*p*-value	Fe-ELE vs Fe+AMB	*p*-value	Fe-ELE vs Fe-AMB	*p*-value
1.1.1	PS. Light reaction. Photosystem II	–	–	DOWN	$1.9E^{-04}$	–	–	–	–
1.1.4	PS. Light reaction. Photosystem I	–	–	DOWN	$3.0E^{-02}$	–	–	–	–
2.1	Major CHO metabolism. Synthesis	–	–	DOWN	$1.0E^{-02}$	–	–	UP	$1.2E^{-04}$
3.4	Minor CHO metabolism. Myo-inositol	–	–	–	–	–	–	UP	$2.0E^{-02}$
4.1	Glycolysis. Cytosolic branch	UP	$2.0E^{-03}$	–	–	–	–	UP	$2.0E^{-02}$
11.9.3.3	Lipid metabolism. Lipid degradation. Lysophospholipases. Glycerophosphodiester phosphodiesterase	–	–	DOWN	$1.7E^{-03}$	–	–	UP	$3.5E^{-03}$
13.2.4	Amino acid metabolism. Degradation. Branched chain group	–	–	UP	$3.3E^{-02}$	–	–	–	–
16.8	Secondary metabolism. Flavonoids	–	–	UP	$6.1E^{-04}$	–	–	–	–
20.1	Stress. Biotic	UP	$6.1E^{-03}$	UP	$3.7E^{-04}$	UP	$1.6E^{-07}$	–	–
20.2.1	Stress. Abiotic. Heat	–	–	DOWN	$2.7E^{-03}$	–	–	–	–
26.9	Misc. Glutathione S-transferases	–	–	UP	$3.8E^{-02}$	–	–	–	–
26.12	Misc. Peroxidases	–	–	UP	$1.3E^{-04}$	UP	$1.8E^{-05}$	–	–
28.1.3	DNA. Synthesis/chromatin structure. Histone	DOWN	$1.5E^{-02}$	UP	$4.1E^{-02}$	–	–	DOWN	$2.4E^{-02}$
29.2.1	Protein. Synthesis. Ribosomal protein	DOWN	$2.8E^{-14}$	UP	$1.9E^{-13}$	–	–	DOWN	$9.5E^{-14}$
29.2.2	Protein. Synthesis. Ribosome biogenesis	DOWN	$9.8E^{-03}$	–	–	–	–	DOWN	$3.8E^{-03}$
29.3.4	Protein. Targeting. Secretory pathway	–	–	–	–	–	–	DOWN	$2.8E^{-03}$
29.5.1	Protein. Degradation. Subtilases	–	–	–	–	–	–	UP	$2.3E^{-03}$
29.5.3	Protein. Degradation. Cysteine protease	–	–	UP	$2.5E^{-03}$	UP	$4.1E^{-03}$	–	–
30.5	Signaling. G-proteins	–	–	–	–	DOWN	$4.2E^{-02}$	–	–
33.1	Development. Storage proteins	UP	$6.5E^{-06}$	UP	$8.3E^{-03}$	–	–	DOWN	$1.4E^{-02}$
34.9	Transport. Metabolite transporters at the mitochondrial membrane	DOWN	$7.8E^{-03}$	–	–	–	–	–	–

6.5. Discussion

To cope with Fe-stress and eCO_2, soybean plants have evolved complex signaling and metabolic processes at the cellular, organ, and whole-plant levels. Elevated CO_2 promoted plant growth under Fe-sufficient and Fe-limited conditions, as shown in Table 6.1. Similarly, this “fertilization effect” was reported in tomato (Jin et al., 2009) and barley plants (Haase et al., 2008) grown under low levels of Fe supply and eCO_2. However, plant growth decreased under Fe-limitation, particularly at aCO_2, suggesting that eCO_2 could mitigate the Fe deficiency responses. Moreover, Pn was not affected by eCO_2 but reduced in Fe-limited plants promoting leaf chlorosis (Table 6.1). The statistical analysis also revealed a significant interaction between eCO_2 and Fe-stress on plant growth but not in Pn. Elevated CO_2 significantly increased the sugar content in root and leaf tissues (Table 6.1). At eCO_2, plants might surpass what they are capable of, using or distributing to sinks, increasing the carbohydrate content and possibly leading to feedback inhibition of photosynthesis (Thompson et al., 2017). Sugars are recognized to crosstalk with hormones acting on gene regulation and therefore modify nutrient uptake and transport, among other functions (Thompson et al., 2017). Lin et al. (2016a) suggested that sucrose acts as a signaling molecule, causing an increase in auxin and a subsequent increase in nitric oxide, leading to the FIT-mediated transcriptional regulation of FRO2 and IRT1 genes inducing Fe-uptake mechanisms. Although physiological aspects of soybean responses to eCO_2 or Fe-limitation are well studied, proteomic profiling helps understand the molecular basis of soybean adaptation to the upcoming changing climate. Thus, several DEPs were found, in root and leaf tissues, due to eCO_2 and Fe-limitation (Figures 6.1 and 6.4). Exposure to eCO_2 and Fe-stress leads to changes in the expression of genes involved in the carbon metabolism in soybean seedlings, especially the expression of genes related to glycolysis, starch and sucrose metabolism, and Myo-inositol metabolism. Elevated CO_2 under Fe-limited conditions stimulated starch and sucrose biosynthesis. Therefore, protein abundance of most enzymes involved in starch (e.g., starch synthase, granule-bound starch synthase, starch-branching enzyme, and 1,4-alpha-glucan branching enzyme) and sucrose biosynthesis (e.g., sucrose-phosphate synthase), were upregulated by eCO_2 conditions (Table 6.3). The central role of glycolysis is to breakdown glucose, produce ATP and generate precursors such as fatty acids and amino acids for anabolism (Chen et al., 2016a). Enzymes involved in glycolysis, such as enolase, pyruvate kinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglucomutase, were upregulated by eCO_2 under Fe-limited conditions (Fig. S6.3). Together, these findings led us to suppose that increased energy production via carbohydrate metabolism maintains energy homeostasis. Therefore, eCO_2 increased the transcript levels of genes encoding enzymes involved in foliar cellular respiration, suggesting an improved flux through glycolysis driven by higher carbohydrate bioavailability (starch and sucrose) at eCO_2. Ainsworth et al. (2006) also found that growth at eCO_2 led to the stimulation of foliar respiration in soybean plants. This adaptive balance enables plant growth even in the case of Fe-limitation.

In the present study, we found evidence of photosynthesis-related genes in roots (Table 6.2). DEPs enriched in photosynthesis were upregulated by Fe-limitation and by the interaction of eCO_2 with Fe-limitation. Similarly, Kobayashi et al. (2012) reported increased levels of transcription factors responsible for the coordinated expression of genes in chloroplast biogenesis in the roots of *Arabidopsis thaliana*. Accumulation of two golden-2-like transcription factors known to improve phototrophic performance and increase photosynthesis-related proteins in wheat roots under Fe deficiency was reported by Kaur et al. (2019). We hypothesize that this might be a strategy to cope with Fe-stress, thereby increasing CO_2 fixation and demonstrating the possibility of root photosynthesis to improve plants' carbon utilization. In leaves, consistent with protein expression levels, Fe-limitation depressed leaf photosynthesis, which agrees with other studies (Jiang et al., 2007, Therby-Vale et al., 2021, Andaluz et al., 2006, Briat et al., 2015). The Fe-AMB treatment produced a higher reduction in plant biomass when compared to the Fe-ELE treatment (Table 6.1). These results indicated that eCO_2, particularly under Fe-limitation, increased the accumulation of photoassimilates due to eCO_2-induced sugar metabolic pathways. This process is stimulated during adaptation to eCO_2 to generate energy used to maintain leaf growth.

We found many genes involved in flavonoid biosynthesis upregulated after exposure to Fe-stress in leaves and some downregulated in soybean roots. The modulation of genes involved in the biosynthesis of flavonoids suggests that secondary metabolism also plays a role in Fe-stress responses. Several studies reported the effect of various stresses on secondary metabolism in plants (Austen et al., 2019, Ramakrishna and Ravishankar, 2011). Flavonoids are secondary plant products that are biologically active and perform different functions in plants as defense mechanisms against abiotic and biotic stresses (Falcone Ferreyra et al., 2012). Ahmed et al. (2021) demonstrated that drought stress induced the expression of flavonoid biosynthesis genes in hybrid poplar plants and increased the accumulation of phenolic and flavonoid compounds with antioxidant activity. Iron deficiency also increased the expression of crucial enzymes in the flavonoid pathway in *Arabidopsis thaliana* roots (Lan et al., 2011). Besides, the emission of phenolic complexes into the rhizosphere, involved in Fe deficiency induced responses, is considered a component of the strategy I plants. It was demonstrated in Arabidopsis roots that coumarins participate in Fe-chelation under Fe deficiency (Perkowska et al., 2021). We found overexpression of feruloyl-CoA 6′-hydroxylase and coumarin synthase involved in coumarin biosynthesis under Fe-limitation in root tissues (Table S6.3). The above results reinforced the role of coumarins in plant responses to Fe-stress. Table 6.2 also reveals that a considerable fraction of the carbon flowing through the glycolysis pathway is diverted to secondary metabolism, particularly flavonoid and isoprenoid biosynthesis, which increased at eCO_2 under Fe-limited conditions in root tissues. Under Fe-stress, most proteins included in lignin biosynthesis, such as phenylalanine ammonia-lyase and peroxidase, were downregulated in the roots of soybean plants. Lignin provides mechanical strength to the plant's secondary cell walls, which protect cells from abiotic stresses and serve as the structures that first perceive and respond to environmental stresses. Hence, the downregulation of phenylpropanoid biosynthesis (Figure S6.4) could save carbon and energy for other metabolic processes (Jia et al., 2019).

The capacity of cellular redox regulation is crucial to maintaining the activity of many physiological processes (Zhang et al., 2018b). Oxidative stress is often neutralized by enzymatic and non-enzymatic antioxidative systems (Kapoor et al., 2019). The expression of glutathione S-transferase proteins was higher in soybean plants exposed to Fe-limitation (Tables 6.2 and 6.3). Glutathione S-transferases are involved in several plant functions as detoxification of xenobiotic, secondary metabolism, growth and development, tetrapyrrole metabolism, and against biotic and abiotic stresses (Dalton et al., 2009, Vaish et al., 2020). Höhner et al. (2013) showed that Fe deficiency increased the expression levels of glutathione S-transferases in *Chlamydomonas reinhardtii*. Similarly, Fe deficiency-induced changes in the protein profile of *Arabidopsis thaliana* roots and glutathione S-transferases were considered highly expressed proteins (Lan et al., 2011). Moreover, the interaction of eCO_2 and Fe-limitation induced the ascorbate-glutathione pathway in roots. Among the antioxidant defense mechanisms, the ascorbate-glutathione pathway is crucial in mitigating further damage to soybean plants caused by reactive oxygen species and derivatives produced during metabolic activity.

Plant growth and development occur due to the global balance between protein synthesis and degradation (Zhang et al., 2018b). Our proteomic analysis showed that many enzymes involved in protein synthesis were downregulated by eCO_2, under Fe-sufficient and Fe-limited conditions, in root and leaf tissues. These results could be associated with the fact that increased levels of carbohydrates can affect gene expression through their role as signaling molecules. Sugars could be involved in photosynthetic acclimation, whereby the additional carbohydrates formed under eCO_2 conditions might cause the downregulation of photosynthetic gene transcripts and suppress protein synthesis (Thompson et al., 2017). However, photosynthetic acclimation does not always completely negate the positive results eCO_2 has on plant growth. The positive effects of eCO_2 on plant growth are well studied, but the role of hormone pathways in regulating the growth responses under eCO_2 is slightly understood. Gibberellins are a class of diterpenoid hormones involved in several growths and developmental processes, including stem elongation, leaf expansion, flower development, and germination (Ribeiro et al., 2012). Biosynthesis of gibberellins includes 2-oxoglutarate/Fe(II)-dependent dioxygenases that are upregulated at eCO_2, under Fe-limited and Fe-sufficient conditions, in this study. An increase in gibberellins expression under eCO_2 has also been reported in species such as *Ginkgo biloba* (Li et al., 2002), *Arabidopsis thaliana* (Teng et al., 2006), and *Populus tomentosa* (Liu et al., 2014*)*. Our finding suggests that eCO_2 might play a role in signaling, allowing higher plant growth rates.

Most enzymes involved in the stress responses, including heat-shock proteins, had low expression levels under Fe-limitation in soybean plants. The role of heat-shock proteins is to manage protein folding and promote cellular protection, protein homeostasis, and cell survival against several environmental and metabolic stresses (Bai et al., 2021, Donnini et al., 2010). We infer that soybean plants had lower levels of protein structure protection under Fe-limited conditions. In addition, proteins involved in cell-wall organization, including UDP-glucose 6-dehydrogenase, cellulose synthase, and xyloglucan endotransglucosylase were downregulated by Fe-stress and by the interaction of eCO_2 with Fe-stress in root tissues (Table S6.3). The reduced level of cell wall-modifying genes could limit cell expansion as plant growth decreased under Fe-limitation. The extent and severity of abiotic stresses or the crops selected are crucial in determining the effects of eCO_2 and Fe-stress. Therefore, eCO_2 and Fe-stress influence the growth and yield of plants and their subsequent adaptation to future climate changes and should require more attention from the scientific community.

6.6. Conclusions

Elevated CO_2 and Fe-stress had profound effects on plant biomass, sugar content, and Pn, with interactive effects of eCO_2 and Fe-stress on plant biomass. Overall, the CO_2-induced increase in biomass was not significantly different between Fe+ELE and Fe-ELE treatments. We performed proteomic analysis to analyze DEPs affected by the interaction of Fe-limitation and eCO_2 in soybean plants. Overall, root and leaf tissues contained 705 and 589 DEPs, respectively. Based on pathway enrichment analysis, cell wall organization, glutathione metabolism, photosynthesis, stress-related proteins, and biosynthesis of secondary compounds changed in roots to cope with Fe-stress. Moreover, the enhanced plant growth by eCO_2 supplied with sufficient or insufficient Fe was associated with the increased abundance of proteins involved in glycolysis, starch and sucrose metabolism, biosynthesis of gibberellins, and decreased levels of protein biosynthesis. The understanding of plant productivity and adaptation to future climate changes will also improve through future studies created to compare responses across various cultivars and during different periods of stress exposure.

PART IV - Conclusions and Future Perspectives

CHAPTER 7 - Conclusions

Elevated CO_2 and limited availability of Fe to plants are issues farmers are experiencing nowadays that will exacerbate in the future due to climate changes. Several studies in recent decades have been conducted based on the effects of climate change on yield parameters and nutritional quality. However, there is a lack of knowledge involving the impact of the interaction of eCO_2 and Fe-limitation on legume species. In this way, a better understanding of the physiological and molecular processes is essential and will help to improve the yield and nutritional quality of grains subjected to climate change. Thus, it was our intention with this doctoral program to study the effects of CO_2 enrichment and Fe-limitation in legumes and try to understand the response and adaptation mechanisms of the plants to these abiotic factors.

In summary, we showed that genetic variation in the seed yield response to eCO_2 under controlled conditions does exist among legume species. At maturity, eCO_2 increased seed protein concentration in beans and did not affect protein concentration in soybeans. Lipid concentration was not affected by eCO_2 in either legume species. However, mineral concentrations were differently affected by eCO_2 conditions.

In FACE conditions, we demonstrated again a variation among soybean genotypes and that genetic background has the potential to adapt to the upcoming atmospheric CO_2 concentrations. Elevated CO_2 increased soybean yield but decreased the grain content of protein, minerals, and antioxidant capacity. The response in the number of pods and the increased number of seeds were the main drivers of the improvement in seed yield. Therefore, the *Winsconsin Black* genotype emerged as the best candidate to breed soybean in future atmospheric conditions based on yield increase and reduced nutrient losses.

In chapter 4, we performed a transcriptomic analysis highlighting significant pathways and metabolic processes changed after exposure to eCO_2 in soybean plants. Several DEGs were founded in leaf (3083 genes) and root (828 genes) tissues, suggesting that leaves were more sensitive to eCO_2. Further analysis showed DEGs enriched in pathways mainly associated with energy and metabolic processes and probably responsible for the adaptation to eCO_2 conditions. Moreover, several TFs were identified and might be involved in the response of soybean plants to eCO_2. Our results highlight the molecular mechanisms in soybean under eCO_2, which will provide a valuable resource for further research on soybean response to CO_2 enrichment.

In chapters 5 and 6, soybean plants were exposed simultaneously to eCO_2 and Fe-limitation. Elevated CO_2 concentration and Fe-supply induced morphological, physiological, and molecular responses in soybean plants. Biomass increased under eCO_2 irrespective of Fe-supply, implying that eCO_2 alleviated symptoms of Fe deficiency. Improved acquisition of minerals in Fe-sufficient plants indicates that eCO_2 can support the translocation of minerals to the leaves. Moreover, eCO_2 mitigated the Fe deficiency-induced chlorosis and improved Fe-acquisition mechanisms in Fe-deficient conditions. In order to examine DEPs impacted by the interaction between Fe-limitation and eCO_2, we also conducted a proteome analysis. The proteomic analysis showed that pathways enriched in cell wall organization, glutathione metabolism, photosynthesis, stress-related proteins, and biosynthesis of secondary compounds changed significantly in roots to cope with Fe-stress. Furthermore, exposure to eCO_2 in Fe-sufficient and Fe-limited plants increased the expression of proteins related to glycolysis, synthesis of gibberellins, starch, and sucrose metabolism but decreased protein synthesis.

CHAPTER 8 – Future Perspectives

In this section, our purpose is to highlight some points that might be interesting to explore and complete in the future. Given the importance of legumes as a source of high-quality food, increasing biomass and yield while maintaining quality will be essential to food security as our worldwide population will rise in the coming years. Exploring genetic diversity in crops under eCO_2 conditions can help mitigate the negative impacts of climate change by anticipating what we can expect in the future and improving crop yields. Even though the number of CO_2-based experiments is large and the findings of the effects on crops are unambiguous, more experimental studies are still needed. Therefore, it would be advisable to use a broader range of germplasm from other sources to improve the geographical representativeness, different maturity groups, and different growth habits to maximize seed yield response to future CO_2 levels. It would be even more interesting to evaluate sensitive and tolerant genotypes to Fe-limitation to identify mechanisms and potential candidate genes for the interplay between Fe-tolerance and CO_2 enrichment. However, field trials in a high CO_2 environment are expensive, and space and time constraints limit the number of genotypes tested in a growth chamber experiment. In addition, more experimental studies on changes in crop quality and nutrition are required for a broader range of crops to represent the threat to human health.

Concerning the interaction of eCO_2 and Fe-limitation experiments, it will be meaningful to understand if the observed effects are persistent under longer-term exposure or during different growth stages. Moreover, it would be interesting to mimic the CO_2-based responses of soybean plants grown in alkaline and limestone soils that can induce Fe deficiency chlorosis. Since considerable gaps remain in our understanding of the interactions between CO_2 and abiotic stresses in plant response and resilience, it will be interesting to evaluate the role of phytohormones such as ethylene, jasmonic acid, and salicylic acid in terms of abiotic stress tolerance.

The rhizosphere microbiome composition of the soybeans changed in response to eCO_2, associated with an increased abundance of nitrogen-fixing bacteria, as reported by Wang et al. (2017). At the same time, Fe is considered a vital nutrient for the function of symbiotic root nodules in legumes (Slatni et al., 2014). Accordingly, it will be interesting to evaluate the effects of eCO_2 and Fe-limitation on the soil microbiome composition through an rRNA-based barcoding approach and to link these differences to environmental factors. Moreover, it will be interesting to analyze the root nodulation mechanisms to discriminate some physiological parameters involved in the response of nodules under eCO_2 and Fe-limited conditions.

Supplementary material

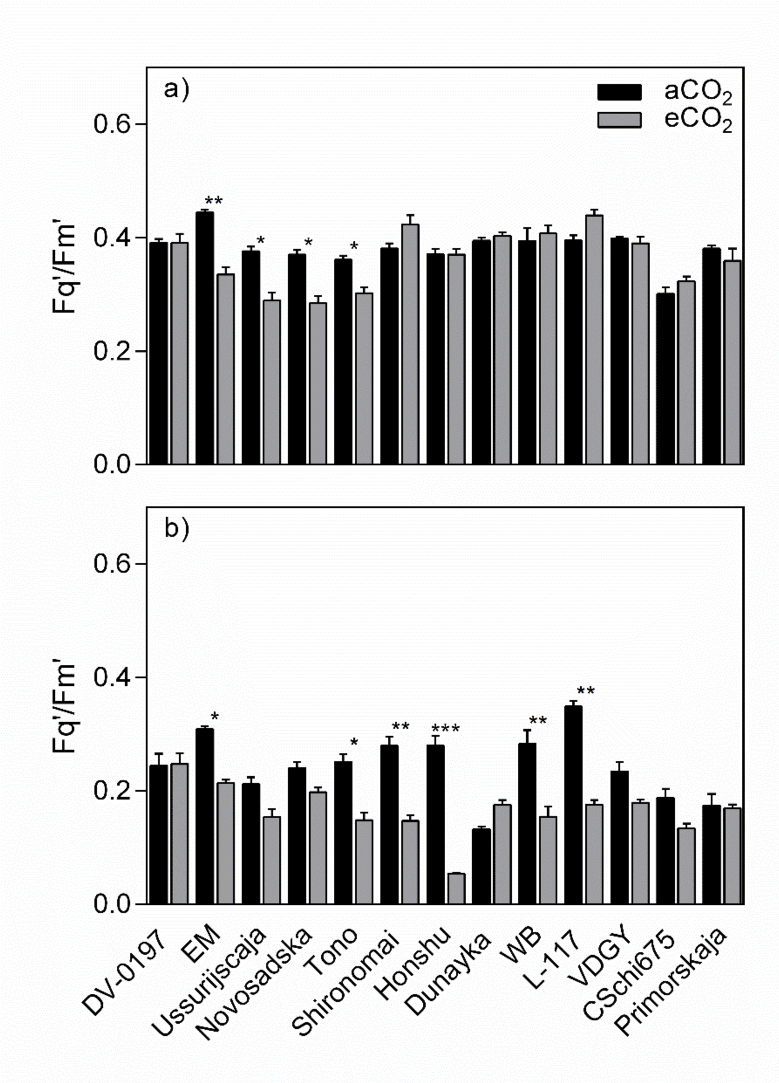


Figure S3.1 – Photosynthetic efficiency (Fq'/Fm') of 13 soybean genotypes exposed to aCO_2 (400 ppm) and eCO_2 (600 ppm) measured by the light induced fluorescence transient (LIFT) method. Values are the mean value ± SE of the measurements made at (a) vegetative and (b) pod filling stages. Three plants from each subplot were sampled to assess the photosynthetic efficiency. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

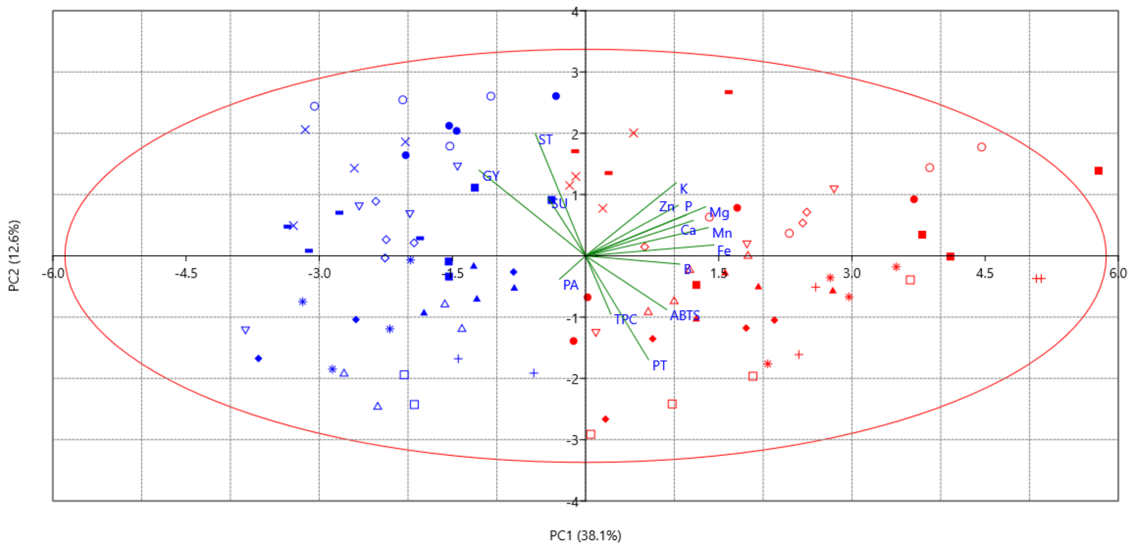


Figure S3.2 - PCA biplot showing two principal components with loadings of different variables (minerals, phytochemicals, and yield) and factor loadings of aCO_2 (400 ppm) and eCO_2 (600 ppm) treatments for soybean plants grown at the FACE facility in 2018. Variables: B = Boron; Fe = Iron; Mn, Manganese; Zn = Zinc; Ca = Calcium; K = Potassium; Mg = Magnesium; P = Phosphorous; SU = Sugar; PA = Phytic acid; ABTS = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; TPC = Total phenolic content; ST = Starch; PT = Protein; GY = Grain yield. Blue = eCO_2; Red = aCO_2. □, DV-0197; +, Early Mandarin; ♦, Ussurijscaja; Δ, Novosadska; ∇, Tono; –, Shironomai; ■, Honshu; ◊, Dunayka; ×, Winsconsin Black; •, L117; ▲, Van Dieckman Green-Yellow; ∗, Cschi675; o, Primorskaja.

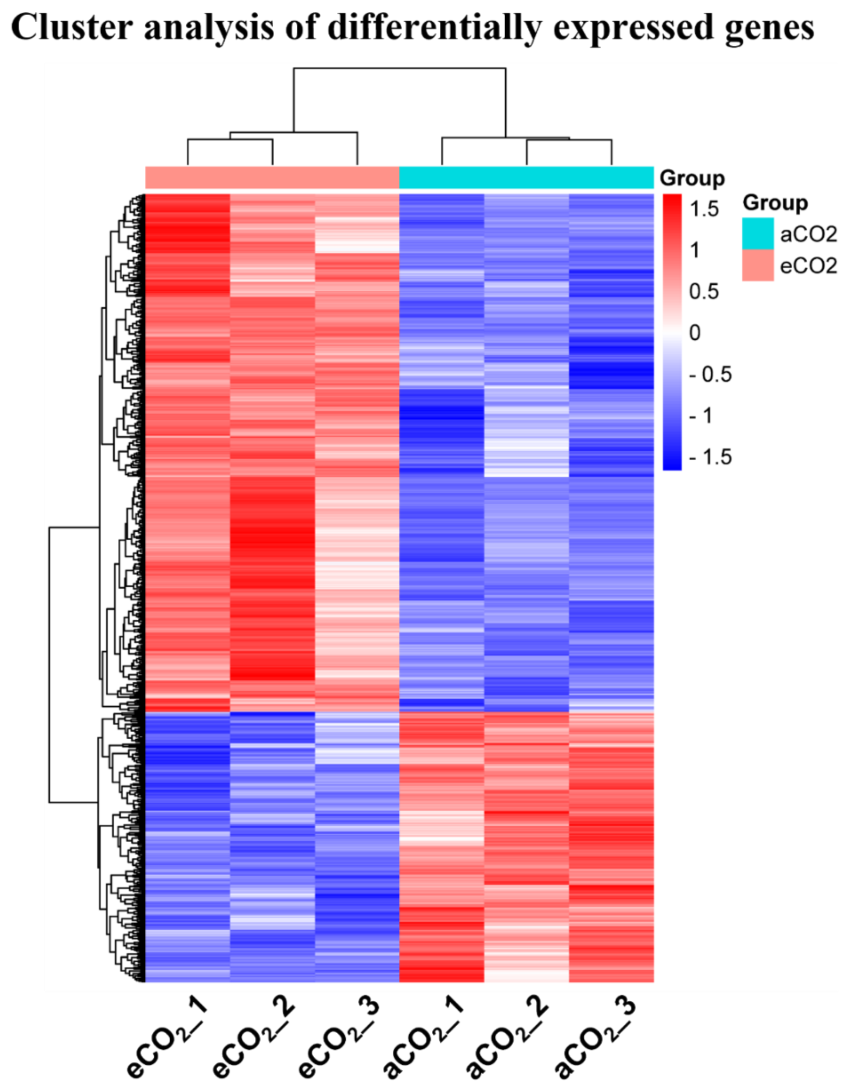


Figure S4.1 - Hierarchical cluster analysis map presenting differential gene expression in the root of soybean grown under hydroponic conditions at ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2).

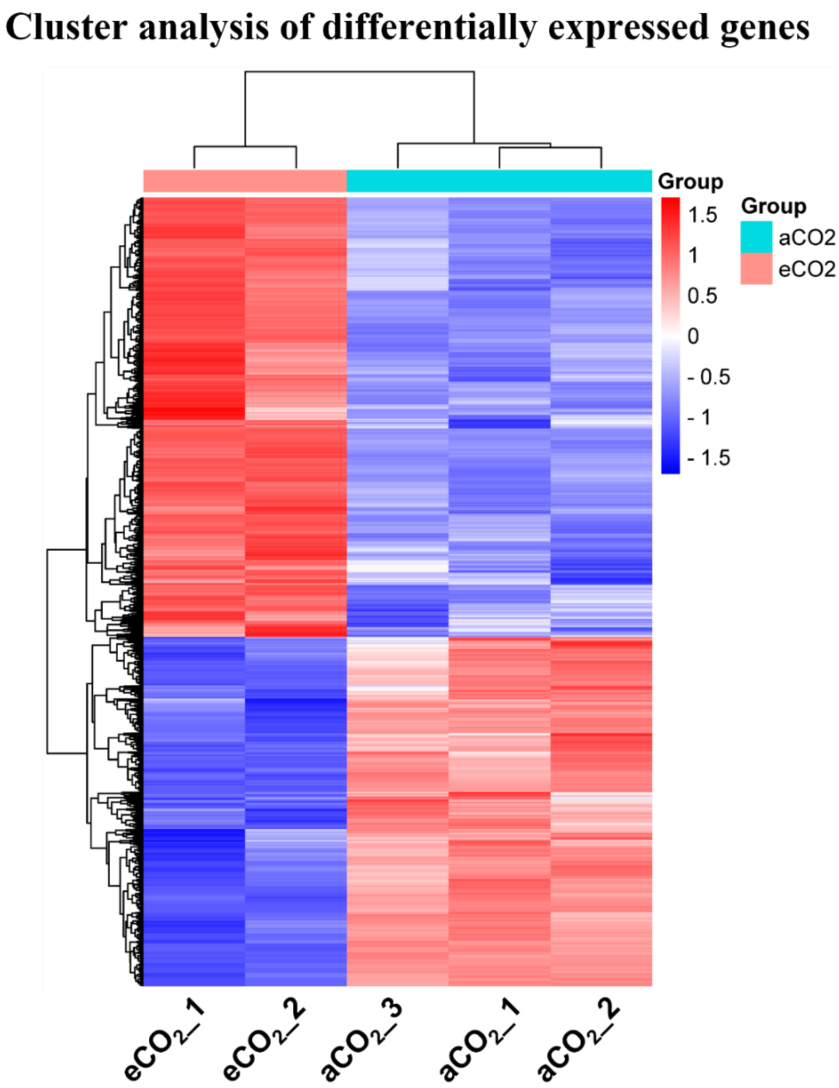


Figure S4.2 - Hierarchical cluster analysis map presenting differential gene expression in the leaf of soybean grown under hydroponic conditions at ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2).

Additional files from Chapter 4 (Tables S4.1, S4.2, S4.3, S4.4, S4.5, S4.6, S4.7, and S4.8) can be downloaded from:

https://figshare.com/s/9b751152ce5a656baa77

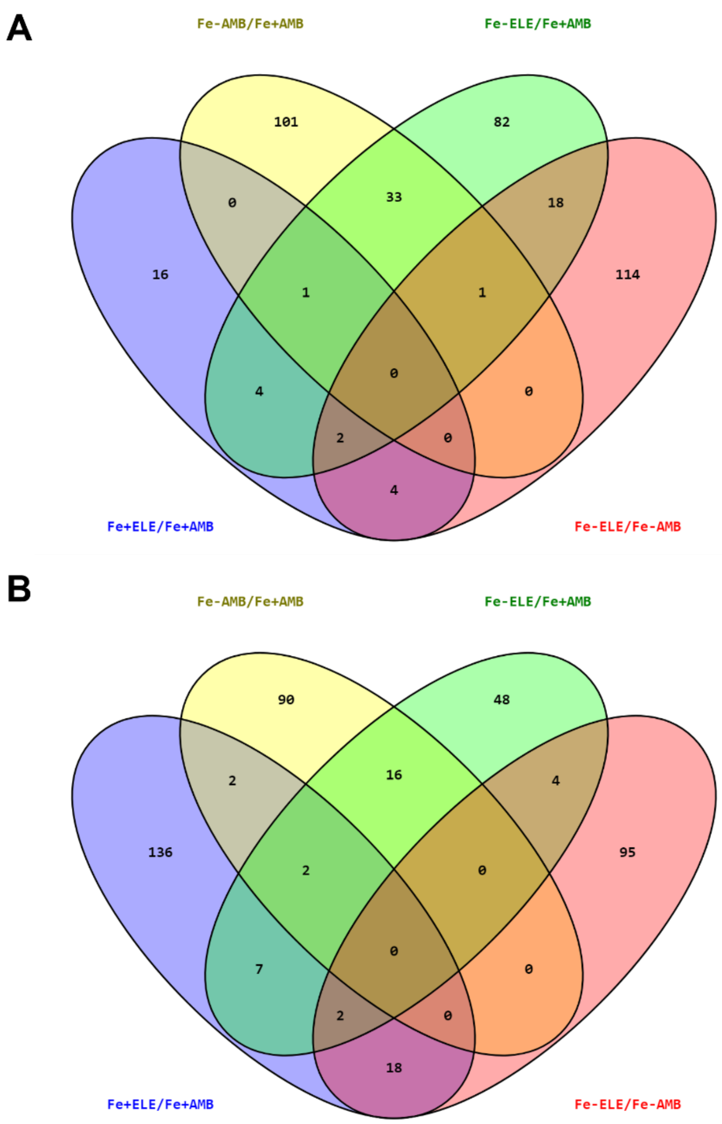
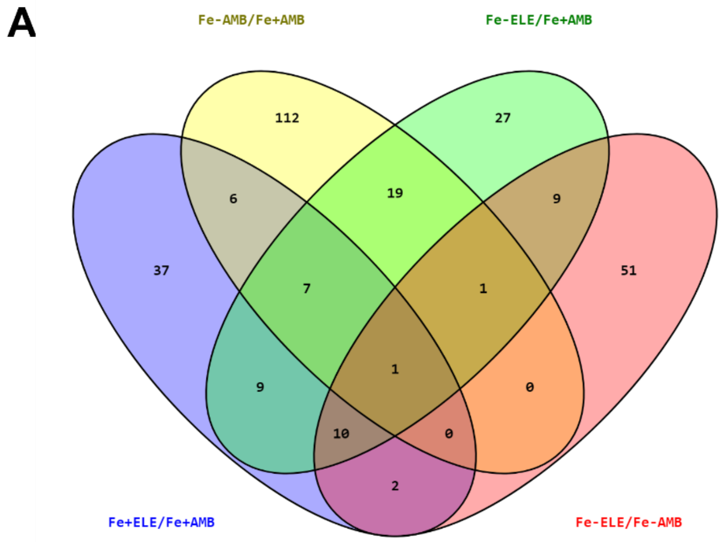


Figure S6.1 - Venn diagram significantly displaying (a) upregulated and (b) downregulated proteins in response to eCO_2 and Fe-limitation in roots. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2.



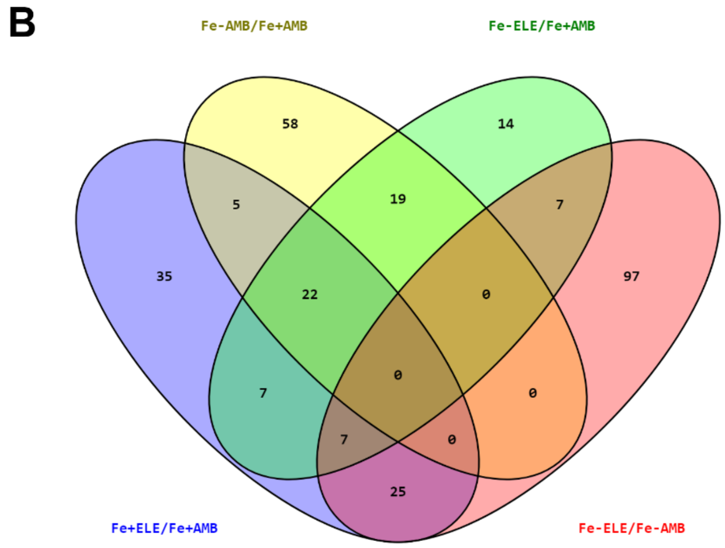
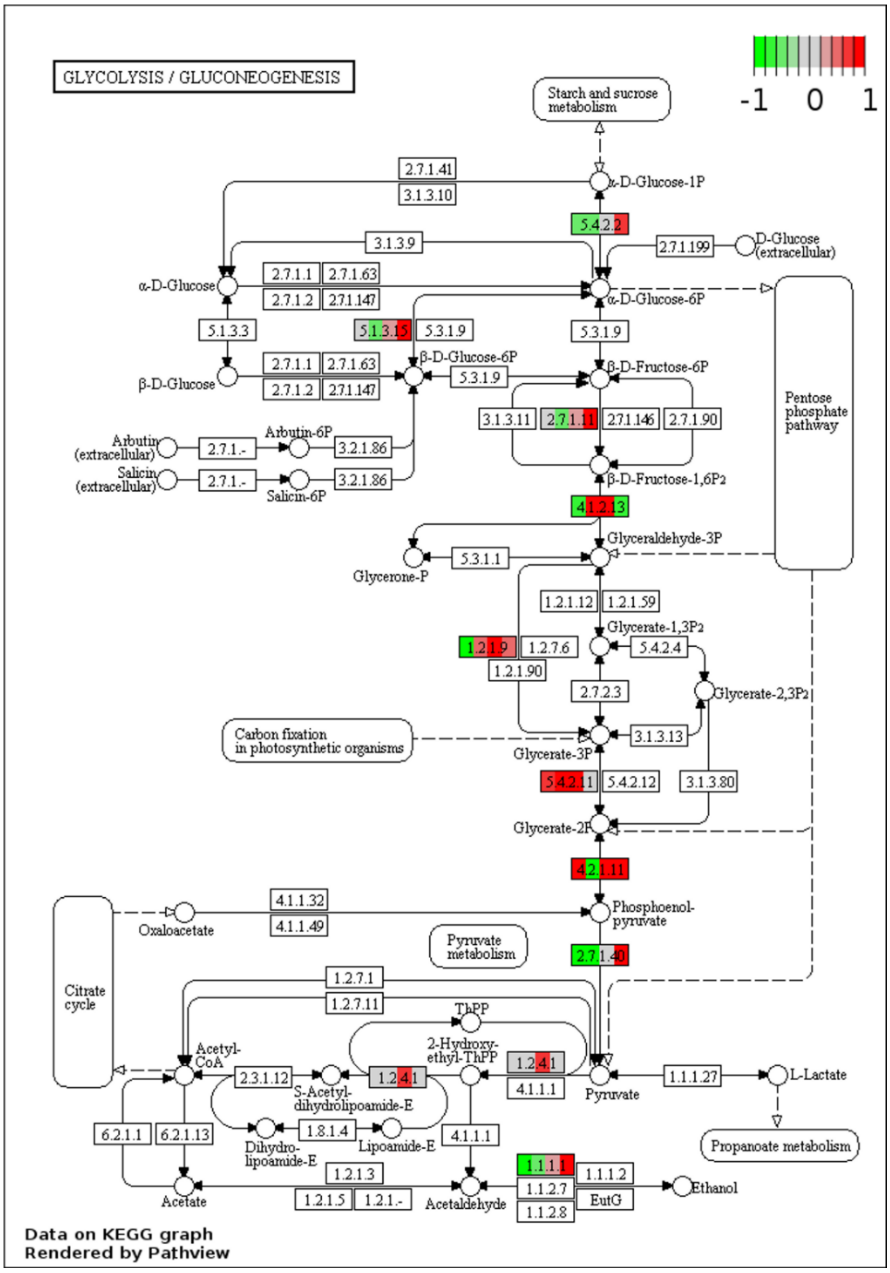


Figure S6.2 - Venn diagram significantly displaying (a) upregulated and (b) downregulated proteins in response to eCO_2 and Fe-limitation in leaves. Fe+AMB, Fe-sufficient + aCO_2; Fe+ELE, Fe-sufficient + eCO_2; Fe-AMB, Fe-limitation + aCO_2; Fe-ELE, Fe-limitation + eCO_2.

A


GLYCOLYSIS / GLUCONEOGENESIS
-1 0 1
Starch and sucrose metabolism
α-D-Glucose-1P
2.7.1.41
3.1.3.10
5.4.2.2
3.1.3.9
2.7.1.199
D-Glucose (extracellular)
α-D-Glucose
2.7.1.1
2.7.1.63
2.7.1.2
2.7.1.147
α-D-Glucose-6P
5.1.3.3
5.1.3.15
5.3.1.9
β-D-Glucose
β-D-Glucose-6P
β-D-Fructose-6P
Pentose phosphate pathway
3.1.3.11
2.7.1.11
2.7.1.146
2.7.1.90
Arbutin (extracellular)
2.7.1.-
Arbutin-6P
3.2.1.86
Salicin (extracellular)
Salicin-6P
β-D-Fructose-1,6P2
4.1.2.13
Glyceraldehyde-3P
Glycerone-P
5.3.1.1
1.2.1.12
1.2.1.59
Glycerate-1,3P2
1.2.1.9
1.2.7.6
1.2.1.90
5.4.2.4
2.7.2.3
Glycerate-2,3P2
Carbon fixation in photosynthetic organisms
Glycerate-3P
3.1.3.13
5.4.2.11
5.4.2.12
3.1.3.80
Glycerate-2P
4.2.1.11
Citrate cycle
Oxaloacetate
4.1.1.32
4.1.1.49
Phosphoenol-pyruvate
Pyruvate metabolism
2.7.1.40
1.2.7.1
1.2.7.11
ThPP
Acetyl-CoA
2-Hydroxy-ethyl-ThPP
2.3.1.12
1.2.4.1
S-Acetyl-dihydrolipoamide-E
4.1.1.1
Pyruvate
1.1.1.27
L-Lactate
6.2.1.1
6.2.1.13
1.8.1.4
Dihydro-lipoamide-E
Lipoamide-E
Propanoate metabolism
1.1.1.1
1.1.1.2
1.2.1.3
1.1.2.7
EutG
Ethanol
Acetate
1.2.1.5
1.2.1.-
Acetaldehyde
1.1.2.8
Data on KEGG graph
Rendered by Pathview

B

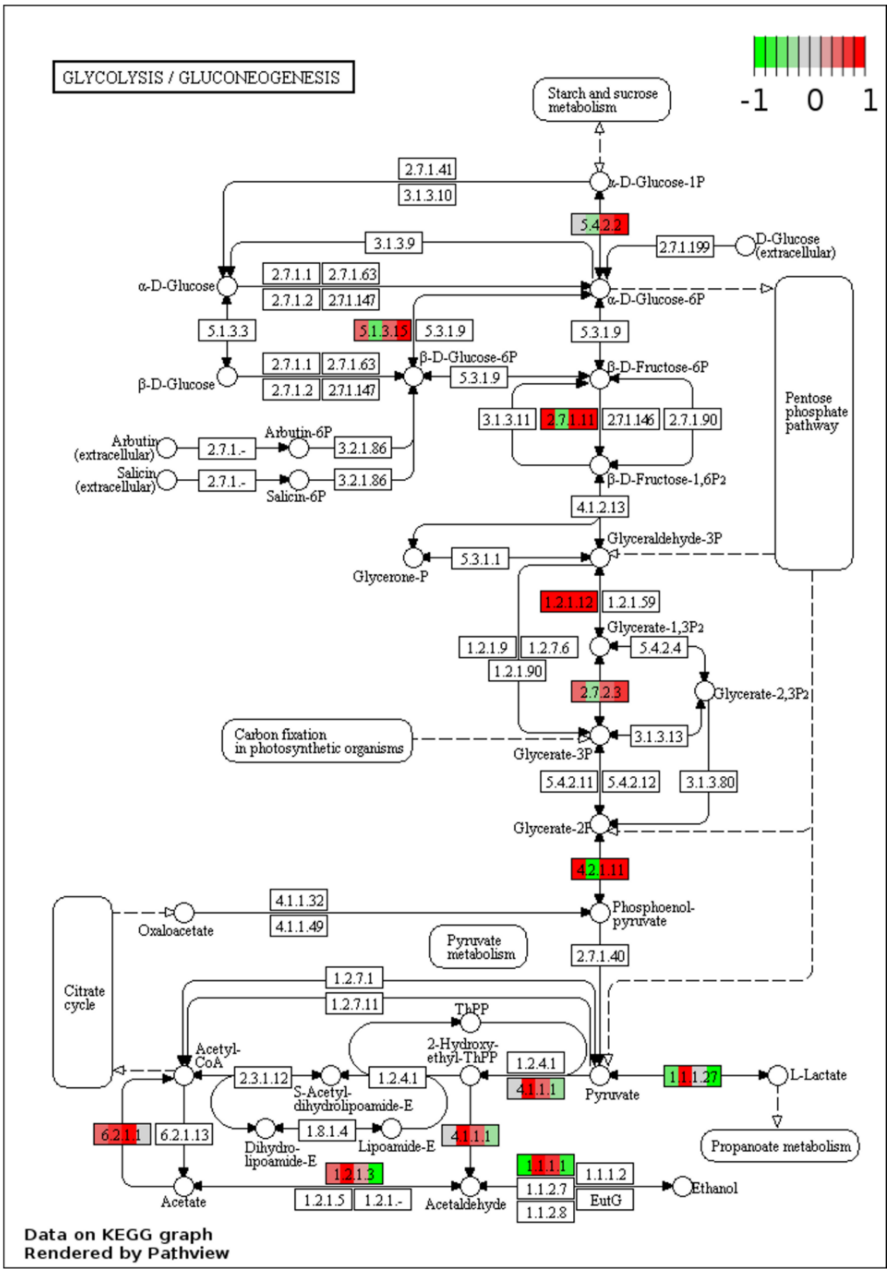
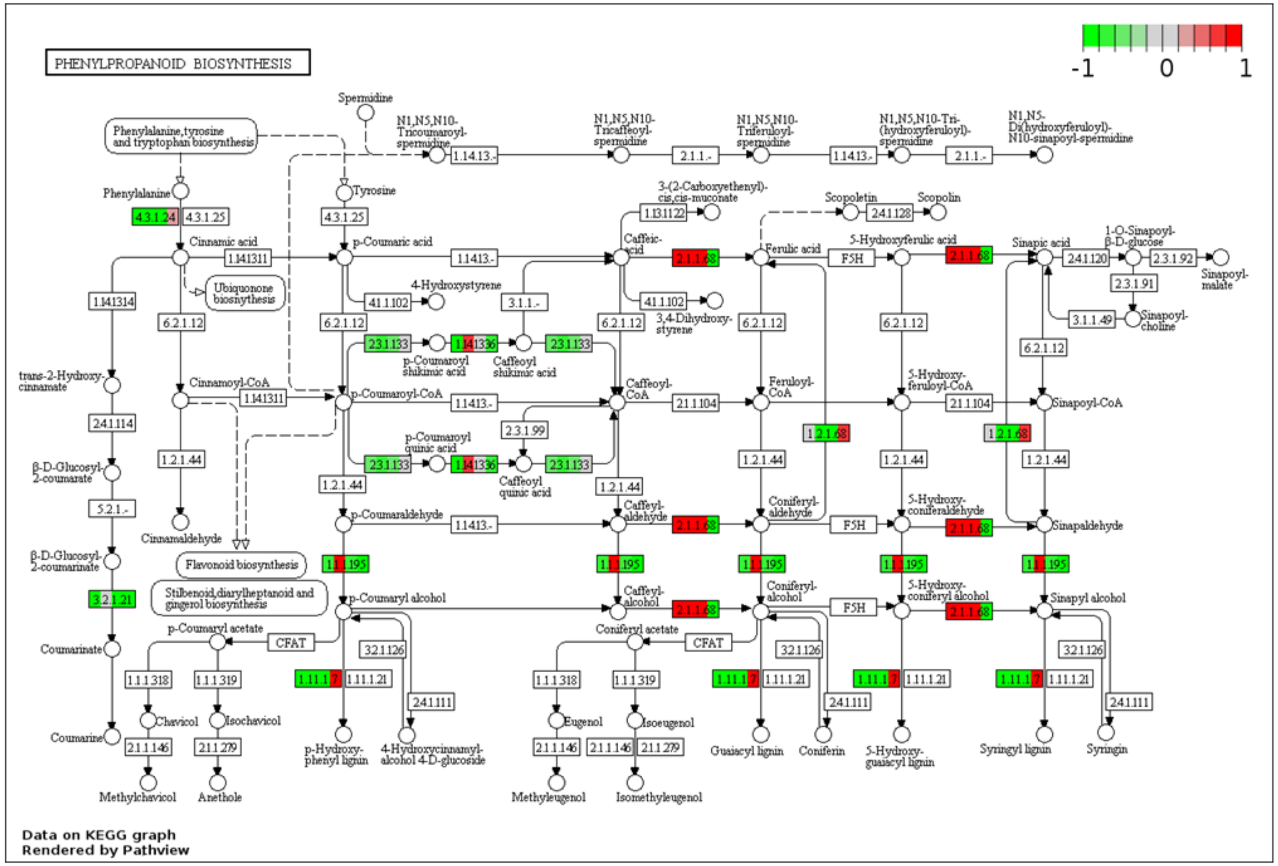


Figure S6.3 – Differential transcript expression in the 'glycolysis/gluconeogenesis' KEGG pathway in the response to eCO_2 and Fe- limitation in root (a), and leaf tissues (b). Each enzyme contains four color bars indicating the mean expression of Fe+ELE/Fe+AMB (first), Fe-AMB/Fe+AMB (second), Fe-ELE/Fe+AMB (third), and Fe-ELE/Fe-AMB (fourth) treatments. Red color indicating increased expression, and green indicating decreased expression. 1.1.1.1 - alcohol dehydrogenase; 1.1.1.27 - L-lactate dehydrogenase; 1.2.1.3 - aldehyde dehydrogenase; 1.2.1.9 - NADP-dependent glyceraldehyde-3-phosphate dehydrogenase; 1.2.1.12 - glyceraldehyde 3-phosphate dehydrogenase; 2.7.1.11 - ATP-dependent 6-phosphofructokinase; 2.7.1.40 - pyruvate kinase; 2.7.2.3 - phosphoglycerate kinase; 4.1.1.1 - pyruvate decarboxylase; 4.1.2.13 - fructose-bisphosphate aldolase; 4.2.1.11 – enolase; 5.1.3.15 - glucose-6-phosphate 1-epimerase; 5.4.2.2 – phosphoglucomutase; 5.4.2.11 - 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; and 6.2.1.1 - acetyl-CoA synthetase .

A


PHENYLPROPANOID BIOSYNTHESIS
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0
1
Data on KEGG graph
Rendered by Pathview

B

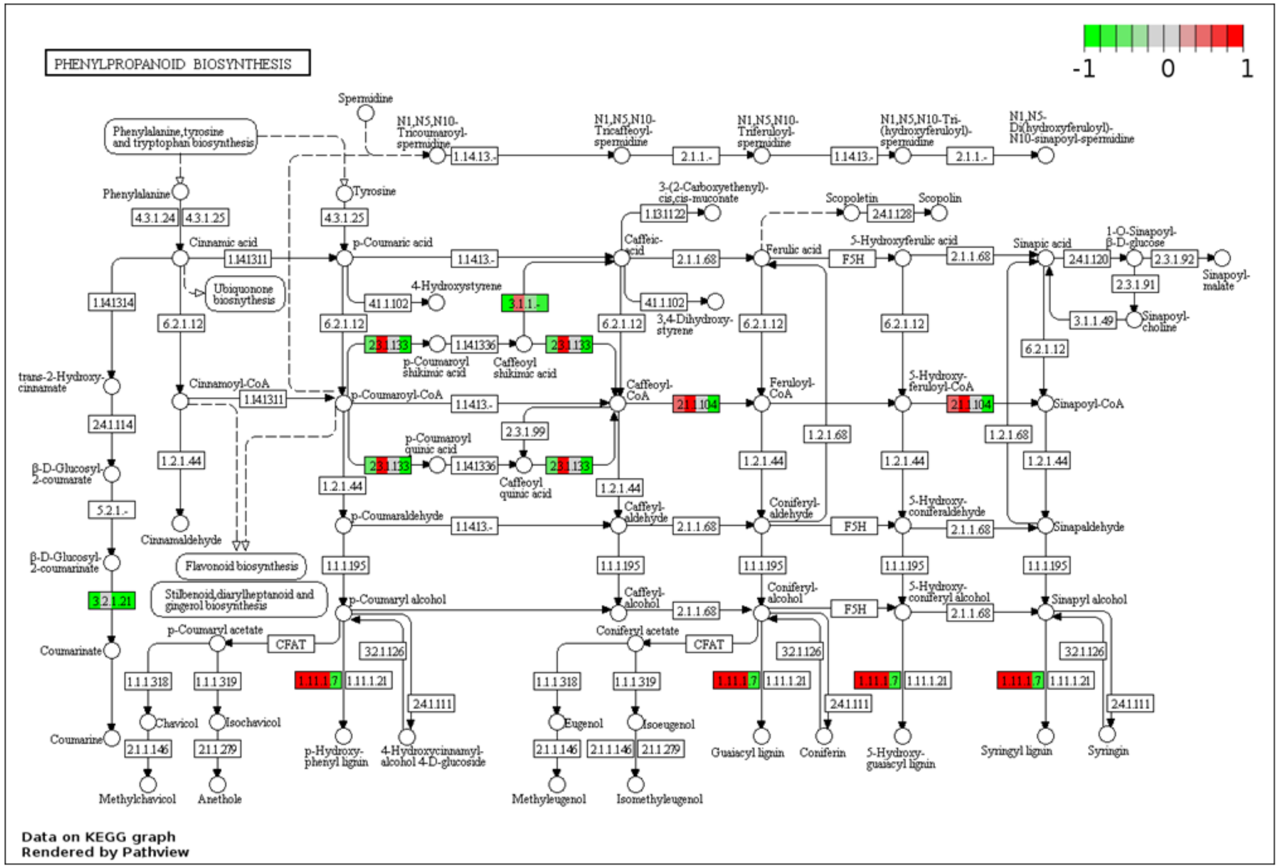

Figure S6.4 – Differential transcript expression in the 'phenylpropanoid biosynthesis' KEGG pathway in the response to eCO_2 and Fe- limitation in root (a), and leaf tissues (b). Each pathway component contains four color bars indicating the mean expression of Fe+ELE/Fe+AMB (first), Fe-AMB/Fe+AMB (second), Fe-ELE/Fe+AMB (third), and Fe-ELE/Fe-AMB (fourth) treatments. Red color indicating increased expression, and green indicating decreased expression. 1.1.1.195 - cinnamyl-alcohol dehydrogenase; 1.2.1.68 - aldehyde dehydrogenase; 1.11.1.7 – peroxidase; 1.14.14.96 - cytochrome P450; 2.1.1.68 - caffeic acid 3-O-methyltransferase; 2.1.1.104 - caffeoyl-CoA O-methyltransferase; 2.3.1.133 - shikimate O-hydroxycinnamoyltransferase; 3.1.1.- - caffeoyl shikimate esterase; 3.2.1.21 - vicianin beta-glucosidase; and 4.3.1.24 - phenylalanine ammonia-lyase.

Additional files from chapter 6 (Tables S4.1, S4.2, S4.3, and S4.4) can be downloaded from:

https://figshare.com/s/9b751152ce5a656baa77

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