CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

UNRAVELLING THE MOLECULAR AND PHYSIOLOGICAL COMPONENTS THAT CONTRIBUTE TO IRON DEFICIENCY CHLOROSIS

Thesis submitted to the *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology, with specialization in Environmental Sciences and Engineering

Carla Sofia Sancho dos Santos

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Carla Sofia Sancho dos Santos

Under the supervision of Marta W. Vasconcelos, Ph.D.
Under the co-supervision of António O. S. S. Rangel, Professor
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A espantosa realidade das cousas
É a minha descoberta de todos os dias.
Cada cousa é o que é,
E é difícil explicar a alguém quanto isso me alegra,
E quanto isso me basta.

Alberto Caeiro, in "Poemas Inconjuntos"

Abstract

Iron (Fe) deficiency chlorosis (IDC) is a serious condition affecting plants which are grown under calcareous or water logged soils. Under such conditions, Fe forms insoluble oxides and becomes unavailable for plant uptake, leading to stunted growth and severe yield reduction, causing aggravated agricultural losses. In the past years, efforts have been made to increase plant Fe content (so-called plant biofortification), in order to reduce the incidence of iron deficiency anaemia (IDA) prevalent around the world. To this end, legume grains and cereals, due to their rich nutritional profile and high worldwide intake by the population, have gained an important role in biofortification studies, which depend on the available molecular and physiological data for their successful implementation. The aim of this thesis was to contribute to the understanding of the molecular, physiological and biochemical mechanisms associated to Fe uptake and transport in Fe-stressed plants and to test a new class of Fe chelates as an efficient tool to prevent IDC.

With the purpose of understanding the transcriptomic response to Fe deficiency in a set of different legume species, a non-targeted analysis was performed using Illumina technology. Transcriptome analysis was performed in the roots of soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and barrel medic (*Medicago truncatula*) grown in Fe deficiency and Fe sufficiency, and 114,723 annotated genes were obtained for all samples. Four IDC-related gene families were up-regulated in common by the three species and can be considered key players involved in the IDC response, namely, metal ligands, transferases, zinc ion binding and metal ion binding genes. Also, amongst the most highly expressed genes were genes of the isoflavonoid pathway and, on the other hand, oxidoreductases were the most down-regulated genes.

Still on the search for IDC molecular players, two targeted genetic analyses were performed, one on *G.max* and *M. truncatula* and another on rice (*Oryza sativa*). Both studies involved the growth of plants under Fe sufficiency and Fe deficiency in order to compare the regulation of IDC related genes. Soybean and barrel medic are strategy I-crops, which means that, before uptake, they need to reduce Fe(III) to Fe(II) via an enzyme encoded by the FRO2 gene and, afterwards, Fe(II) is transported to the roots via a metal transporter encoded by the IRT1 gene. The expression of these two genes was analysed and both behaved similarly between species, appearing to be co-regulated. Moreover, the Fe transporters YSL1 and VIT1 and the main Fe storage protein-encoding gene – *ferritin* –

were up-regulated in the presence of Fe. The NRAMP3 gene, responsible for Fe remobilization from the vacuoles, was up-regulated under Fe deficiency, as was the GCN2 gene, indicating a putative role of the latter in Fe metabolism and homeostasis. The targeted study performed in rice, a strategy II cereal that releases phytosiderophores in order to chelate and absorb Fe, involved the analysis of two rice cultivars with distinct susceptibilities to IDC – cv. Nipponbare and cv.Bico Branco. This different susceptibility was confirmed by their contrasting leaf chlorosis development and tissue nutrient accumulation patterns. The cv. Nipponbare, that showed lower IDC susceptibility, was able to induce higher levels of the key reduction enzyme activity (Fe reductase) and showed higher levels of expression of the strategy I-OsFRO2 gene in roots. In contrast, cv. Bico Branco induced more genes involved in strategy II, specially, the transcription factor OsIRO2 and the phytosiderophore precursor OsTOM1.

The screening for tolerant genotypes to IDC is an important tool in plant breeding programs. The most common IDC indicator is the degree of chlorosis development, which is quantified using a numerical scale. Therefore, after gathering the molecular data, the physiological mechanisms triggered by IDC were studied. The model crop *G. max* was selected, as it comprises lines well characterized according to their IDC-susceptibilities. To this end, two studies were performed. In the first study we aimed at understanding if the ability to partition Fe could be related to Fe-efficiency. We concluded that IDC susceptible lines, when compared to efficient lines, have lower ability to translocate Fe to the shoots, having about two fold higher Fe content at the root level, and they have lower capacity to induce the ferric reductase enzyme, having about three fold lower enzyme activity. In the second study the regulation of the antioxidant and tetrapyrrole systems under Fe deficiency was analysed for the first time and we inferred that higher levels of oxidative stress might induce the oxidation of the tetrapyrrole heme into hemin, which leads to the induction of the heme-containing catalase enzyme and the reduction of ferric reductase activity. Taken together, the previous results indicate that low ferric reductase activity and Fe accumulation in the root tissue could be added as new IDC-related physiological markers.

The application of fertilizers and Fe chelating agents is one of the most frequently used tools to manage IDC. However, most of them are ineffective, too expensive or recalcitrant in the environment. Hence, the search for new Fe chelates is of utmost importance. In the last step of this thesis, we investigated the potential of a *tris*(3-hydroxy-4-pyridinonate) Fe(III) complex (Fe(mpp)$_3$, which has never been utilized in agricultural context) as an Fe fertilizer. Soybean plants were grown hydroponically under Fe deficiency

and with Fe(mpp)$_3$ or FeEDDHA supplementation. Results of both physiological and molecular markers showed that the new Fe complex led to healthier plants with increased growth by 24%, 42% higher SPAD units and lower Fe retention in the roots.

In general, the results presented in this thesis have contributed to a better understanding of the IDC-associated mechanisms and elucidated the key factors to be considered when analysing Fe deficient plants and their defence responses.

Resumo

A clorose por deficiência de ferro (Fe) é uma condição grave que afeta plantas em solos calcários ou alagados. Sob estas condições, o Fe forma óxidos insolúveis e torna-se indisponível para absorção pelas plantas, o que conduz a um crescimento diminuído e a uma redução severa na produção, resultando em perdas agronómicas agravadas. Nos últimos anos, têm sido desenvolvidos estudos no sentido de aumentar o conteúdo de Fe nos tecidos vegetais (biofortificação), de forma a reduzir a incidência da anemia por deficiência de Fe prevalente no mundo. Com este objetivo, as leguminosas e os cereais, dado o seu perfil nutricional rico e o seu alto consumo pela população mundial, têm ganho particular enfoque nos estudos de biofortificação, cujos resultados dependem da informação molecular e fisiológica disponível. O objetivo do presente trabalho foi contribuir para a compreensão dos mecanismos moleculares, fisiológicos e bioquímicos associados à absorção e transporte de Fe, bem como o estudo do potencial de uma nova classe de quelantes de Fe como uma ferramenta eficaz na prevenção da clorose férrica.

Com o objetivo de compreender a resposta transcritómica à deficiência de Fe num conjunto de diferentes espécies de leguminosas, foi realizada uma análise não-direcionada com recurso à tecnologia Illumina. A análise transcritómica foi realizada nas raízes de soja (*Glycine max*), feijão (*Phaseolus vulgaris*) e luzerna-cortada (*Medicago truncatula*), crescidas em deficiência ou suficiência de Fe. Deste estudo, identificaram-se 114.723 genes para todas as amostras. Quatro famílias de genes, nomeadamente ligandos de metais, transferases, proteína quinase e genes de ligação a metais e iões de zinco, foram sobre-expressas pelas três espécies e podem ter um papel relevante na resposta à clorose férrica. Entre os genes específicos mais expressos em deficiência de Fe, identificaram-se também genes da via dos isoflavonóides. Por outro lado, entre os genes cuja expressão foi diminuída sob deficiência de Fe, identificaram-se genes codificantes de oxidoreductases.

Realizaram-se também dois estudos direcionados, um em *G. max* e *M. truncatula* e outro em arroz (*Oryza sativa*). Ambos os estudos implicaram o crescimento de plantas com e sem suplementação de Fe, por forma a comparar a regulação de genes relacionados com a clorose férrica. A soja e a luzerna-cortada são leguminosas que utilizam a estratégia I, o que significa que, antes da absorção pelas raízes, elas necessitam de reduzir o Fe(III) a Fe(II) utilizando uma enzima codificada pelo gene FRO2 e, depois deste passo, o Fe(II) é transportado por um transportador de metais codificado pelo gene IRT1. A expressão

destes dois genes foi estudada e verificou-se que ambos comportaram-se de forma semelhante entre espécies, sugerindo que a sua expressão é co-regulada. Estudaram-se também os transportadores de Fe YSL1 e VIT1, e o gene codificante da principal proteína de armazenamento de Fe – a *ferritina* – tendo sido todos sobre-expressos na presença de Fe. O gene NRAMP3, responsável pela remobilização do Fe dos vacúolos, foi sobre-expresso na deficiência de Fe, tal como o gene GCN2, o que sugeriu um possível papel deste último no metabolismo e homeostasia do Fe. No estudo realizado com o arroz, um cereal que utiliza a estratégia II e que liberta fitosideróforos para quelatar e absorver o Fe, analisaram-se duas cultivares de arroz com suscetibilidades distintas à clorose férrica – cv. Nipponbare e cv. Bico Branco. A suscetibilidade diferencial foi confirmada pelo padrão oposto obtido nos resultados do desenvolvimento da clorose férrica e da acumulação de nutrientes nos tecidos. A cv. Nipponbare, que demonstrou menor suscetibilidade à clorose férrica, induziu níveis mais altos da enzima reductase férrica nas raízes, responsável pela redução de Fe(III), assim como do gene correspondente, OsFRO2, típico da estratégia I. Pelo contrário, a cv. Bico Branco induziu maiores níveis dos genes envolvidos na estratégia II, em particular, o fator de transcrição OsIRO3 e o precursor de fitosideróforos OsTOM1.

A seleção de cultivares tolerantes à deficiência de Fe é uma ferramenta importante para programas de melhoramento de plantas. O indicador de clorose férrica mais comum é o grau de desenvolvimento de clorose, que é quantificado com uma escala numérica. Assim, após reunir os dados moleculares, estudaram-se os mecanismos fisiológicos associados à clorose férrica. A soja foi selecionada como espécie-modelo pelo facto de incluir diversas linhas amplamente caracterizadas de acordo com a sua suscetibilidade à clorose férrica. Deste modo, este estudo foi dividido em duas análises principais. Na primeira análise, o objetivo foi compreender se a capacidade de partição de Fe podia ser relacionada com a eficiência de Fe. Concluiu-se que as linhas suscetíveis, em comparação com as linhas eficientes, tiveram uma capacidade menor de translocação do Fe para a parte aérea da planta, acumulando cerca do dobro do conteúdo de Fe nas raízes e, mais ainda, estas linhas tinham também níveis três vezes mais baixos de atividade da enzima reductase. Na segunda análise estudou-se, pela primeira vez, a regulação dos sistemas antioxidante e tetrapirrólico na deficiência de Fe e observou-se que níveis superiores de stress oxidativo podem induzir a oxidação da molécula heme em hemina, que resulta na indução da enzima catalase e na redução da atividade da enzima reductase, sendo que ambas possuem o grupo heme na sua estrutura. Em suma, os resultados anteriores indicam que uma atividade baixa

da enzima reductase férrica e acumulação de Fe nas raízes podem ser novos indicadores fisiológicos para a clorose férrica.

A aplicação de fertilizantes e de agentes quelantes de Fe é uma das estratégias mais utilizadas para tratar a clorose férrica. Porém, muitos destes produtos são ineficazes, dispendiosos ou recalcitrantes no ambiente. Como tal, o desenvolvimento de novos quelatos de Fe é de extrema importância. Na última parte desta tese investigou-se o potencial de um complexo do grupo *tris*(3-hydroxy-4-pyridinonate) Fe(III) (Fe(mpp)$_3$, nunca utilizado em contexto agronómico) como um fertilizante novo de Fe. Plantas de soja foram crescidas em hidroponia sob deficiência de Fe ou suplementadas com Fe(mpp)$_3$ ou FeEDDHA. Quer os resultados dos marcadores fisiológicos, quer dos moleculares demonstraram que, com o novo complexo de Fe, as plantas desenvolveram-se de forma mais saudável, obtendo um crescimento superior em 24%, 42% maior acumulação de clorofilas e menor retenção de Fe nas raízes.

Em geral, os resultados apresentados nesta tese contribuíram para uma melhor compreensão dos mecanismos associados à clorose férrica e esclareceram alguns dos fatores chave a considerar na análise das respostas de defesa de plantas sob stress de ferro.

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List of abbreviations

ALA	5-Aminolevulinic Acid
APX	Ascorbate Peroxidase
CAT	Catalase
Chl	Chlorophyll
DMA	Deoxymugineic Acid
DMAS	Deoxymugineic Acid Synthase
DW	Dry Weight
EDDCHA	Ethylenediamine di(5-carboxy-2-hydroxyphenylacetic) acid
EDDHMA	Ethylenediamine di(2-hydroxy-4-methylphenylacetic) acid
EDDHSA	Ethylenediamine di(2-hydroxy-5-sylfophenylacetic) acid
EDDS	Ethylenediamine disuccinic acid
EDTA	Ethylenediamine tetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FeEDDHA	Iron (III) complex of ethylenediamine-N,N'-bis(o-hydroxyphenyl)acetic acid
$Fe(mpp)_3$	*Tris*(2-methyl-3-hydroxy-4-pyridinonate) iron(III)
FRO	Ferric Reductase Oxidase
GE	Genetic Engineering
GM	Genetically Modified
GR	Glutathione Reductase
HBED	*N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid
HIDS	Hydroxyiminodisuccinate
HO	Heme Oxygenase
3,4-HPO	3-hydroxy-4-pyridinone ligands
HY2	Phytochromobilin Synthase
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometer
IDA	Iron Deficiency Anaemia
IDC	Iron Deficiency Chlorosis
IDEF	Iron Deficiency Responsive Element Binding Factor
IRO	Iron Oxidase

IRT	Iron Regulated Transporter
MA	Mugineic Acid
MDA	Malondialdehyde
MES	2-(N-morpholino)ethanesulfonic acid
NA	Nicotianamine
NAS	Nicotianamine Synthase
NRAMP	Natural Resistance-Associated Macrophage Protein
PCA	Principal Component Analysis
Proto	Protoporphyrin IX
PS	Phytosiderophores
PSI	Photosystem I
ROS	Reactive Oxygen Species
RT-qPCR	Real Time-quantitative Polymerase Chain Reaction
SPAD	Soil and Plant Analyzer Development
TALEN	Transcription Activator-Like Effector Nuclease
TOM	Transporter of MA
VIT	Vacuolar Membrane Transporter
WHO	World Health Organization
YSL	Yellow-Stripe Like
ZFN	Zinc Finger Nucleases

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CHAPTER 1

General Introduction

In this chapter the importance of Fe in human and plant metabolism will be presented, with closer attention to the latter for which, more specifically, the mechanisms involved in Fe uptake, transport and homeostasis will be reviewed. Also, the importance of legume plants in modern agriculture will be analysed, as well as the damage caused by Iron Deficiency Chlorosis (IDC). Current alleviation strategies utilized for IDC prevention and/or correction will be discussed. Finally, the scope and outline of this thesis will be presented.

[Some parts of this chapter were taken from]
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1.1. Introduction

Iron (Fe) deficiency anaemia (IDA) is one of the most prevalent nutrient deficiencies in humans and has deleterious consequences that can range from fatigue to reduced work capacity or premature infant death. Over 30% of the world's population is affected by IDA. Depicted in Fig. 1.1, is the world's distribution of IDA-affected preschool aged children (World Health Organization, 2008) which, alongside with women of child bearing age, constitute the most affected groups by this disease. The occurrence of IDA reaches severe levels in most countries of Africa and in several countries in South America and South Asia.

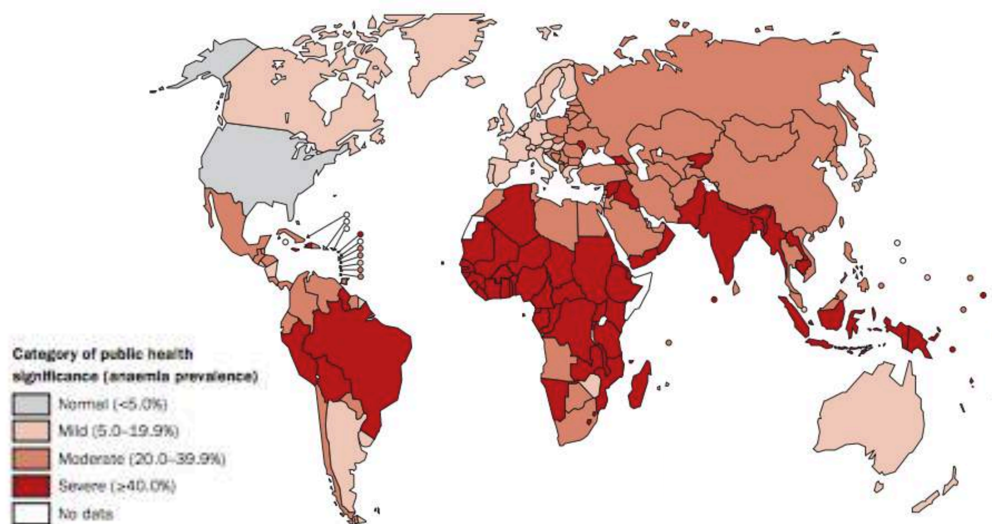

Fig. 1.1. World distribution of anaemia prevalence in preschool aged children (adapted from World Health Organization, 2008).

Plants provide the major part of human food intake, with the majority of energy being provided by cereals and other starchy staples (Mathers, 2006). In some cultures, either by choice or by economic constraints, plant-based nutrition comprises 100% of the diet (Auestad and Fulgoni, 2015). Amongst the most commonly consumed plant foods worldwide is rice, a crop that has shaped the cultures, the diets and societies of many countries around the world. Even though Asia is still the biggest rice consumer, rice is also highly consumed in European, North American and South American diets. Other traditional plant foods that have a high worldwide consumption are cassava, wheat, potato, soybean, pearl millet, sorghum, bean and maize. All these crops have been targets of conventional and modern technological processes that aim at increasing their nutritional

value. Amongst these processes, plant-based food biofortification programs have been developed, in order to increase minerals and vitamins in food staples through use of conventional plant breeding, transgenic techniques or the application of mineral fertilizers (Carvalho and Vasconcelos, 2013; Vasconcelos *et al*., 2017; de Steur *et al*., 2017). Plant breeding is one of the most used approaches to develop new varieties with specific agronomic traits and improved nutritional qualities (Farnham *et al*., 1999; Unnevehr *et al*., 2007). However, classical breeding approaches have many limitations because the crossing can only be done between closely related specie or genus, and therefore it uses available genetic diversity and existing traits to obtain new varieties. Over the last decade, significant progress has been made in the development of new and efficient transformation methods in plants, which have allowed us to develop plants expressing desired traits unattainable by conventional breeding. In fact, plant genetic engineering has become one of the most important molecular tools in the modern molecular breeding of plant foods (Barampuram and Zhang, 2011). With these transgenic techniques, several crops have been biofortified with different metabolites. Recent successful examples include the case of the provision of Fe-biofortified pearl millet to Fe deficient school-aged Indian children, that led to a significant increase in ferritin concentrations and in total body Fe concentration (Finkelstein *et al*., 2015); and the inclusion of Fe-biofortified beans in the diet of women in Rwanda that significantly improved their Fe status, reducing IDA prevalence (Haas *et al*., 2016). Despite the promising findings on Fe biofortified crops contribution to IDA control (Finkelstein *et al*., 2017), it is important to refer that biofortification programs still have some limitations given that, depending on the nutritional status or the prevalence of the micronutrient deficiency, a biofortified crop may not be sufficient to meet the iron requirements (Bouis *et al*., 2011). Moreover, public opinion on transgenic organisms for consumption is yet to be completely favourable.

It is important to refer that, in order to perform biofortification studies there must be large genetic and phenotypic variation in the target germplasm, which is very much achievable through the existence of germplasm collections in various national genetic resource centres. For example, a total of 93706 soybean accessions with different genotypic traits are available at the Institute of Crop Sciences of the Chinese Academy of Agricultural Sciences, the National Plant Germplasm System from USA and the Asian Vegetable Research and Development Center (Foyer *et al*., 2016). At the present time, biofortification is one of the most sustainable methods to overcome human micronutrient deficiencies (Manwaring *et al*., 2016). However, it implies a successful manipulation of

the nutrient's content in the edible parts of the biofortified crops. To build effective programs for Fe biofortification there must be a deep understanding on the processes underlying this mineral's uptake mechanisms and metabolism in plants.

1.2. Iron metabolism in plants

Iron is one of the most important nutrients among transition metals, being the second most abundant in the earth's crust (Broadley *et al*., 2012). However, it is mostly present in soils in the form of insoluble ferric oxides, which are not bioavailable for plants' uptake. It has an essential role in plant metabolism, being fundamental in biochemical activities, namely, respiration, photosynthesis and chlorophyll biosynthesis (Nenova, 2006). This nutrient is part of several constituents of the electron transport chain in mitochondria and of the photosynthetic complexes found in chloroplasts (Zocchi *et al*., 2007) and, when it is not present in sufficient amounts, it inhibits the biosynthesis of essential cofactors, impairing the biogenesis of thylakoid complexes and inducing dysfunction of electron transport and enzyme reactions (Briat *et al*., 2015). Iron-sulfur (Fe-S) clusters, e.g. have a key role in photosynthesis, are the most abundant in photosynthetic organisms and include ferredoxins as the most well known Fe-S proteins (Johnson *et al*., 2005). Ferredoxins are involved in several redox reactions, mediating electron transfer from photosystem I (PSI) to enzymes involved in different pathways, like glutamate synthase, sulphate reductase, nitrite reductase and ferredoxin-thioredoxin oxidoreductase (Knaff and Hirasawa, 1991). Therefore, PSI - due to its high Fe content – appears to be the main target of Fe deficiency and, consequently, both chloroplasts and mitochondria are the most affected organelles, since Fe is required for their structural and functional integrity (Scheumann *et al*., 1998) and its low availability leads to high energy requests to support the higher need for Fe uptake (Vigani *et al.*, 2013). In fact, mutants lacking mitochondrial Fe-S clusters also show low aconitase activity, another Fe-S protein that belongs to a family of hydratases/dehydratases responsible for the isomeration of citrate (an Fe chelator) to isocitrate (Beinert *et al*., 1996).

Fe is also an essential cofactor for proteins belonging to the tricarboxylic acid (TCA) cycle and when Fe is deficient, citric and malic acids tend to increase, as well as the production of other organic acids (which are intermediate compounds of the TCA cycle). Although it is not still clear why, it was proposed that these alterations in organic acid metabolism may be necessary for the maintenance of Fe in soluble forms within the plant (Abadía *et al*., 2002; Vigani *et al*., 2013).

Other Fe-containing constituents are heme proteins and chlorophyll, which belong to the tetrapyrrole group of compounds. The tetrapyrrole cycle (Fig. 1.2) occurs in the chloroplast and starts with the formation of 5-aminolevulinic acid (ALA) from glutamate that, after a series of reactions of linear polymerization, is transformed in the intermediate product protoporphyrin IX (Proto) (Tanaka *et al.*, 2011). At this point, the tetrapyrrole cycle is divided in two different branches, the 'Fe-branch' and the 'magnesium (Mg)-branch' (Brzezowski *et al.*, 2015). The first leads to heme biosynthesis and involves the activity of a ferrochelatase (FC) that catalyses the insertion of Fe^{2+} into Proto. The resulting heme molecules can be inserted in hemoproteins or may be further degraded by heme oxygenase (HO) into the biliverdin IXα molecule for the phytochromobilin synthesis via the enzyme phytochromobilin synthase (HY2) (Tanaka *et al.*, 2011). Cytochromes, peroxidases, catalases and Fe reductase are the best example of heme enzymes, which are highly involved in the antioxidant system and act as defence agents against ROS (Briat *et al.*, 2007).

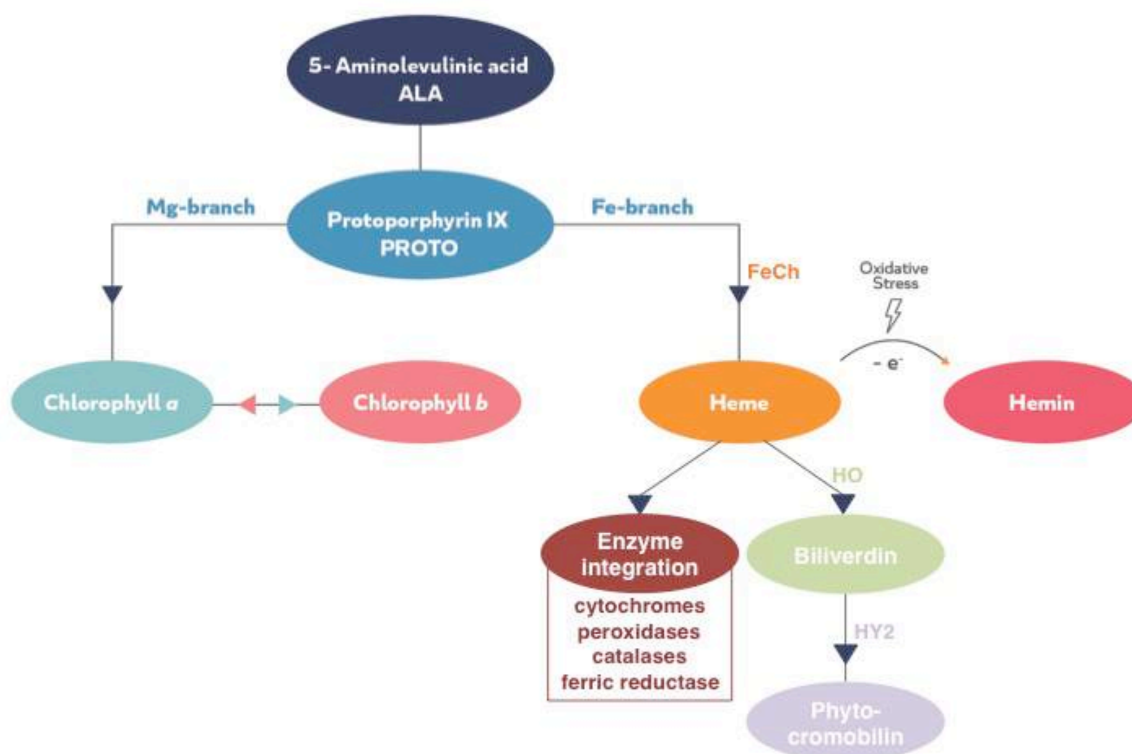

Fig. 1.2. Schematic overview of the tetrapyrrole cycle (adapted from Chapter 3.2 - Santos et al., 2017).

In the 'Mg-branch', Mg^{2+} is inserted into Proto that suffers a series of modifications to synthesize chlorophyllide *a*, which is esterified to form chlorophyll (Chl) *a*. As there are two species of Chl, a second cycle occurs, with the interconversion of Chl *a* in Chl *b* (Tanaka *et al.*, 2011).

The protoporphyrin biosynthesis pathway is highly Fe-dependent and its regulation has a direct impact on plant's metabolism (Briat *et al.*, 2015) since, as mentioned above, electron transport, chloroplast development, acquisition of the photosynthetic capacity and various catalytic processes appear to be influenced by Fe status in the cells (Papenbrock *et al.*, 2000; Hamza and Dailey, 2012). Despite all these facts, how Fe deficiency affects the photosynthetic process at these various levels is still not fully understood, although evidences showing that it may lead to complete destruction of the photosynthetic machinery (Msilini *et al.*, 2013), not only at the thylakoid level but also at the stromal and lumenal levels (Terauchi *et al.*, 2010).

1.3. Iron uptake mechanisms

In order to acquire Fe from the soil, for long it has been thought that higher plants have evolved Fe uptake strategies, depending on the ionic state of Fe, that historically were divided into: Strategy I to acquire Fe(II) and Strategy II to acquire Fe(III) (Brown, 1978). Recently, studies have suggested that there may be an overlap between these two strategies, depending on crop species and environmental conditions (Ricachenevsky and Sperotto, 2014).

Strategy I, also referred to as 'Reduction Strategy', is utilized by all dicotyledonous and non-graminaceous plants (Fig. 1.3a). The first engaged step consists on proton release via H^+-ATPases in order to decrease rhizosphere's pH and, consequently, increase Fe solubility (Colangelo and Guerinot, 2004). After the acidification step, Fe^{3+} is reduced to Fe^{2+} by a root ferric chelate reductase. In Arabidopsis, this enzyme is encoded by ferric reductase oxidase 2 (AtFRO2), which is composed of two intramembrane heme groups, and is induced in the root epidermis to transfer electrons across the plasma membrane (using NAD(P)H as an electron donor), performing the reduction step (Robinson et al., 1999). Genes encoding the FRO enzyme include eight members that are differentially expressed at the tissue levels, being not only important for metal acquisition from soil, but also for intracellular distribution of Fe (Jain *et al.*, 2014). More specifically, FRO1 was characterized in pea to have 74% of overall similarity to AtFRO2 (Waters *et al.*, 2002); AtFRO5, AtFRO7 and AtFRO8 do not seem to be Fe-regulated and AtFRO3 is expressed

in Fe deficient leaves (Jeong and Connolly, 2009); AtFRO6 overexpression in tobacco plants enhanced ferric reductase activity in the leaves (Li *et al*., 2011). Alongside with FRO, other compounds have been proposed to have a key role in the reducing step, such as phenolics, organic acids, sugars and flavins (López-Millán *et al*., 2000; Rodríguez-Celma *et al*., 2011) and recent reports identified scopoletins, a class of phenolic-type compounds, to be secreted under Fe deficient conditions and have an important role in plant Fe nutrition (Fourcroy *et al*., 2014; Schmid *et al*., 2014).

After Fe^{3+} is reduced, Fe^{2+} is transported into the root by iron-regulated transporter 1 (IRT1), which belongs to the zinc-regulated transporter/IRT-like protein (ZIP) family (Guerinot, 2000). IRT1 was described to be expressed only under Fe deficient conditions (Connolly *et al*., 2002), but it can also transport other divalent metals and it has been shown that the overexpression of *AtIRT1* induces metal overload (Barberon *et al*., 2011). Other studies showed that when the peanut *AhIRT1* gene was introduced in tobacco and rice, it had a dual function: besides being responsible for Fe absorption, it could also be responsible for Fe translocation, as the transgenic plants increased their tolerance to Fe-deficiency and, even under Fe-sufficiency, Fe concentration was enhanced in roots and shoots (Xiong *et al*., 2014).

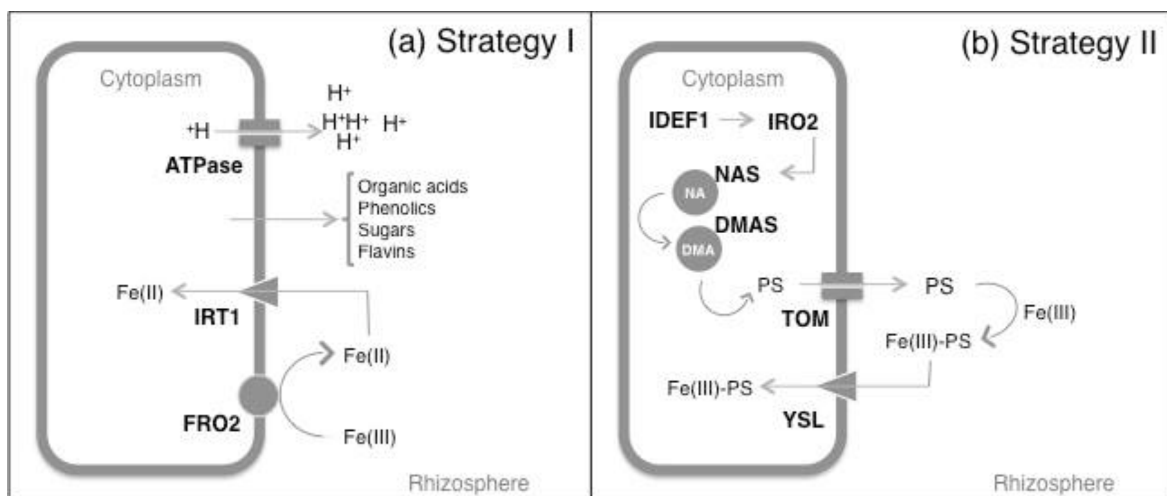

Fig. 1.3. Schematic representation of mechanisms for iron (Fe) acquisition in plants: (a) Strategy I or the reduction strategy - proton pump (ATPase), Fe transporter (IRT1) and ferric reductase (FRO2); (b) Strategy II or the chelation strategy – transcription factors IDEF1 and IRO2, nicotianamine synthase (NAS), deoxymugineic acid synthase (DMAS), phytosiderophore (PS); PS effluxer (TOM) and PS influxer (YSL).

Graminaceous plants, like barley, rice and maize utilize Strategy II (a 'Chelation Strategy') for Fe uptake (Fig. 1.3b). In order to increase uptake, plants release phytosiderophores (PSs) to the rhizosphere which act as chelators with high affinity for Fe^{3+}. The primary member of the PSs family is deoxymugineic acid (DMA), and nicotianamine (NA) is the main precursor for its synthesis (Morrissey and Guerinot, 2009). Two transcription factors seem to have an essential role in DMA and NA synthesis, namely, IRO2 that regulates their synthesis by influencing DMA and NA synthases (DMAS and NAS) expression (Ogo *et al*., 2007); and an Fe Deficiency-responsive Element-binding Factor 1 (IDEF1) that intervenes in this synthesis by positively regulating the expression of IRO2 (Kobayashi *et al*., 2009). Phytosiderophores are effluxed to the rhizosphere via TOM1, a transporter whose expression levels augment under Fe-deficient conditions (Nozoye *et al*., 2011). Once in the rhizosphere, the complex Fe^{3+}-PS is formed and is taken up into the root cells by transmembrane proteins of the yellow-stripe1 (YS1) family (Curie *et al*., 2001). YS1 transporters have been identified in several grass species, and, interestingly, non-graminaceous plants also have YS1-like (YSL) genes that encode proteins essential in metal-NA complexes transporting (Inoue *et al*., 2009).

Although this classic division is mostly true, there are few studies showing that some Strategy II plants could use Strategy I mechanisms, as is the example of rice (Bughio *et al*., 2002; Ricachenevsky and Sperotto, 2014). Evidences suggest the use of a 'combined strategy', where rice plants besides absorbing Fe(III) via the chelation strategy, also take up Fe(II) directly by the induction of the strategy I transmembrane transporters IRT1/IRT2 (Sperotto *et al*., 2012).

1.4. Iron transport and homeostasis

After entering the root cells, Fe can be transported to the aboveground organs via the xylem (Conte and Walker, 2011). This transport has for long been associated to the formation of complexes between Fe and citrate, which seemed to be the preferential form for Fe loading in the xylem (Tiffin, 1966). In the meantime, studies confirmed this theory (Green and Rogers, 2004) and a ferric reductase defective 3 (FRD3) protein, belonging to the multidrug and toxin efflux (MATE) family has been described to be necessary for efficient Fe translocation (Durrett *et al*., 2007). Despite being predominantly transported through the xylem (Lopéz-Millán *et al*., 2000), Fe can also be transported through the phloem, complexed with NA, as this metabolite, although not secreted by non-graminaceous plants, is synthetized and chelates Fe (Stephan and Scholz, 1993; Takahashi

et al., 2003). When Fe reaches the leaves it is putatively unloaded in the apoplastic space via the YSL transporters (Waters *et al*., 2006).

Free Fe is toxic, therefore, it must be incorporated in storage structures. Ferritins, for example, store Fe in excess for detoxification and maintain the mineral available for protein synthesis (Briat *et al*., 2010). Ferritins can be found in most of the cellular compartments, but the main storage organelle is the chloroplast (Briat et al., 2010). It is generally established that under Fe supply genes of the ferritin family are usually over-expressed (Lescure *et al*., 1991; Wu *et al.*, 2016). The majority of the Fe pool is mainly located in chloroplasts (Roschzttardtz *et al*., 2013) and, although the method for influx is still not well described (López-Millán *et al*., 2016), it is thought to require a reduction-based mechanism, mediated by a member of the FRO family, probably FRO7, both in strategy I and strategy II plants (Solti *et al*., 2014). The other major reservoir for inactive Fe is the vacuole and Fe is imported via a vacuolar membrane transporter, VIT1 (Kim *et al*., 2006) and remobilized by the NRAMP3 and NRAMP4 transporters (Lanquar *et al*., 2005), which also have a role in Mn trafficking in the vacuoles of the mesophyll cells (Lanquar *et al*., 2010). Moreover, these proteins have been shown to have a conserved role in Fe transportation and homeostasis in different crops, as is the case of VIT expression in rapeseed (Zhu *et al*., 2016) and of Arabidopsis VIT1 expression in cassava that showed promising results for biofortification programs development (Narayanan *et al*., 2015); other examples include AhNRAMP1 in peanut (Xiong *et al*., 2012) and MxNRAMP1 in apple (Pan *et al*., 2015). Figure 1.4 summarizes the main players in the Fe trafficking pathway described above.

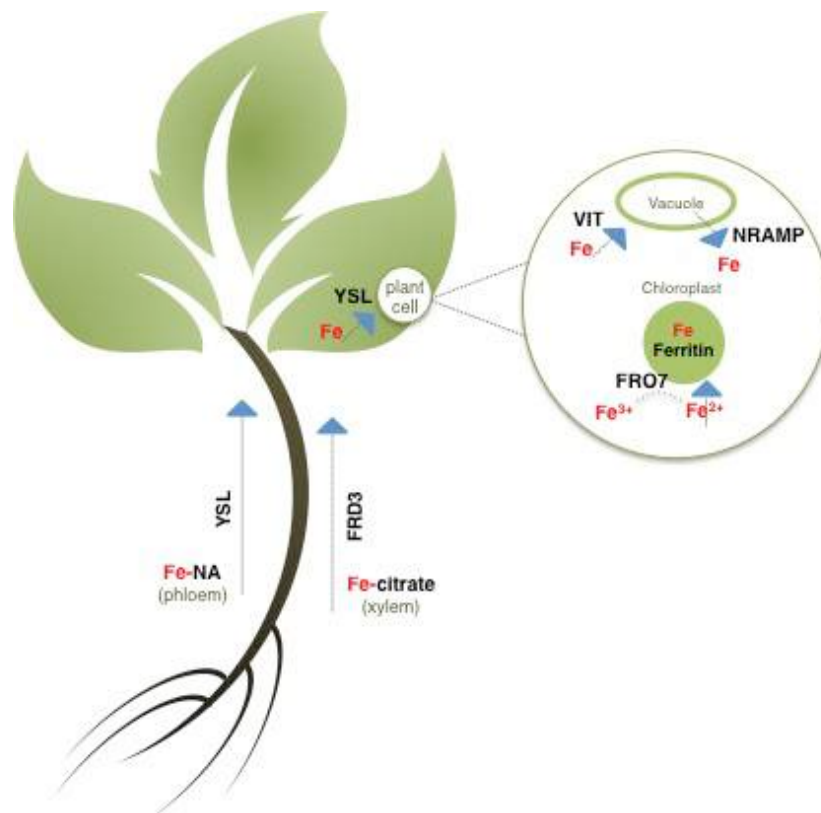

Fig. 1.4. Main proteins involved in the Fe transport and homeostasis mechanisms. Transport movement is indicated by a blue arrow; all transport proteins and chelators are depicted in black.

Fe homeostasis is tightly regulated in plants and requires different signals and regulators, having an ultimate implication on photoassimilate partitioning, due to its source-sink control (Marschner *et al*., 1996; Lemoine *et al*., 2013). Shoots have always been pointed as the main responsible organ for signalling the need for increased Fe uptake at the root level (Brown *et al*., 1961; Schmidt, 2003). A negative feedback control for Fe uptake has been proposed, where Fe sufficiency represses the synthesis of the ferric chelate reduction system (Maas *et al*., 1988); however, a positive regulation has also been proven to exist, where a long-distance signal for Fe deficiency in the shoots induces Fe uptake in the roots (Enomoto *et al*., 2007). More recently, a combined network-system for the activation of physiological Fe-stress responses has been suggested (García-Mina *et al*., 2013). In this model, together with a predominant shoot to root signal, which is dependent not on the Fe conditions at the root level, but on the development of Fe-stress symptoms in the leaves, a local Fe-sensing is also present in the roots, which corresponds to the triggering of FRO and IRT1 genes in response to Fe stress at the root level, independently of the Fe conditions presented by the leaves.

Molecules with the potential to regulate Fe accumulation could also be an interesting target for biofortification strategies development, and some have been identified, namely, the aforementioned IDEF1 in graminaceous plants, that is a transcription factor which positively regulates IRO2 gene under Fe deficiency (Fig. 1.3b), but whose expression is not affected by this stress (Kobayashi and Nishizawa, 2014); Hemerythrin motif-containing Really Interesting New Gene (RING)- and Zinc-finger proteins (HRZs) / BRUTUS (BTS) ubiquitin ligases, which negatively regulate Fe deficiency responses in both graminaceous and non-graminaceous plants, controlling Fe uptake and translocation under Fe-sufficiency to prevent Fe excess caused damage (Kobayashi *et al*., 2013; Matthiadis and Long, 2016); or metal tolerance proteins (MTP), identified in wheat grains as good biofortification candidates (Vatansever *et al*., 2017) due to their role in divalent metals effluxing out of the cytoplasm and involvement in metal tolerance under Fe deficiency stress (Eroglu *et al*., 2016).

Although most of the abovementioned genomic studies are typically performed on model crops, such as Arabidopsis and maize, in the past decade, genomic studies in legume plants have become abundant, due to their great nutritive, economic and environmental value (reviewed by O'Rourke *et al*., 2014).

1.5. The importance of grain legumes in modern agriculture

Grain legumes can be divided into pulses and non-pulses. Food and Agriculture Organization of the United Nations (FAO) defines pulses as annual leguminous crops yielding from one to 12 grains or seeds of variable size, shape and colour within a pod, which include dry beans, peas, lentils and lupins (among others), but exclude soybean and peanut (non-pulses), because these are mainly considered as oil crops (Duranti, 2006). The year of 2016 was declared by the United Nations as the International Year of Pulses, in order to implement a plan of action to increase awareness of the importance of legumes in human health in the community and encourage the increase of pulse production by all possible stakeholders (FAO, 2016a). Hence, in the present times, major attention is being directed to grain legumes due to their role in global food security and environmental health.

In what concerns human nutrition, grain legumes have high protein levels as well as essential amino acids, carbohydrates, vitamins and minerals and are a predominant component of traditional diets of many regions throughout the word (Messina, 1999). The consumption of these crops contributes to a healthier diet and decreases nutrient diseases,

obesity risk, as well as cardiovascular disease or type II diabetes risk (Rochfort and Panozzo, 2007). Grain legumes also provide about one-third of processed vegetable oil for human consumption (Graham and Vance, 2003).

Moreover, the production of grain legumes contributes to food security since, in developing countries, it is one of the main sources of income for smallholder farmers. Additionally, given the increased concern with climate change and its future consequences, it is important to note that grain legumes also contribute to climate change adaptation and mitigation, either because they fix atmospheric nitrogen and provide it to the soil, reducing the need for synthetic nitrogen fertilizers (Herrige *et al*., 2008), or because they can be used in inter-cropping systems, increasing crop diversification and biodiversity and economic returns, as shown by the successful case studies with *Trifolium* L. and *Medicago sativa* (Reckling *et al*., 2016).

According to the latest FAO data, dry beans (common beans) have the highest percentage of production between pulses, holding a 32.7% share of global pulse output (FAO, 2016b). In Portugal, from the 4 kg/capita provision of legumes, 3.1 kg/capita corresponded to dry common bean (*Phaseolus vulgaris*) (INE, 2016). Also, heading the list of the most produced crops worldwide, there is soybean (*Glycine max*), which has a high market value for its rich content in vegetable oil, protein, fatty-acids, isoflavones and saponins (Carrera *et al*., 2014). In 2016, in the United States alone, a total of 83.7 million acres were dedicated to soybean production, corresponding to approximately 107 million tons of product, which was 34 % of the global production (314 million tons) (United States Department of Agriculture, 2017). Following the United States, Brazil is the second biggest soybean producer and the other countries with relevance to this market are Argentina, China, India, Paraguay and Canada (Foyer *et al*., 2016).

When compared to other grain legumes, such as bean, chickpea or lentil, soybean has higher levels of protein, fat, calcium and iron and, together with their high content of isoflavones, their consumption has been associated with decreased risk for the development of certain forms of cancer, osteoporosis and heart disease (Messina, 1999). Interestingly, since the introduction of genetically modified (GM) soybean crops in the market in 1996, crops either with increased herbicide tolerance or insect resistance have contributed to lowering production costs and increasing crop yield (Brookes and Barfoot, 2016a), accounting for 75% of the soybean plantings worldwide in 2014 (Brookes and Barfoot, 2016b). Several studies attest the safety of GM soybeans, as recently reviewed (Domingo, 2016). Soybean value in the food industry is further increased due to its various

derived products, many of them associated with oriental soy foods, such as tofu, soy sauce, miso, tempeh and natto, but also other by-products already largely included in the west diet like soymilk, soy sprouts or flour. Besides its importance in human nutrition, soybean has also great impact in animal feed as it is largely incorporated in the rations for dairy cattle.

Similarly to common bean and soybean that are the most important grain legumes with high production and consumption rates, rice is one of the top commodities in the world and is grown in more than a hundred countries, with a total harvested area in 2014 of approximately 163 million hectares, producing more than 700 million tons annually (FAO, 2016c). The main producer in the world is Asia, which accounts for over 90 % of the world production of rice, with China and India producing the most, accordingly with the latest data provided by FAO (2016c). Portugal is the fifth producer in Europe with 167,000 tons of rice produced in 2014 and about 61,000 tons exported in 2013 (FAO, 2016c). As it utilizes a different Fe uptake strategy (strategy II) as compared to legume crops like soybean and bean (that utilize strategy I), rice is also an important model crop for studying Fe uptake mechanisms and related molecular players in comparison to the strategy I utilizing crops.

Despite the general advantages of producing grain legumes, when these are grown under alkaline conditions, they develop a condition named Fe deficiency chlorosis (IDC) (Vasconcelos and Grusak, 2006) caused by Fe unavailability in the soils. This condition deeply affects legumes' economic and social value and efforts must be made to overcome this problem.

1.6. The IDC problem and alleviation strategies

Again, despite the abundance of Fe in soils (it is the fourth most abundant element in the earth's crust), Fe has low solubility and this is a hurdle that leads to Fe deficiency in plants, especially in aerated calcareous soils, which represent one third of cultivated lands of the whole world (Fig. 1.5).

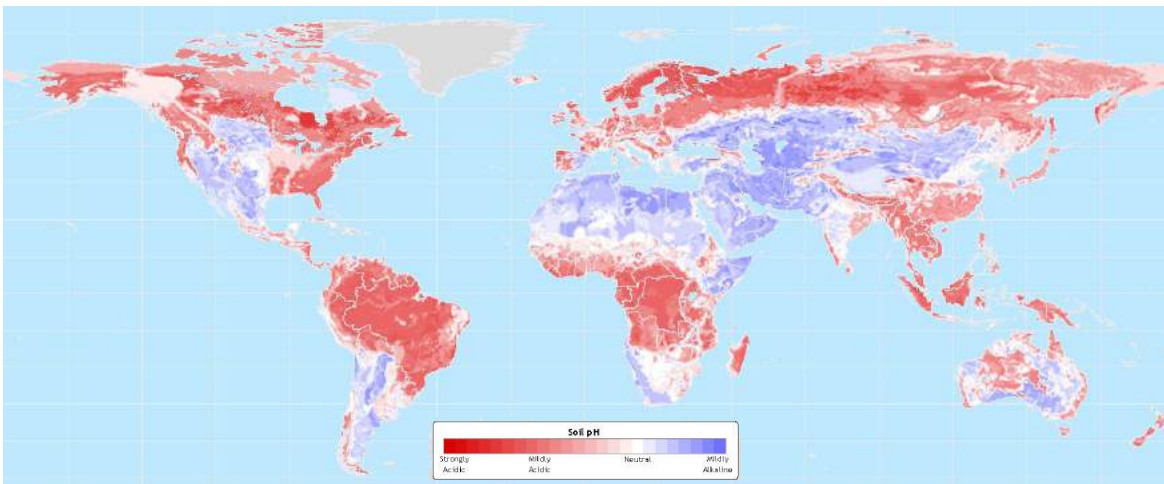

Fig. 1.5. Soil world distribution, according to pH (Adapted from IGBP-DIS Global Soils Dataset, 1998).

In Portugal, calcareous soils correspond to small areas in the south half of the country (Fig. 1.6). In these regions, great areas are attributed for legume plants production, mainly common bean (3193 ha) and chickpea (1630 ha) (INE, 2016), which are highly susceptible crops to calcareous conditions.

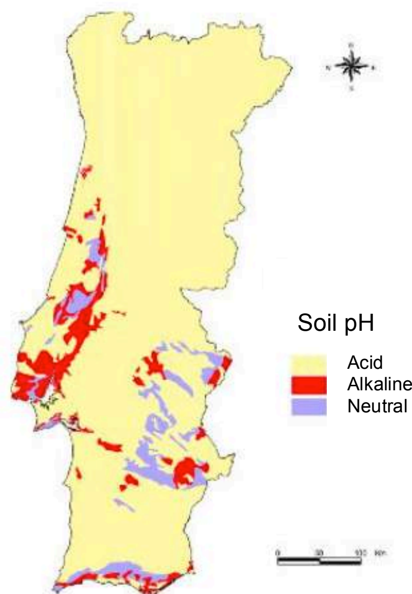

Fig. 1.6. Soil pH distribution in Portugal (Adapted from Ferreira, 2000).

Under such adverse conditions, Fe reduction is hindered (Briat *et al.*, 2015) and the mechanisms for Fe uptake described in Section 1.3 are especially affected, due to the inability of plants to absorb Fe from the rhizosphere (Robinson *et al.*, 1999). IDC is one of

the main consequences of Fe deprivation in plants, and leads to a reduction in photosynthesis and an accumulation of reactive oxygen species, resulting in a visible interveinal leaf chlorosis. One of the most classically implemented tools for IDC screening in the field has been a visual chlorosis score, which ranges from 1, if the plant is completely healthy, to 5, when it is necrotic or dead as a result of Fe stress (Prohaska and Fehr, 1981). If left untreated, IDC leads to stunted growth with reduced total biomass, which together with chlorosis, leads to severe yield losses due to reduced number of seeds per plant and economic problems of great impact amongst farmers (Briat *et al.*, 2015).

IDC is also aggravated by soil water content: when the soils are over-irrigated, bicarbonate concentration increases in the rhizosphere, leading to an augment of soil pH and interfering with the plants defence mechanisms against low Fe (Fleming *et al.*, 1984; Chaney *et al.*, 1992; Zhang *et al.*, 2016). To prevent or treat IDC, farmers have to employ different strategies that are usually expensive and are not always effective.

1.6.1. Genetic manipulation

At the turn of the 21st century, genetic engineering has known a rapid development resulting from the progress made in molecular biology and the better understanding of the DNA and its functions in living organisms. Genetic engineering aims to makeup the genome of a living organism in a laboratory using "recombinant DNA technology" by inserting, altering, removing or switching off specific piece(s) of DNA containing the gene(s) of interest. As results, crops developed through genetic engineering are commonly known as transgenic or GM crops (Datta, 2013; Desmond and Nicholl, 1994). GE allows transferring specific and targeted genes from close or distant related plant species to the targeted species, and therefore obtaining a "new" plant with desired agronomic traits. The two most interesting benefits of GE are (i) the possibility to obtain a plant with specific agronomic traits difficult to obtain in the case the trait is not present in the germplasm of the crop, and (ii) the long time needed to introduce that trait in the targeted crop using conventional breeding (Desmond and Nicholl, 1994; Giddings *et al*., 2000).

In order to increase Fe in plant tissues through genetic manipulation, the levels of siderophores, chelating agents, reducing agents, enzymes and transporter proteins could be increased with positive outcomes (Zimmermann and Hurrell, 2002). Amongst the DNA delivery methods, the two most frequently applied are biolistics, where the gene of interest is bombarded through the plant cell wall, so that the genetic information is delivered into the plants genome (Taylor and Fauquet, 2002) and *Agrobacterium*-mediated

transformation, which is based on the presence of a tumor inducing (*A. tumefaciens)* or root inducing (*A. rhizogenes*) species, that allow the utilization of the plasmids as vectors for genes of interest (Klee et al., 1987). Using biolistic transformation, efforts have been made towards the increase of ferritin in crops with low available Fe, successfully achieving increased total Fe pool (Vasconcelos *et al*., 2003). The same result has been obtained, more recently, using interbreeding techniques between a ferritin-overexpressing transgenic soybean and another high-yielding soybean cultivar (Paul *et al*., 2014). On the other hand, through Agrobacterium-mediated transformation, the expression of Fe(III) reductase-encoding genes was increased in Strategy I species with low activity of this enzyme (Connolly et al., 2003), as was the synthesis and exsudation of phytosiderophores in Strategy II plants (Masuda *et al*., 2012), consequently increasing Fe accumulation in both cases. Furthermore, with this approach, not only the content of Fe can be increased, but also the IDC-sensitivity in general can be attenuated, as shown in a study where barley NA synthase 1 was overexpressed in soybean plants (Nozoye *et al*., 2014). However, the major obstacle associated to these techniques of genetic modification is still overcoming Fe loading and unloading in/from the seed, which is the edible part of these plants (Briat *et al*., 2015). A comprehensive review on the topic can be obtained in Vasconcelos *et al.* (2017).

Nowadays, newer technologies have evolved such as oligo-directed mutagenesis, reverse breeding, RNA-directed DNA methylation and sequence-specific nuclease technology or 'genome editing' (Schaart *et al*., 2015). Particularly, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspersed short palindromic repeats (CRISPR)-associated protein 9 nuclease (Cas9) system, are powerful tools for developing new traits in plants (Gupta and Shukla, 2016). ZFNs and TALENs are artificially designed restriction enzymes for genome editing: the first are composed by the DNA-binding domain zinc finger protein derived from eukaryotic transcription factors, which makes them highly sequence-specific, and the Fok I cleavage domain from *Flavobacterium okeanokoites*, that has high cleavage activity (Miller *et al*., 2011); the second, is a highly specific tool, with high targeting efficiency and has been successfully applied to genome editing in rice for herbicide resistance (Li *et al*., 2016c). CRISPR-Cas9 is the most recent and revolutionary technique, which allows multiplexed gene editing without compromising the targeting efficiency (Gupta and Shukla, 2016). This tool origin is the defense system of bacteria that helps to detect and destroy a pathogen invasion in the cells (Mojica *et al*., 2009) and has been applied, for

example, to targeted mutagenesis in soybean (Sun *et al*., 2015) and to yield traits manipulation in rice, such as grain number and size and panicles formation (Li *et al*., 2016a), holding great potential for improved crops for their commercial and nutritional value.

Since these new gene editing methods target specific regions of the genome, it is very precise and regulations like the ones associated to GM organisms are yet to be applied, putatively making them generally more well accepted (Abdallah *et al*., 2015). However, the unpredictable resulting phenotypes, as well as the lack of sufficient diversity in cultivars used in the breeding programs, consumer resistance and the safety of genetically modified crops are still obstacles delaying the market and public acceptance of GM organisms (White and Broadley, 2005).

1.6.2. Screening for tolerant genotypes

The susceptibility to IDC may be influence by genetic and environmental factors. In general, there are species that are more susceptible to IDC than others and, differences between cultivars have also been reported, which is an important characteristic for breeding for tolerant cultivars in order to reduce IDC's incidence (Boodi *et al*., 2016). Soybean, in particular, comprises cultivars that, when grown on calcareous soils, exhibit little foliar chlorosis (Fe-efficient or tolerant) and other cultivars that express severe leaf yellowing or even plant death (Fe-inefficient or susceptible) (Vasconcelos and Grusak, 2014), making this crop an exceptional model for Fe-related studies.

Although Fe-efficiency mechanisms are still far from being totally understood, Fe-efficient plants usually have the ability to induce biochemical reactions that make Fe available in a useful form, while Fe-inefficient plants do not (Brown and Jolley, 1989). Furthermore, Fe-efficient plants should also have higher ability to grow under Fe stress conditions, which implies a better metabolic use of the Fe pools inside the plant (García-Mina *et al*., 2013). This is not always true and, due to this fact, when under Fe sufficient conditions, soybean growers may prefer to grow an IDC susceptible line because they frequently have higher yields than IDC tolerant lines (Atwood *et al*., 2014).

Two soybean lines, equal in phenotype but with different Fe efficiencies, were compared in terms of ferric reductase induction and gene expression profiling. Studies showed that the Fe-inefficiency trait was correlated with low levels of ferric reductase enzyme activity under Fe deficient conditions and with the up-regulation of genes of signalling and regulatory pathways in order to maintain cellular homeostasis (O'Rourke *et*

al., 2007); Fe-efficiency could be linked to the ability of the plants to induce energy controlling pathways to promote nutrient recycling and stress responses to Fe deficiency (Atwood *et al*., 2014) and due to a greater capacity of Fe translocation to the aboveground organs (Roriz *et al*., 2014).

Other species have also been studied for their Fe-efficiency. For example, sugar beet plants that accumulate flavin in roots appear to have higher efficiency levels (López-Millán *et al*., 2000). In tomato, the accumulation of citrate and malate seem to modulate their level of efficiency (López-Millán *et al*., 2009), as well as the induction of IRT, FRO, SOD, APX and CAT gene expression (Muneer and Jeong, 2015). Quince and pear fruits have also been compared in terms of efficiency and the degree of metabolic responses activation varied between genotypes (Donnini *et al*., 2008). In *Prunus* genotypes, Fe-inefficiency was associated with increased oxidative stress and reduced antioxidant defence (Cellini *et al*., 2011) and, in two rice genotypes, the one proven to be more Fe-efficient was able to acidify the rhizosphere, as well as exudate higher rates of phytosiderophores and induce the expression levels of the genes OsIRO2, OsIRT1, OsNAS1 and OsNAS2, and OsYSL2 and OsYSL15 (Li *et al*., 2016b).

Given the diverse type of responses obtained in different crops and genotypes, it is important to establish specific tools that allow the clear distinction between tolerant/efficient and susceptible/inefficient cultivars. This is important not only to select cultivars for field production, but also to better understand and identify the most important traits in the efficiency trait and use them in biofortification programs.

1.6.3. Fertilizers and chelating agents

Since the main causal agent of IDC is the bio-unavailability of Fe in soils, one attractive option to prevent and/or treat this physiological disease could be managing the bioavailability of Fe in the soil. Lowering the soil pH is a possible strategy but, besides being very expensive, it cannot be performed effectively since the calcareous soils are severely buffered (Morgan, 2012). Hence, fertilization is a more widespread agronomic approach for IDC management. Iron fertilizers are mainly grouped in three distinct categories, specifically: 1) natural Fe-complexes; 2) inorganic Fe compounds; and 3) synthetic Fe-chelates (Table 1.1).

Natural Fe-complexes consist of organic materials that naturally contain sufficient amounts of Fe to act as an Fe source for plants (Shenker and Chen, 2005). These comprise peat, coal, lignite, manure, humic substances and by-products of wood processing wastes,

like polyflavonoids and lignosulfonates. These types of ligands have a special role on Fe bioavailability in coastal and oceanic waters (Kuma *et al*., 1999), while in field conditions, to be effective, these organic compounds must be applied in high amounts, which are not practical and have more economic costs associated (Shenker and Chen, 2005). There are, however, studies showing that, when applied to Fe-deficient plants, some humic substances can lead to a more effective and quicker restoration of the Fe content in the leaves than a synthetic chelate (Kovács *et al*., 2013) or when compared to other organic substances like phytosiderophores (Zanin *et al*., 2015). Natural complexes may also be applied to the leaf in order to overcome their low soil stability (Carrasco *et al*., 2012) and new organic compounds have been tested, such as, a natural hetero-ligand Fe chelate, which application, although depended on the presence of a synthetic Fe-starter fraction, was as effective as that of 100% synthetic chelate in total leaf Fe accumulation (Fuentes *et al*., 2012).

Among inorganic Fe compounds are Fe salts and Fe oxide-hydroxides. If, on one hand, these compounds have low cost and are easily applicable to the soil, they react with $CaCO_3$ to form Fe oxides, and their high insolubility hinders the value of this type of ligand as plant Fe source *via* the soil, being usually applied *via* foliar fertilization with more successful results (Wei *et al*., 2012). However, foliar application could have poor results, due to the low penetration rates of Fe in the leaves, which have thick cuticles' and to the limited translocation of this form of Fe within the plant (Shenker and Chen, 2005). In order to overcome these constraints, a recent study utilized Fe oxide nanoparticles to deliver Fe to peanut plants, comparing this to the supplementation with a synthetic chelate, and showed that both had similar outcomes in terms of Fe accumulation and chlorosis development, but with the Fe oxide nanoparticles better adhering to soil particles, reducing nutrient loss (Rui *et al*., 2016). Nevertheless, synthetic Fe-chelates are considered the most effective soil fertilizers, even in calcareous conditions (Lucena, 2006; Abadía *et al*., 2011; Briat *et al*., 2015).

Table 1.1. Main types of Fe chelates, principal characteristics and examples

	Fe chelates		
	Natural Complexes	Inorganic Salts	Synthetic
Characteristics	◆ Require high dosage ◆ Low soil stability ◆ Environmental friendly	◆ Insoluble ◆ Low cost ◆ Foliar application	◆ Soluble in soil ◆ Recalcitrant ◆ High cost
Examples	peat, coal, lignite, manure, humic substances, lignosulfonates	Ferrous sulfate, iron oxide, iron oxide nanoparticles	EDTA, EDDHA, EDDS, HBED

Synthetic Fe-chelates are generally composed of two or more functional groups that have an unshared electron pair. By electron sharing, they form a coordination link with a centrally located Fe, resulting in metal-chelate molecules with different stability constants, depending on structural configuration and other parameters (Shenker and Chen, 2005). Differently of the other types of Fe fertilizers, Fe-chelates contain the Fe, but also the chelating agent. A mechanism has been proposed where, once Fe is delivered to the plant, the 'free' chelating agent could take more native Fe from the soil, dissolving Fe from oxides that would otherwise be unavailable for plants' uptake, creating a "shuttle effect" (Lucena, 2003). On the other hand, it has been shown that metal-EDTA complexes might be absorbed by the plant roots via the apoplastic pathway, as these complexes have been found intact in the xylem of barley plants (Collins *et al*., 2001).

The majority of the studied synthetic Fe-chelates are derivatives of the ethylenediamine-carboxylic acids family (Briat *et al*., 2015). Non-phenolic compounds, such as ethylene diamine tetraacetic acid (EDTA) have low stability in calcareous soils and are unable to maintain Fe in solution (Rodríguez-Lucena *et al*., 2010). A naturally occurring derivative of EDTA, [S,S]-ethylenediaminedisuccinate, [S,S]-EDDS, has also been studied for its ability to maintain Fe in water-soluble form and, although it was more biodegradable than EDTA holding potential for lower environmental impact Fe solubility was lower than that of EDTA (Ylivainio, 2010). The most effective chelating agents to deliver Fe under calcareous conditions are diamino-diphenolic-dicarboxylic acids, such as ethylene diamine-*N,N'*-bis(2-hydroxy phenyl) acetic acid (EDDHA) (Lucena, 2006). Studies have also shown that the isomeric form of EDDHA is important and influences its

capability to provide Fe to plants, being the *ortho-ortho* (*o,o*) isomer the most effective (Rojas *et al*., 2008).

Lately, the search for new, more efficient, less expensive and environmentally friendly synthetic chelates has stimulated the introduction of different products that seem promising for agricultural purposes. However, the formulation of new fertilizing compounds must have into account a set of characteristics that determine their effectiveness (Fig. 1.7). An iron chelate must be able to maintain Fe in soil solution, in order to permit the reduction of Fe(III) by the roots (Rojas *et al*., 2008), which implies that it must be stable enough in calcareous conditions so that Fe is not exchanged by a competing cation and that it doesn't get adsorbed to the soil solid phase. On the other hand, the stability of the complex should not be too high, so that the ligand is able to release Fe in the rhizosphere for plant uptake (Hasegawa *et al*., 2012). Chlorosis assessment is the most informative tool to assess the effectiveness of a certain chelate (Fig. 1.7) and, between field or controlled conditions, either type of experiment is valid to do the assessment, only depending on the question and the means to address it (El-Jendoubi *et al*., 2011).

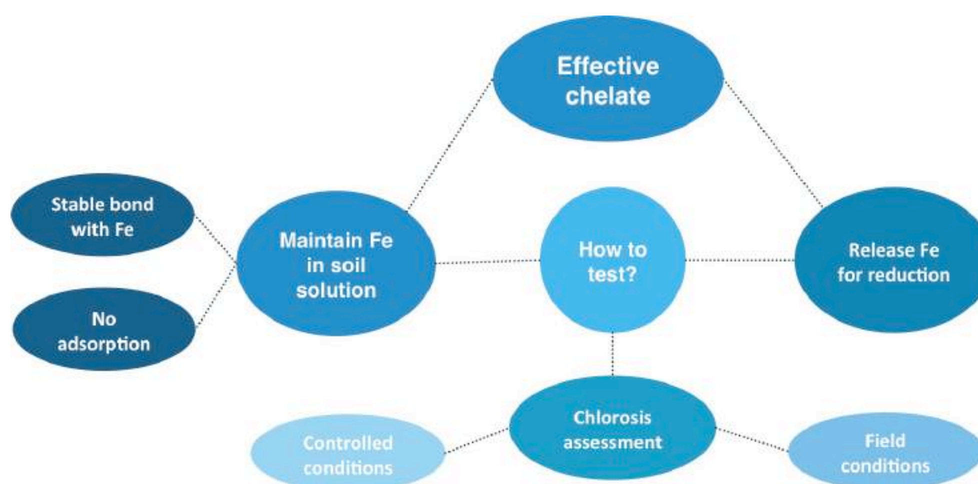

Fig. 1.7. Main characteristics of an iron-chelate to be considered as effective in supplying Fe to the plant.

Several structurally analogous molecules to FeEDDHA have been tested for their potential as effective Fe-chelates, namely, ethylenediamine di(2-hydroxy-4-methylphenylacetic) acid (EDDHMA), ethylenediamine di(2-hydroxy-5-sylfophenylacetic) acid (EDDHSA) or ethylenediamine di(5-carboxy-2-hydroxyphenylacetic) acid (EDDCHA), but despite having similar efficacy to FeEDDHA for chlorosis treatment, the

latter continues to be preferred (Cantera *et al*., 2002; Álvarez-Fernández *et al*., 2005; Lucena and Chaney, 2006). Another Fe(III) chelating agent with a similar structure to EDDHA is *N,N'*-bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED), that has higher stability in soil that EDDHA (López-Rayo *et al*., 2009) and induces chlorophyll production over time (Bin et al., 2016), but requires higher concentrations to obtain the same Fe absorption by the plant as FeEDDHA (Nadal *et al*., 2012). New generation biodegradable complexing agents, such as hydroxyiminodisuccinate (HIDS), have also been proposed as good alternatives to EDTA and EDDHA (Hasegawa *et al*., 2012; Rodríguez-Lucena *et al*., 2010).

1.7. Scope and outline of the thesis

This study aimed at understanding IDC in plants and contributes to avoid the processes involved in its development. Firstly, using different crops in order to understand the common responses to Fe deficiency at a molecular level and, secondly, looking at soybean lines with different efficiencies to the unavailability of Fe, we aimed at unveiling the physiological and biochemical parameters related with higher tolerance to this phenomenon. The three main goals of this research programme were: 1) studying the molecular mechanisms triggered by IDC; 2) understanding the physiological mechanisms behind IDC; and 3) finding a possible solution to prevent IDC development in legume plants. To achieve these goals, more specific objectives comprised: *i*) identifying IDC-relevant genes using transcriptomic analysis; *ii*) gaining insight into the transcriptome dynamics that are associated with IDC response in different grain crops; *iii*) studying the regulation of IDC-related genes in soybean cultivars with different Fe-efficiencies; *iv*) evaluating physiological and plant growth aspects associated to IDC-tolerance, to develop reliable tools for IDC-cultivars selection; *v*) correlating IDC-responses with the tetrapyrrole and antioxidant systems; and *vi*) evaluating the potential of a novel iron chelate (3,4-HPO) as a chlorosis corrector.

In this thesis, an option was made to present the results in the form of printed articles as they were published or submitted in international, peer-reviewed journals.

Even with the most recent technologies and studies that combine them, to understand Fe-deficiency responses in legume plants, there is still the need to identify new genes capable to confer better adaptation to this abiotic stress (Amaral *et al*., 2016) and that can be used as targets in biofortification programs (Manwaring *et al*., 2016). In Chapter 2 a molecular approach was undertaken to compare the transcriptome of three

different species, in order to understand which common genes are triggered in response to Fe deficiency (**section 2.1**). In Chapter 2 we also investigated the regulation of a set of IDC-related genes in two model legume species – soybean and barrel medic -, with a special emphasis on a new Fe metabolism-associated gene (**section 2.2**) and in a cereal crop - rice (**section 2.3**).

As previously described in the Introduction section, several physiological responses have been associated to IDC. Also, the selection of IDC-tolerant cultivars is one of the most commonly used tools to prevent agricultural losses due to the unavailability of Fe in the soils. Therefore, it is of the upmost importance the association of specific traits of Fe metabolism regulation to IDC-tolerance. To that end, a first study was performed where, among other physiological mechanisms, the Fe-translocation ability was studied in two soybean cultivars with different Fe-efficiency (**Chapter 3, section 3.1**). Furthermore, as new metabolic players are being identified as key factors in Fe reduction and uptake (e.g. coumarins), we focused on the influence of Fe deficiency on tetrapyrrole metabolism and antioxidant system (**Chapter 3, section 3.2**), which is an unprecedented point of view in this research area. After gathering molecular and physiological information on IDC responses, we aimed at finding a new fertilizing compound that could be an efficient alternative to the commercially used products (**Chapter 4**). The results and main conclusions of each previous chapter are discussed in **Chapter 5**, as well as the identification of knowledge gaps and future perspectives in Fe research. Finally, a critical reflection concerning the nutritional value of legume plants in face of the challenges ahead in the future of agriculture is presented (**Chapter 5, section 5.2)**. A graphic representation of the outline of this thesis is represented in Fig. 1.8.

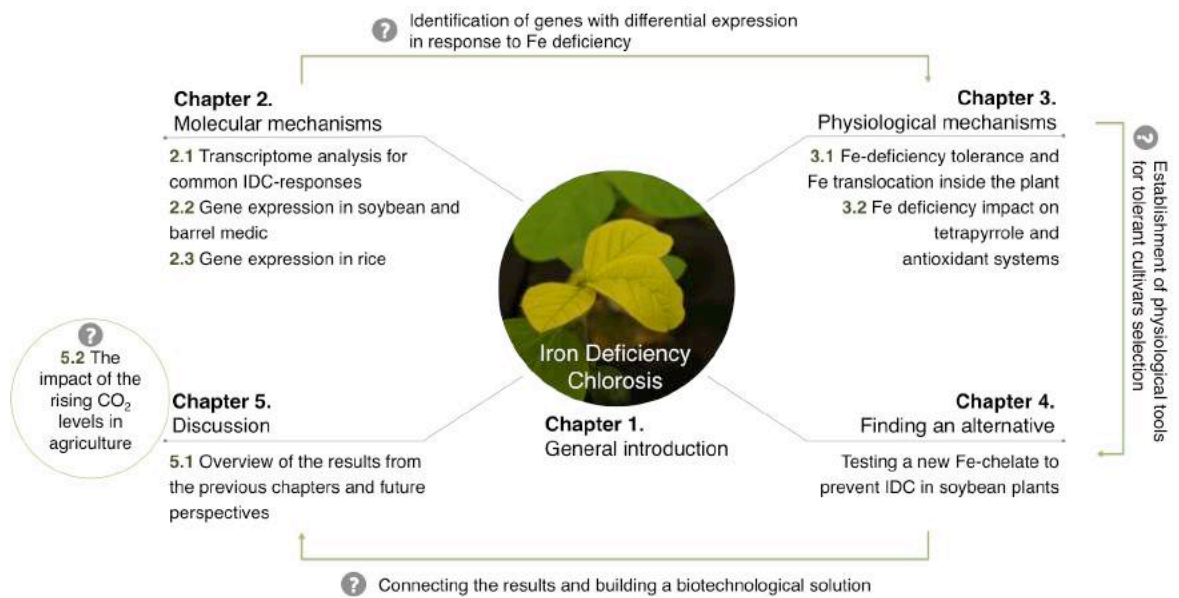

Fig. 1.8. Schematic representation of the thesis outline. Question marks represent the main goals achieved in each chapter.

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CHAPTER 2

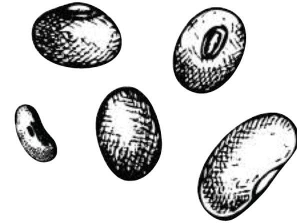
Molecular mechanisms associated with IDC

In this chapter the molecular mechanisms associated with IDC will be studied. Firstly, in section **2.1**, a non-targeted approach will be presented, where an Illumina study was conducted in order to obtain the common molecular responses to three cultivars with agronomic or research interest, namely *Phaseolus vulgaris*, *Glycine max* and *Medicago truncatula*.

A second, targeted, approach was undertaken. While in section **2.2** the regulation of specific genes, including a new gene that could have preponderance in Fe metabolism (*GCN2*), was studied in both *Glycine max* and *Medicago truncatula* cultivars, in section **2.3** genes preponderant in Fe uptake from both strategy I and II were analysed in two rice cultivars.

CHAPTER 2

section 2.1



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Transcriptomic analysis of iron deficiency related genes in the legumes

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ABSTRACT

Among the mineral elements required by humans, iron (Fe) is the most common cause of nutritional deficiencies, particularly anaemia. Legume plants are extremely important in the world's diet and they are major sources of mineral nutrients. However, when these plant foods are grown in calcareous soil, their production is severely affected by Fe deficiency chlorosis (IDC), and when less Fe is available for absorption, less amount of this element will be available for accumulation in the edible plant parts. As Fe plays critical roles in photosynthesis and respiration, when lacking this element, plants develop chlorosis and their growth is drastically reduced. IDC morphological symptoms were monitored in soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and the model crop barrel medic (*Medicago truncatula*). When compared to the other two legumes, *G. max* presented lower Fe-reduction rates and severe chlorosis, associated with lower SPAD values. Transcriptome analysis was performed in roots of the three species when grown in Fe deficiency and Fe sufficiency, and 114,723 annotated genes were obtained for all samples. Four IDC-related genes were up-regulated in common by the three species and can be considered key players involved in the IDC response, namely, metal ligands, transferases, zinc ion binding and metal ion binding genes. With regards to the genes most highly expressed under iron deficiency individually by each species, we found that the most highly expressed genes were a defensin in *P. vulgaris*, a phosphatase in *M. truncatula* and a zinc ion binding gene in *G. max*.

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1. Introduction

Out of the world's 6 billion people, one third of them suffer from mineral deficiencies. Since most of the world's population does not ingest enough Fe to meet daily dietary requirements, Fe deficiency is one of the most common nutritional deficiencies and the leading cause of anaemia (Zimmermann & Hurrell, 2002). Among the main risk groups are pregnant women and women of childbearing age (Krafft, Murray-Kolb, & Milman, 2012), as well as children, both infants and teenagers (Toutain, Le Gall, & Gandemer, 2012). This constitutes a serious problem, since anaemia can cause poor pregnancy outcome and children morbidity, as well as diminished work productivity in adults (WHO, 2001).

Grain legumes are cultivated primarily for their seeds which are rich in starch and dietary protein. Legumes have an important socio-economical role in the Mediterranean diet. Their benefits on human health are diverse, ranging from their high protein content, to high concentration in micronutrients, such as Fe and zinc (Vasconcelos & Grusak, 2006). However, the low bioavailability of Fe in alkaline soils, where this nutrient is often insoluble, together with the cultivation of susceptible genotypes causes drastic economic damage due to the reduced crop viability (Zamboni et al., 2012). When Fe lacks in plant metabolism, several processes are affected, like photosynthesis, respiration, nitrogen fixation, DNA synthesis, hormone production, chlorophyll formation (Vasconcelos & Grusak, 2006), among others. This generally leads to the development of Fe deficiency chlorosis (IDC), characterized by the yellowing of the upper leaves, interveinal chlorosis and stunted growth, with the plant's yield severely affected (Prasad, 2003). To cope with this, non-grass plants use a two-step mechanism for Fe uptake: firstly, Fe(III) is reduced to Fe(II) by a plasma membrane-bound ferric reductase, and the latter is subsequently released from the chelate and then transported into the cytoplasm via a transport protein (Jeong & Connolly, 2009). Since increasing the Fe uptake in the roots can augment Fe concentrations in the leaves, it is possible that some of this additional Fe may be re-mobilized to the grains, which would help in biofortification efforts that aim at enhancing Fe seed levels. However, the increased Fe translocation from shoots to seeds still remains one of the major bottlenecks in most biofortification programs (White & Broadley, 2005).

Common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) are rich in protein, which makes them valuable crops for worldwide consumption, and they are both susceptible to IDC. Specifically, *P. vulgaris* total production exceeds 23 million metric tonnes (MT) and consists a major staple of eastern and southern Africa, as well as of Latin America (Broughton et al., 2003). Moreover, *G. max* is a good crop to study IDC molecular mechanisms since, in 2010, its genome was sequenced, assembled and published (Schmutz et al., 2010). This species had a production yield of 2567 kg/ha in 2002 in Brazil

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and, besides contributing for vegetable oil production, they are also rich sources of dietary protein for chicken and pork industries (Graham & Vance, 2003). On the other hand, barrel medic (*M. truncatula*) represents a good model for Strategy I plants, since when this plant is challenged with Fe shortage, the most important root physiological responses induced by Fe deficiency are developed, including the yellowing of root tips (Andaluz, Rodríguez-Celma, Abadía, Abadía, & López-Millán, 2009).

Different sequencing technologies have given us some insight regarding the legume plants response to Fe deficiency. A few examples include: 1) genome-wide transcriptional analysis in tomato roots, that identified genes potentially involved in Fe starvation and root response to nutrient deficiency (Zamboni et al., 2012); 2) microRNA (miRNA) survey of genes related to Fe deficiency in *Arabidopsis*, where 24 miRNA genes were found to contain Fe deficiency responsive cis-Element 1 and 2 in their promoter regions (Kong & Yang, 2010); 3) Solexa sequencing, a high throughput sequencing technology that allowed to isolate 1,563,959 distinct *M. truncatula* sequences and to predict target genes for novel miRNAs (Szittya et al., 2008); 4) high-throughput sequencing analysis of miRNA associated with stress response in *G. max*, from which 133 expressed conserved miRNAs were identified, putatively inducible in response to certain stresses like alkalinity (Li et al., 2011).

In this study, specific genes associated with plant mineral metabolism were identified by high throughput sequencing (Illumina Hiseq 2000). Root samples of *Glycine max*, *Phaseolus vulgaris* and *Medicago truncatula* grown hydroponically under Fe-sufficiency and Fe-deficiency were analyzed to further our knowledge on legume nutrition and abiotic stress. Identification of these genes can help us understand the common and individual regulatory mechanisms of iron uptake in the legumes and assist in plant biofortification programs.

2. Materials and methods

2.1. Plant growth conditions

All plants (*Medicago truncatula* cultivar Luzerna revilheira, *Glycine max* cultivar Williams 82 and *Phaseolus vulgaris* ecotype PMB-0121 [Rodiño, Monteagudo, Santalla, & De Ron, 2001]) were grown in an Aralab Fitoclima 10000EHF with 16 h day/8 h night photoperiod. The temperature was kept at 20 °C during the light period, with 70% of relative humidity and 350 μmol s^{-1} m^{-2} of photon flux density, and at 18 °C during the dark period, with 80% of relative humidity.

Scarified seeds of *M. truncatula* were germinated in 1.2% Agar inside the chamber, and seeds of *G. max* and *P. vulgaris* were rolled in filter paper and placed vertically in a solution of 250 mM $CaCl_2$, for 7 days in the dark. The 7 day old seedlings were transferred to hydroponic solution with different Fe treatments.

The standard solution for hydroponical growth of *M. truncatula* contained: 3 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.5 mM $NH_4H_2PO_4$; 0.75 mM K_2SO_4; 25 μM $CaCl_2$; 25 μM H_3BO_3; 2 μM $MnSO_4$; 2 μM $ZnSO_4 \cdot H_2O$; 0.5 μM $CuSO_4.H_2O$; 0.5 μM MoO_3; 0.5 μM $NiSO_4$. The conditions used for *G. max* and *P. vulgaris* included: 1.2 mM KNO_3; 0.8 mM $Ca(NO_3)_2$; 0.3 mM $MgSO_4 \cdot 7H_2O$; 0.2 mM $NH_4H_2PO_4$; 25 μM $CaCl_2$; 25 μM H_3BO_3; 0.5 μM $MnSO_4$; 2 μM $ZnSO_4 \cdot H_2O$; 0.5 μM $CuSO_4.H_2O$; 0.5 μM MoO_3; 0.1 μM $NiSO_4$. All hydroponic solutions were buffered with the addition of 1 mM MES, pH 5.5.

Five plants of each species were maintained for 14 days in Fe sufficient (10 μM Fe(III)-EDDHA [ethylenediamine-N,N′bis(o-hydroxyphenyl) acetic acid]) and Fe deficient (0 μM Fe(III)-EDDHA) conditions. During the time of the experiment, pH and conductivity were measured daily and solutions were changed every 2 days.

Soil and Plant Analyzer Development (SPAD) readings were taken on the last day of the assay with a chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan) from at least four random trifoliate leaves.

Table 1
Forward and reverse primer sequences used in quantitative real time PCR analyses.

Gene	5′-3′ Forward primer	5′-3′ Reverse primer
18S-rRNA	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA
Metal ion binding	ACTAACGGTGACGGGAGAGA	GACATCTGGTGGCTTCGTTT
Glucan 1, 3-β-glucosidase	TACGCCGCTCTTGAAAAAGT	CAATTGCTCCGGGTCTCTTA
Phosphotransferase	GCAAGCACGTTCACAGAAAA	TCTGCTGCAACGACCTAATG
UGT	CAACACCACCAGATCATTGC	TTCCCAAACTCCAGGTCTTG

2.2. Fe reductase localization in roots

As previously performed by Vasconcelos et al. (2006) a gel composed by nutrient solution (6 mM KNO_3; 4 mM $Ca(NO_3)_2$; 1.5 mM $NH_4H_2PO_4$; 1 mM $MgSO_4$; 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 $NiSO_4$), 100 mM agarose (SeaPlaque, Duchefa Biochemie, The Netherlands), 100 mM MES Buffer, 100 μM Fe(III)-EDTA and 100 μM BPDS (bathophenanthroline disulfonic acid) was prepared. Intact roots were carefully laid in the mixture and left for 45 min in the dark, before visualization of a pink coloration around the roots was observed, indicating Fe(II)-$BPDS_3$ formation. These assays were performed using 2-week-old plants grown hydroponically as described above.

2.3. Root Fe reductase measurements

Reduction was measured in intact roots via the spectrophotometric measurement of Fe^{2+} chelated to BPDS. To measure Fe reduction, roots were submerged in 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 and 100 μM BPDS. All nutrients were buffered 1 mM MES, pH 5.5. The assays were conducted under low light conditions at 20–22 °C and were terminated after 45 min by removal of the roots. Absorbance values were obtained spectrophotometrically at 535 nm, and an aliquot of free-roots solution was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}.

2.4. Statistical analysis

Student's *T*-test corrected for multiple comparisons using the Holm–Sidak method was used to analyse statistical significant differences between samples (Prism 6 — GraphPad Software, Inc).

2.5. RNA extraction

The roots of the five plants of each treatment were polled together and grounded with liquid nitrogen, until a fine powder was obtained. To extract the RNA, Qiagen RNeasy Plant Mini Kit (USA, #74904) was used. Possible DNA contamination was removed using the Turbo DNA-free kit (Ambion, Austin, TX, USA), according to manufacturer's instructions. RNA quality and quantity were checked with UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal).

The RNA was sent for transcriptome analysis with high throughput sequencing (Illumina Hiseq 2000, Fasteris, Switzerland).

2.6. Bioinformatic analysis

After Illumina sequencing, high-quality small RNA reads were extracted from raw reads through filtering out the low quality tags and eliminating contamination of adaptor sequences. Each sample

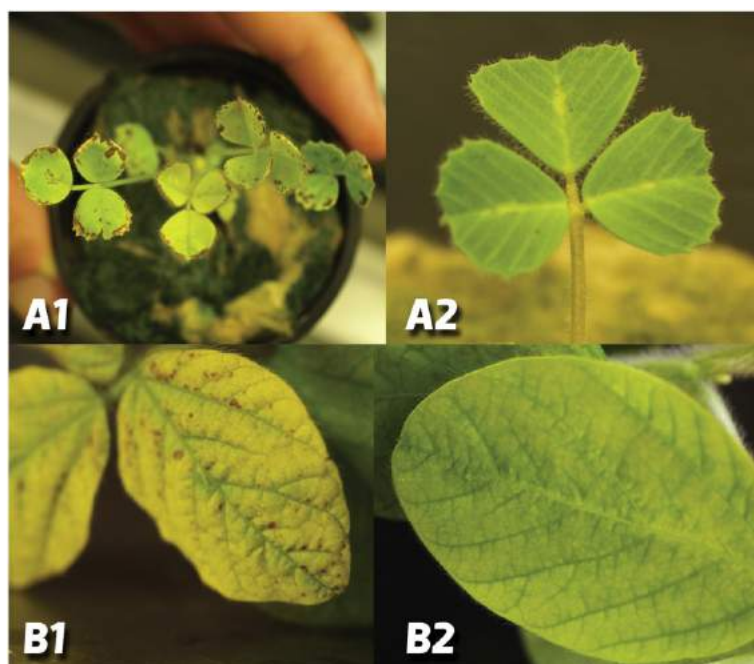

Fig. 1. IDC symptom development in: *M. truncatula* grown at (A1) 0 μM Fe(III)-EDDHA and (A2) 10 μM Fe(III)-EDDHA; and in *G. max* grown at (B1) 0 μM Fe(III)-EDDHA and (B2) 10 μM Fe(III)-EDDHA, in hydroponic solution.

data were merged, normalized and mapped using Burrows–Wheeler Aligner (BWA) mapping on references.

BWA is a program that aligns relatively short nucleotide sequences against a long reference sequence. It implements two algorithms, bwa-short and BWA-SW. The algorithm bwa-short is used for query sequences shorter than 200 bp and the BWA-SW for longer sequences up to around 100 kbp. BWA is used to map the reads with a maximum set at two mismatches in the first 32 bases of the sequences, and a maximum of n mismatches in total (Li & Durbin, 2010).

The GeneConv statistical tests for detecting gene software were utilized to calculate gene abundance for each species separately and by finding the most likely candidates for aligned gene conversion between pairs of sequences in the alignment. The program can also look for gene conversion events from outside of the alignment and candidate events are ranked by multiple-comparison corrected P-values and listed in an output file (http://www.genconv.org).

In order to analyze the quality of the high throughput sequence data, all sequences were submitted to FastaQC software (www.bioinformatics.babraham.ac.uk). This created a comprehensive report about the composition and quality of a high throughput sequence library and information was gathered about number of reads and GC content. Per base sequence quality information of each sample is presented in supplementary data.

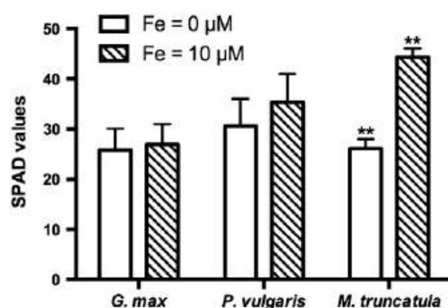

Fig. 2. SPAD readings, at the end of 14 days of assay, in *G. max*, *P. vulgaris* and *M. truncatula* grown in Fe-deficient (0 μM Fe(III)-EDDHA — darker shade) and Fe-sufficient (10 μM Fe(III)-EDDHA — lighter shade) hydroponic conditions. SPAD values were taken from at least four random leaves. Results show a mean and a ± SE of 5 plants. Significant differences between iron treatments for each species are indicated by an asterisk (P-value < 0.001).

2.7. Functional annotation

Genes with abundance lower than 1000 were eliminated from the analysis. For the remaining genes, the abundance of those with the same function was summed and a ratio of expression between Fe-sufficient and Fe-deficient root samples was calculated. Only those with a ratio higher than one were considered as up-regulated genes in the analysis, resulting in a final subset of 223 genes.

The selected sequences were converted from fastaq to fasta format file using Galaxy platform (https://main.g2.bx.psu.edu/). Sequences were then aligned by BLASTx to NCBI non-redundant protein (nr) database (E-value < 0.001). With nr annotation, Blast2GO program was used to retrieve Gene Ontology annotation, InterPro identification and sequence description.

2.8. Confirmation of differential expression

Candidate genes were selected according to the bioinformatics analysis described above, and according to their established role on Fe metabolism.

The same plant material that was used for the Illumina sequencing technique was used for the quantitative real-time PCR (qPCR) to assess and quantify the relative expression of the candidate genes.

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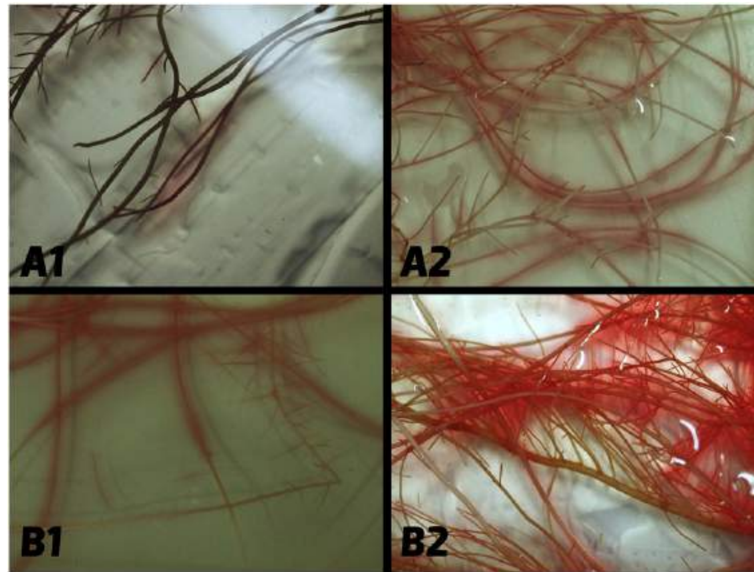

Fig. 3. Root iron reductase activity localization in roots of *G. max* and *P. vulgaris* grown at (A1) and (B1) 0 μM Fe(III)-EDDHA and at (A2) and (B2) 10 μM Fe(III)-EDDHA: formation of the reddish colored Fe(II)-BPDS3 product indicates the location of Fe reduction.

Primers targeting iron stress related genes were designed using Primer3 (Frodo.wi.mit.edu), specifying an expected PCR product of 100–200 bp and primer annealing temperatures between 56 °C and 58 °C. The sequences are presented in Table 1. qPCR reactions were performed on a Chromo4 thermocycler (Bio-Rad, CA, USA). Amplifications were carried out using 1.25 μM of the specific primers and mixed to 12.5 μM of 2xPCR iQ SYBR Green Supermix (Bio-Rad) and 100 ng of cDNA in a final volume of 25 μl. Three replicates were performed for each gene tested in q PCR reactions, as well as for controls. Melt curves profiles were analyzed for each gene tested. The 18S rRNA gene was used as the housekeeping gene and for normalization of expression of the genes of interest. The comparative CT method (ΔΔCT) (Livak & Schmittgen, 2001) for the relative quantification of gene expression value of iron stress related genes using the 18S rRNA gene as the control transcript (Opticon Monitor 3 Software, Bio-Rad). Data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.

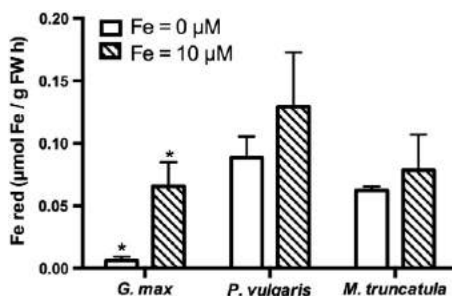

Fig. 4. Roots Fe reduction activity in *G. max*, *P. vulgaris* and *M. truncatula*, when grown in Fe-sufficient (10 μM Fe(III)-EDDHA) and Fe-deficient (0 μM Fe(III)-EDDHA) hydroponic conditions. Plants were assayed for 45 min, absorbance values were obtained at 535 nm and these were applied to calculate reduction rates. Results show the mean and ± SE of 5 plants. Significant differences between iron treatments for each species are indicated by an asterisk (*P*-value < 0.05).

2.9. Data deposition

The Illumina sequencing reads of *G. max*, *P. vulgaris* and *M. truncatula* were submitted to NCBI Sequence Read Archive under the accession numbers of SRS393260, SRS393259 and SRS393261, respectively, in the project number PRJNA189320.

3. Results and discussion

3.1. Morphological responses

M. truncatula and *G. max* plants grown in hydroponics without Fe(III)-EDDHA developed visible symptoms of IDC, namely yellowing of leaves, whereas plants grown with 10 μM Fe(III)-EDDHA were green throughout the experiment (Fig. 1). *P. vulgaris* remained green until the end of the assay, in both treatments.

Roots of plants grown in Fe-deficient conditions developed more secondary structures and showed clear differences of root development when compared to the Fe-supplied plants, which presented longer roots, mainly primary. As previously observed by others (Schmidt, 1999), the plants under Fe-deficient conditions developed swelling of root tips, lateral roots and root hairs, in order to increase root surface and, consequently, Fe uptake.

It is known that young leaves become chlorotic during iron limitation due to inhibition of chloroplast biogenesis and chlorophyll biosynthesis (Henriques et al., 2002). As Fe chlorosis decreases the level of chlorophyll in plant species, Fe deficiency leads to decreased photosynthesis (Prasad, 2003). Soybean was the most susceptible plant to IDC since it showed the lower SPAD values when compared with the other species, even when in Fe-sufficiency (Fig. 2). This could be explained by the fact that the *G. max* cultivar utilized in this study (Williams 82) is very susceptible to Fe shortage. This was the chosen cultivar due to the fact that it was the one used for sequencing the soybean reference genome (Schmutz et al., 2010), and this was the reference genome used in our bioinformatics analysis.

The model crop barrel medic was the only species that showed statistically significant differences between SPAD values, as Fe-deficient

Table 2
Summary of sequence information (with abundance ≥ 1000) in root samples of *P. vulgaris*, *G. max* and *M. truncatula* grown in the presence (+) or absence (−) of Fe.

	P. vulgaris Fe (+)	*P. vulgaris* Fe (−)	*G. max* Fe (+)	*G. max* Fe (−)	*M. truncatula* Fe (+)	*M. truncatula* Fe (−)
Total number of genes	14,260	12,114	24,150	19,091	23,980	21,128
No. of reads	22,146,154	26,617,669	23,052,072	29,447,921	35,617,680	29,728,214
Mapped	15,277,115	21,544,299	16,494,316	21,368,881	17,324,296	14,033,557
% mapped	69.0	80.9	71.6	72.6	48.6	47.2
% GC	44	40	43	43	41	41
Quality (%PF)	86.72	86.49	85.66	85.83	90.26	90.59
% of > = Q30 Bases (PF)	90.20	90.84	90.13	90.11	92.65	92.78

PF stands for "passed filter" that indicates values for quality score cut-off.
Q30 is a score equivalent to the probability of incorrect base call 1 in 1000 times.

to Fe-sufficient samples had an increase of 41%. *G. max* and *P. vulgaris* had an increase of 4% and 13%, respectively, but this was not statistically significant (Fig. 2). *M. truncatula* is considered a model crop since, when in Fe-deficiency, it allows the observation of IDC symptoms development and remains healthy when in Fe-sufficiency.

3.2. Visualization of Fe reductase activity

To localize Fe reductase activity in the roots, an agarose assay with BPDS was performed. After 45 min in the dark, the reddish colored Fe(II)-$BPDS_3$ product was detected along Fe supplied and Fe deficient roots (Fig. 3). The color intensity was higher in the plants grown in the presence of Fe, but the plants grown in Fe deficient conditions, the lateral roots showed higher reductase activity in contrast to the main roots. Once again, this indicates how important the development of these secondary root structures is to increase Fe uptake and how their growth is related to IDC symptoms (Rodríguez-Celma, Vásquez-Reina, et al., 2011).

3.3. Root Fe reductase activity

As shown in Fig. 4, the reductase activity in all species, when grown in the presence of Fe is always higher than when grown in the absence of this element; however this was only statistically true for *G. max*. Cohen, Norvell, and Kochian (1997) argue that the induction of plasma membrane ferric reductase is a response specific to Fe-deficiency, since this condition plays an exclusive role in eliciting elevated activity of this enzyme in intact root systems of various legume species. But in the present work, as in Vasconcelos et al. (2006) experiments, plants grown in the absence of Fe, exhibited poor Fe reduction rates.

G. max had the lowest levels of reduction, in opposition to *P. vulgaris*, which had 93% and 50% higher rates of reduced Fe at 0 μM Fe(III)-EDDHA and 10 μM Fe(III)-EDDHA, respectively.

3.4. Sequence analysis

A total of 114,723 annotated genes were obtained for all samples (Table 2). More specifically, we obtained 23,052,072 and 29,447,921 quality reads expressed by *G. max*; 22,146,154 and 26,617,669 quality reads expressed by *P. vulgaris*; and, finally, 35,617,680 and 29,728,214 quality reads expressed by *M. truncatula*, grown in Fe-sufficient and in Fe-deficient conditions, respectively.

All samples had an average of 40% of GC content and, considering the default Illumina criteria, *G. max* sequences had 85.7% of quality, *P. vulgaris* 86.6% and *M. truncatula* 90.4%. These values represent the high quality of the samples and allow a better and more accurate analysis of gene expression levels.

3.5. Functional annotation and classification

For validation and annotation of assembled genes, a sequence similarity search was conducted against nr database using BLASTX algorithm with an *E*-value threshold of 10^{-3}.

The data from the six samples in study were compiled and the amino acid sequences were grouped into different functional sub-categories within the Cellular Component, Molecular Function and Biological Process GO organizing principles.

Within the Biological Process category, "cellular process" and "metabolic process" were prominently represented (Fig. 5). In Cellular Component the majority of the sequences corresponded to "cell", "organelle" and "membrane" terms (Fig. 5). Furthermore, the matches for Molecular Function were most prevalent within "binding", "catalytic activity" and "transporter activity" (Fig. 5).

3.6. Gene expression in response to Fe stress

Abiotic stress such as nutrient deficiency in the soil is one of the primary causes of crop losses worldwide. Also, the amount of nutrients which are accumulated by plant foods will be influenced by their availability in the soil. Therefore, unravelling the molecular response underlying stress resistance of economically important plants has profound implications. Fe is a crucial participant in biological redox processes like photosynthesis and respiration (Rodríguez-Celma, Lattanzio, et al., 2011) and, when lacking this nutrient, it is expected that plants suffer from its absence. To cope with this problem, as referred in Introduction, legumes and other plants have developed a strategy that allows them to increase their Fe uptake capacity.

The transcriptomic sequences of *G. max* and *P. vulgaris* were compared to the sequences of the model plant *M. truncatula* and between each other in order to find the common features displayed by these species to antagonize Fe deficiency. Of the total sequences, only those with an abundance higher than 1000 (both in Fe-deficiency and Fe-sufficiency) were analyzed. Four sequences were commonly up-regulated in Fe-deficiency (when compared with the control treatment) to all three species and about 10% of the sequences were common between *M. truncatula* and *G. max*, between *G.max* and *P. vulgaris* and between *M. truncatula* and *P. vulgaris*.

Fig. 6A and Table 3 show the four common gene families that were up-regulated by the three legume species. It can be seen that the four genes commonly up-regulated by the three species were a protein kinase (GO:0030295), a heavy metal ion binding (GO:0046872), a transferase (GO:0080089) and a zinc ion binding (GO:0008270). Fig. 6B shows the five common gene families that were down-regulated by the three legume species. These were an oxidoreductase (GO:0016629), a nucleoside-triphosphatase (GO:0017111), a copper ion binding (GO:0005507), a thioredoxin (GO:0009055), and a carboxylic ester hydrolase (GO:0052689).

In order to control mineral homeostasis, plants have evolved a complex network of events directed by numerous genes. This

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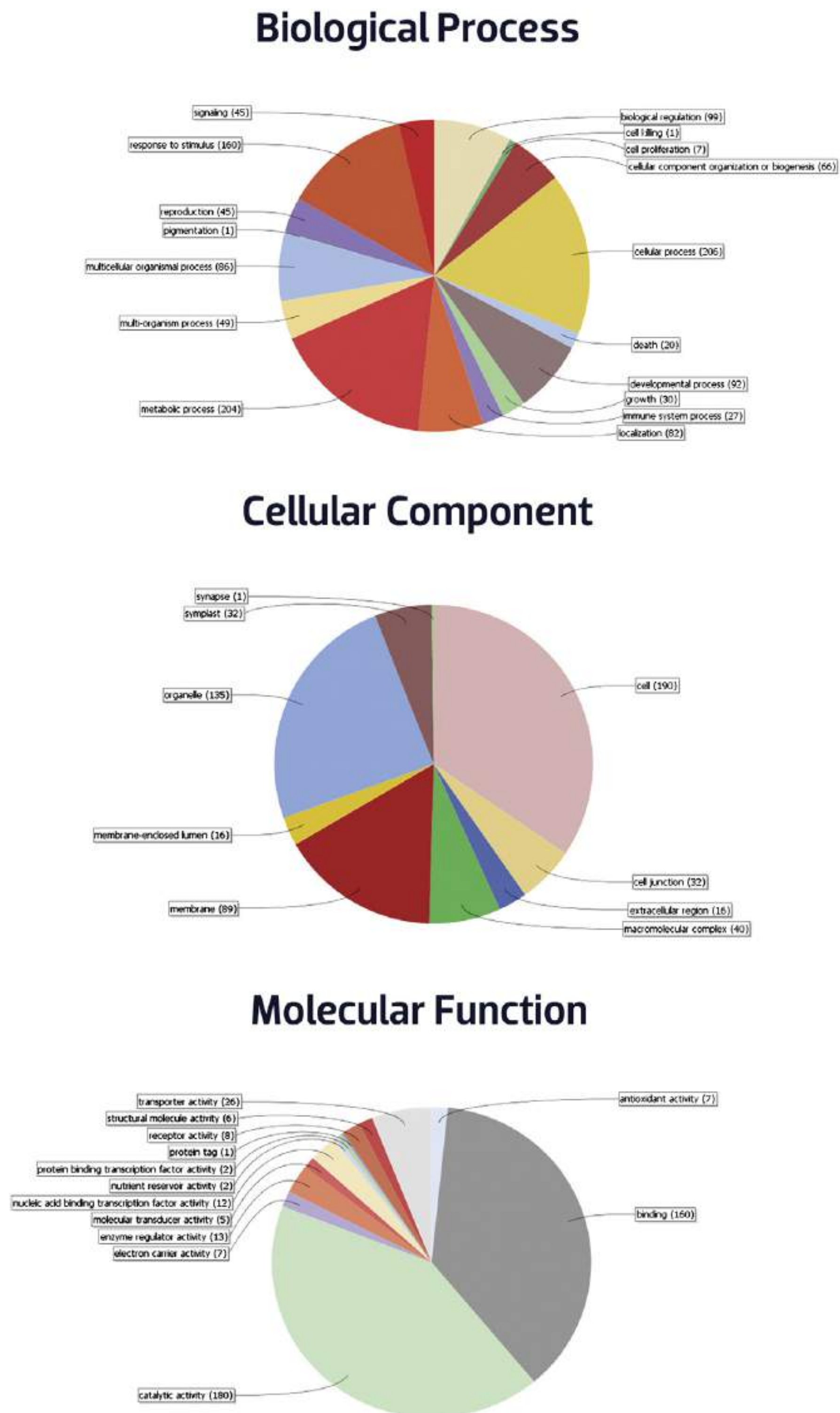

Fig. 5. Gene ontology classification of the annotated amino acid sequences accordingly to three main categories: biological process, cellular component and molecular function.

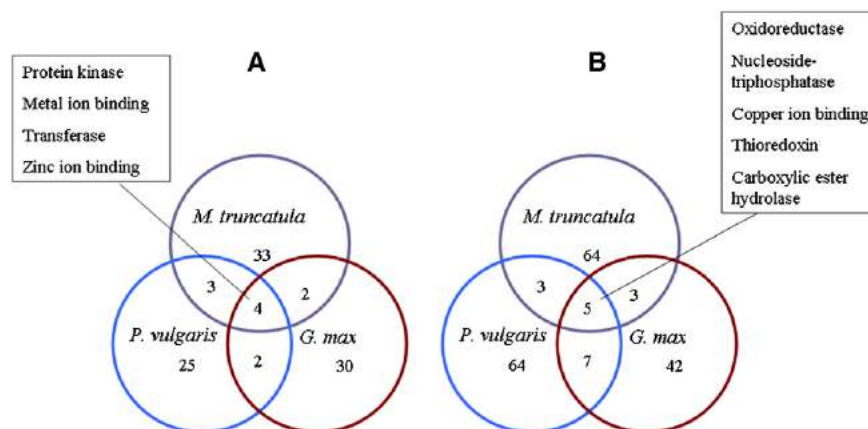

Fig. 6. A Venn diagram showing the comparisons of the (A) up-regulated and (B) down-regulated sequences that are in common between *Glycine max*, *Phaseolus vulgaris* and *Medicago truncatula* grown at 0 μM Fe(III)-EDDHA. All sequences with abundance higher than 1000 were compared and sequences with the same identification in databases were considered common.

network includes metal ligands with different substrate specificities, transferases and regulatory proteins such as protein kinases (Ghandilyan, Vreugdenhil, & Aarts, 2006), all commonly expressed by the legumes under our study.

Metal ion binding proteins such as the rice transcription factor IDEF1 have shown to directly bind to iron and other divalent metals for sensing cellular iron status (Kobayashi et al., 2012). Studies in *Dunaliella salina* have also shown that iron deficiency induces a large enhancement of iron binding capacity (Paz, Shimoni, Weiss, & Pick, 2007). The authors suggest that the major parameter that is modulated by iron deficiency is iron-binding capacity, and they propose that excessive iron binding in iron-deficient cells serves as a temporary reservoir for iron that is subsequently internalized. The metal ion binding gene family up-regulated in our study could have a parallel function in the legumes and could be a key gene in iron deficiency responses.

Transferases are enzymes which catalyze the transfer of functional groups from donor to receptor molecules. As post-transcriptional regulation, including phosphorylation and methylation, have been hypothesized as key events in modulating Fe deficiency responses (Lan, Li, Wen, & Schmidt, 2012), the common up-regulation of this gene family by the three legumes under study further cements this hypothesis.

Table 3
Gene ontology identity of up-regulated genes to *G. max*, *P. vulgaris* and *M. truncatula* (Gm x Pv x Mt), to *P. vulgaris* and *G. max* (Pv x Gm), to *P. vulgaris* and *M. truncatula* (Pv x Mt) and to *G. max* and *M. truncatula* (Gm x Mt) and of only *P. vulgaris*, only *G. max* and only *M. truncatula* presented in Fig. 6.

	Common genes		*G. max*	*P. vulgaris*	*M. truncatula*
Gene ontology ID		GO:0046872	GO:0016567	GO:0030414	GO:0016791
		GO:0080089	GO:0009505	GO:0046872	GO:0043295
	Gm x Pv x Mt	GO:0030295	GO:0019953	GO:0015103	GO:0004372
		GO:0008270	GO:0009001	GO:0004221	GO:0003674
		GO:0017017	GO:0009269	GO:0047889	GO:0007128
	Pv x Gm	GO:0003723	GO:0004872	GO:0004579	GO:0015238
		GO:0008889	GO:0009651	GO:0008536	GO:0009409
	Pv x Mt	GO:0055085	GO:0004657	GO:0016161	GO:0018580
		GO:0003676	GO:0006559	GO:0047513	GO:0004674
		GO:0071805	GO:0019825	GO:0000398	GO:0008272
	Gm x Mt	GO:0016773	GO:0009703	GO:0005524	GO:0030976
			GO:0030410	GO:0045548	GO:0008756
			GO:0004497	GO:0004462	GO:0016740
			GO:0008237	GO:0042389	GO:0047134
			GO:0017153	GO:0030244	GO:0005765
			GO:0004332	GO:0042349	GO:0016301
			GO:0016165	GO:0031386	GO:0080025
			GO:0009737	GO:0005787	GO:0005509
			GO:0004091	GO:0042493	GO:0048443
			GO:0010279	GO:0009055	GO:0009867
			GO:0005215	GO:0004866	GO:0006352
			GO:0045298	GO:0010181	GO:0004298
			GO:0006417	GO:0003756	GO:0004553
			GO:0003677	GO:0017111	GO:0004713
			GO:0000166	GO:0006508	GO:0004333
			GO:0009611		GO:0006486
			GO:0034969		GO:0015693
			GO:0046872		GO:0004656
			GO:0008289		GO:0004806
			GO:0031225		GO:0045735
					GO:0003993
					GO:0008447

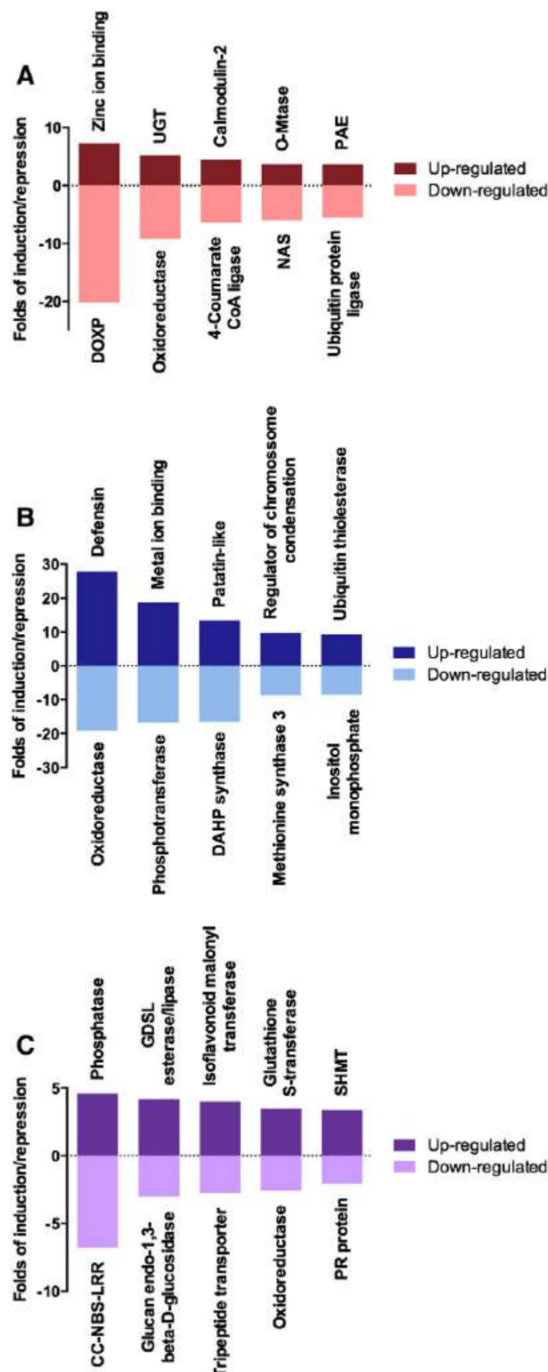

Fig. 7. The five most up and down-regulated genes by (A) *G. max*, (B) *P. vulgaris* and (C) *M. truncatula*, grown in Fe-deficiency (0 μM Fe(III)-EDDHA) compared to Fe sufficiency (10 μM Fe(III)-EDDHA) hydroponic conditions.

Zinc ion binding proteins, which were also found in our study to be commonly up-regulated by the three legumes, have been previously linked to Fe deficiency responses. It is the case, for example of members of the ZIP family of transporters, which are known to bind and transport Fe and Zn (Eng, Guerinot, Eide, & Saier, 1998). It is also the gene family most highly expressed by *G. max* under iron deficiency (Fig. 7).

Finally, the last gene which was commonly up-regulated by the three legumes was a protein kinase. Similar to transferases, protein kinases are involved in post-translational modifications, and, as suggested before, phosphorylation processes can be very important in modulating Fe deficiency responses. Our data seem to indicate that in fact post-translational modifications are key processes in regulating IDC mechanisms.

Fig. 7 represents the five most up- and down-regulated genes in *G. max* (Fig. 7A), *P. vulgaris* (Fig. 7B) and *M. truncatula* (Fig. 7C), when in Fe shortage. With regards to species specific regulation, the most highly expressed gene in *M. truncatula* plants grown under iron deficiency was a phosphatase (GO:0016298). It has been shown before that phosphorylation patterns of several enzymes are altered by Fe starvation in the model plant *Arabidopsis thaliana* (Lan et al., 2012), indicating that this event may also be especially important in *M. truncatula*.

In *P. vulgaris*, a defensin was the gene with highest expression levels under Fe shortage (Fig. 7). Defensins are small cysteine-rich proteins that have a known role in biotic stress defence (De Coninck et al., 2010) and cell-to-cell communication (Takeuchi & Higashiyama, 2012). This species-specific gene may have an important role in Fe deficiency responses in *P. vulgaris*.

In calcareous soil, Fe is abundant in its ferric form, which is not soluble. In order to solubilize Fe, plants had to develop a strategy to transform this micronutrient to its ferrous form (Prasad, 2003). Strategy I plants acidify the soil by proton release to enhance Fe uptake (Grotz & Guerinot, 2006). ATPases are responsible for proton extrusion to the rhizosphere and these proton pumps were detected in all samples. Also, ATPases intervene in redox reactions as several other oxidative stress related genes that were found in all samples, like previously referred. Oxidoreductases (GO:0016629) were highly down-regulated by all species (Figs. 6B, 7, Table S2) and, like in other studies with tomato, redox regulation proteins have particular importance in Fe-deficiency stress adaptation (Brumbarova, Matros, Mock, & Bauer, 2008). Ferric reductases are NADPH-dependent and NAD-related genes (GO:0035798; GO:0009703; GO: 0043295; GO:0004022; GO:0050661) expression was found in all samples too (Tables S1 and S2).

Isoflavonoid pathway appeared to have a significant role in IDC stress response. UDP-glucuronosyltransferase (UGT, GO:0016157) was found to be highly up-regulated by Fe-deficient *G. max* plants (Fig. 7A, Table S1) and enzymes belonging to UGT family seem to be key in the production of isoflavones that participate in stress response induction (Noguchi et al., 2007). With a similar function to UGT, an isoflavonoid malonyl transferase (GO:0016740) was up-regulated by *M. truncatula* (Fig. 7C, Table S1). On the other hand, *M. truncatula* also repressed one gene encoding a β-glucosidase (GO:0042973), an enzyme of the isoflavonoid pathway (Fig. 7C, Table S2). As confirmed by qPCR, UGT was up-regulated by *G. max* and, when in Fe-sufficiency, *M. truncatula* the abundance the enzyme β-glucosidase appears to augment (Fig. 8). Using qPCR we also confirmed the common expression of metal ion binding gene (Fig. 8) and all species presented a higher fold of expression when in Fe-sufficiency, which is coherent since the more Fe quantity is available, more metal ion binding genes will be needed. The glucan 1,3-β-glucosidase gene was also confirmed by qPCR and its transcript levels matched the results obtained by RNAseq. It was undetected in *G. max* and *P. vulgaris* and was repressed in iron deficiency conditions. Lastly, the gene phosphotransferase (GO:0016773) was also monitored by qPCR and results indicate an induction of this gene in *G. max* under iron deficiency. The expression of this phosphotransferase was down-regulated by Fe-deficient *P. vulgaris* sample (Fig. 7B, Table S2) and this was also observed in qPCR results (Fig. 8).

Several genes related to lipid, RNA and DNA binding were detected, which may show that the stress induced by the lack of Fe could result in the modification of such molecules. Interestingly, among the five most

up-regulated genes in *P. vulgaris* (Fig. 7B, Table S1) an ubiquitin thiolesterase encoding gene (GO:0004221) was detected. Although the exact function of this gene is not fully understood, it appears to be important in removing and recycling ubiquitin molecules from degraded proteins and linking together ubiquitin molecules for use in tagging proteins for disposal (Garbarino, Oosumi, & Belknap, 1995). The direct link between these functions and Fe deficiency response can be explored.

4. Conclusion

IDC is a complex phenomenon in which several factors are involved. In general, plants behaved differently in terms of chlorophyll levels, IDC symptoms and reduction of Fe when grown in the presence or absence of Fe, suggesting that legume grains do not all respond to Fe stress equally.

G. max showed acute IDC symptoms and Fe reduction rate had a higher increase than the other species when the samples were supplemented with 10 μM Fe solution in comparison to the Fe deficient samples.

SPAD measurements confirmed that our growth conditions induced iron sufficiency and deficiency, since when in presence of the essential micronutrient the chlorophyll concentration was enhanced and chlorosis was alleviated.

In general, all samples up-regulated several Fe-metabolism related genes and, more interestingly, most of them were related to Fe deficiency control. This work has allowed us to identify key genes necessary in the response to Fe deficiency in the studied legumes: we found genes with functions related to metal ion binding, to protein kinase, to transferase activity and to zinc ion binding activity. These four gene families must have a critical role in Fe-deficiency responses as they were commonly up-regulated by the three legumes. Metal ion binding genes that interact selectively and non-covalently with any metal ion, therefore are likely candidates for this role. The same is true for the zinc ion binding proteins, as Zn and Fe share common transporters and regulatory mechanisms. The role of protein kinases and transferases should be explored, as these genes are not commonly related with IDC, but they indicate an important role of posttranslational modifications of proteins involved in the Fe deficiency response. Also, novel sequences were identified in our studies but not commonly up-regulated by the three legumes, such as lipid, RNA and DNA binding genes — denoting modifications at the molecular level — and several isoflavonoid pathway-related genes were identified, which could indicate that this is an important pathway in antagonizing IDC. In what concerns species-specific responses to Fe-deficiency, the most highly expressed genes for each species were a zinc ion binding gene in *G. max*, a defensin in *P. vulgaris*, and a phosphatase in *M. truncatula*. It is also noteworthy that a member of NRAMP family, directly linked to Fe metabolism, was only detected in *P. vulgaris* samples and an ubiquitin thiolesterase was also very up-regulated; and that UGT was found in the list of the most up-regulated genes of *G. max*, but not in the other species.

Since Fe deficiency is the leading human nutritional disorder in the world today, there is great interest in enhancing the knowledge on Fe metabolism, not only to combat IDC crop devastation and consequent economic damage, but also to increase Fe content in the edible parts of legume plants in order to improve human nutrition and health (Sperotto, Ricachenevsky, Waldow, & Fett, 2012).

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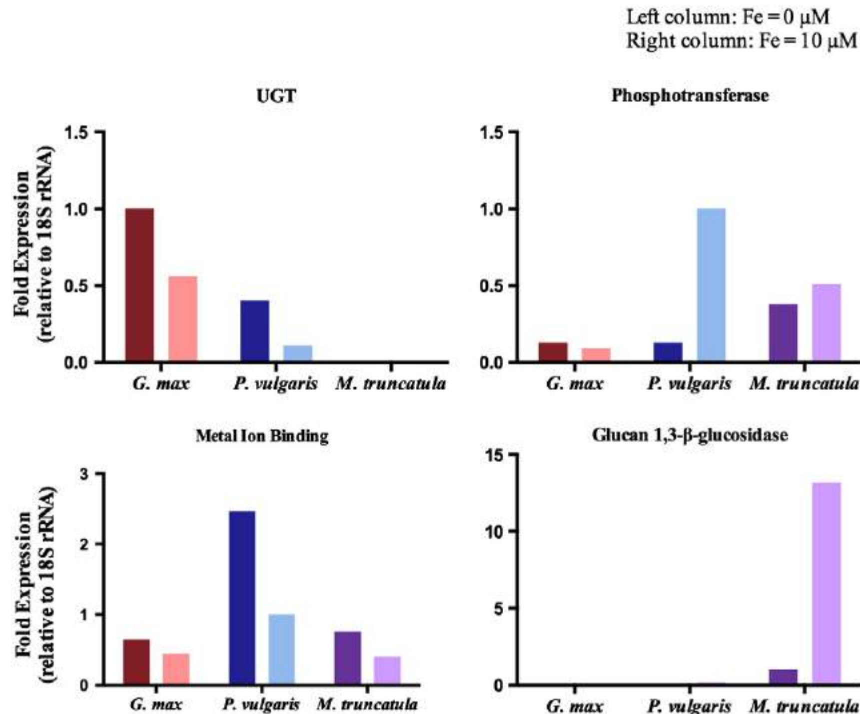

Fig. 8. Quantitative RT-PCR analysis of UGT, phosphotransferase, glucan 1,3-β-glucosidase and metal ion binding gene expression. Abundance of transcripts was normalized using the housekeeping gene 18S-rRNA. Milli-Q water was used as control and no amplification was obtained, therefore it is not represented in the figure.

M. truncatula seeds, respectively, and FCT for funding the project "IMPROVIRON: Improved Productivity and Iron Nutrition in Legume Grains" (PTDC/AGR-GPL/102861/2008). C.S.S. would like to thank FCT for the PhD grant SFRH/BD/78353/2011.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.06.024.

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CHAPTER 2

section 2.2

Title: Comparative analysis of iron deficiency chlorosis responses in soybean (*Glycine max*) and barrel medic (*Medicago truncatula*)

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Comparative analysis of Iron Deficiency Chlorosis responses in soybean (*Glycine max*) and barrel medic (*Medicago truncatula*)

Análise comparativa das respostas à Clorose por Insuficiência de Ferro em soja (*Glycine max*) e luzerna-cortada (*Medicago truncatula*)

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A B S T R A C T

Legume grains have an important socio-economical role, being highly utilized in human and animal nutrition. Although iron (Fe) is abundant in the earth's crust, its limited solubility makes it poorly bioavailable for plants, contributing to iron deficiency chlorosis (IDC). In this work the physiological and molecular mechanisms associated with IDC were studied, namely, the mechanisms involved on Fe deficiency response, as well as a new Fe metabolism related gene in two important legume crops, *Glycine max* and *Medicago truncatula*. Fe deficient plants developed: decreased root and shoot length, increased number of secondary roots and lower chlorophyll levels. Fe shoot content decreased six- and 11-fold for *G. max* and *M truncatula* in Fe-deficiency. Whilst in *G. max* roots no significant differences were detected, in *M. truncatula* roots Fe decreased nine-fold in Fe-deficiency. Genes involved in Fe uptake (*FRO2*-like and *IRT1*-like), were over-expressed in roots of Fe-sufficient *G. max* and in Fe-deficient *M. truncatula*. *VIT1*-like, *YSL1*-like and *ferritin* presented higher expression levels in Fe-sufficient shoots and roots, whereas *NRAMP3*-like and *GCN2*-like showed higher expression values in Fe-deficiency.

Key Words: Ferric reductase, *Glycine max*, *Medicago truncatula*, morphological analysis, RT-PCR.

R E S U M O

As leguminosas têm um importante papel socio-económico, pela sua utilização na nutrição humana e animal. Apesar do ferro (Fe) ser um elemento abundante na crosta terrestre, a sua solubilidade limitada diminui a disponibilidade para as plantas, contribuindo para o desenvolvimento da Clorose por Insuficiência de Ferro (CIF). No presente trabalho, mecanismos fisiológicos e moleculares associados à CIF foram estudados, nomeadamente, os mecanismos de resposta à insuficiência de Fe e um novo gene associado ao metabolismo do Fe, em duas espécies cultivadas com relevância económica, *Glycine max* e *Medicago truncatula*. Plantas deficientes em Fe apresentaram: tamanho diminuído, maior número de raízes secundárias e baixos níveis de clorofila. Em insuficiência de Fe, o conteúdo de Fe na parte aérea diminuiu seis e onze vezes para *G. max* e *M. truncatula*, respetivamente; nas raízes de *G. max* não houve diferenças significativas e nas de *M. truncatula* o conteúdo de Fe diminuiu nove vezes. Genes envolvidos na absorção de Fe (*FRO2-like* e *IRT1-like*) foram sobre-expressos nas raízes de *G. max* em suficiência de Fe e, nas raízes de *M. truncatula*, quando em insuficiência. *VIT1-like*, *YSL1-like* e *ferritina* apresentaram níveis de expressão mais elevados em suficiência de Fe, ao contrário dos genes *NRAMP3-like* e *GCN2-like*, cuja expressão foi aumentada em insuficiência de Fe.

Palavras-chave: Análise morfológica, luzerna-cortada, reductase férrica, RT-PCR, soja.

INTRODUCTION

Legumes represent one of the most important foods, for both humans and animals (Vasconcelos and Grusak, 2006), providing an important source of protein and oil (Libault *et al*., 2010). One of the world's top commodity production is soybean (*Glycine max* L.). In fact, much of the world's protein and oil comes from soybean and this legume contains more protein (40%) and oil (20%) than any other ordinary food source, including meat, cheese and fish (Krishnan, 2005; Bolon *et al*., 2010). The appropriate addition of soy to different products, results in lower calorie alternative food products, with high content of protein, dietary fiber and minerals, preserving the physical and sensory characteristics of the product (Dhingra and Jood, 2001). The genome of soybean was sequenced, assembled and published (Schmutz *et al*., 2010), making it a good model crop to study genetic and molecular mechanisms. Barrel medic (*Medicago truncatula*) has been chosen as a model species for molecular studies in view of its growth and genomic characteristics (Trieu *et al*., 2000). To be convenient as a model for legume genomics, it is also essential that *M. truncatula* exhibit genome conservation with other crop legumes. Detailed comparisons between *M. truncatula* and *M. sativa* – a high feeding value crop used in animal nutrition – have reported that marker relationships were uniformly syntonic and that genes from *M. truncatula* share very high sequence identity to their counterparts from *M. sativa*, so it serves as an excellent model organism for soybean and other economically important legumes (Bell *et al*., 2001; Choi *et al*., 2004).

Besides protein and oil, legumes are also an important source of micronutrients, such as iron (Fe) (Vasconcelos and Grusak, 2006). This mineral is involved in the production of chlorophyll, and is also a component of many enzymes associated with the antioxidant system, energy transfer and nitrogen reduction and fixation. Legumes are very susceptible to Fe deficiency, when grown in adverse conditions, like calcareous soils, due to the low solubility of the oxidized form of Fe (Fe^{3+}) at near neutral and alkaline soil pH (Waters *et al*., 2002; Andaluz *et al*., 2009). Insufficient Fe uptake leads to Fe-deficiency chlorosis (IDC) symptoms, such as yellowing of the younger leaves, interveinal chlorosis and stunted growth, as well as reduction of crop yields (Prasad, 2003; Kim and Guerinot, 2007). IDC lowers the concentrations of Fe in the seeds and other harvested tissues (Grusak, 1999), affecting both farmer profit and the nutritional value of plant products (Vasconcelos and Grusak, 2013).

In order to uptake Fe from the soil, dicotyledonous plants such as soybean and barrel medic, utilize Strategy I, where Fe^{3+} is reduced to Fe^{2+} through the action of a membrane-bound Fe^{3+}-chelate reductase, like the ferric reduction oxidase (FRO). Fe^{2+} is then transported into the plant by specific membrane transporters (Grotz and Guerinot, 2006), such as the Iron-Regulated Transporter 1 (IRT1) (Waters *et al*., 2002). A broad spectrum of transporters have been characterized, such as the Natural Resistance Associated Macrophage (NRAMP) proteins, involved in Fe import into the cytoplasm, the Vacuolar Iron Transporter (VIT), involved in the uptake of Fe^{2+} into the vacuole for storage (Brear *et al*., 2013), and the Yellow Stripe 1-Like (YSL), involved in the transport of Fe^{2+}-NA complexes (Kim *et al*., 2006). Free Fe is toxic since it facilitates the generation of highly reactive oxygen species (ROS). ROS can damage cellular constituents and, therefore, Fe homeostasis needs to be strictly controlled to avoid iron deficiency and toxicity (Liao *et al*., 2012). Therefore, storage proteins, such as Ferritin, play an important role in iron homeostasis, since they assure that ferric Fe is bio-available in case of cellular needs but yet nonreactive with oxygen (Briat *et al*., 2010).

Even though much has been learned about the physiology of Fe uptake in *Arabidopsis*, there is still a limited understanding of the physiology of tolerance to Fe deficiency in soybean and barrel medic, and this has hampered breeding programs (Vasconcelos and Grusak, 2013). There have been few works focusing in the comparative study between these two species (Yan *et al*., 2004), however more information is needed to understand the mechanisms at a molecular level, such as which genes have been selectively conserved or lost between both species. Since increasing the Fe uptake in the roots can augment Fe concentrations in the leaves, it is possible that some of this additional Fe may be remobilized to the grains, which would help in biofortification efforts that aim at enhancing Fe seed levels (Santos *et al.*, 2013,

2015). However, the increased Fe translocation from shoots to seeds still remains one of the major bottlenecks in most biofortification programs (White and Broadley, 2005), and the answer to this may be in the identification of new candidate genes. GCN2 is a protein kinase present in several organisms such as mammals and yeasts (Lageix *et al.*, 2008) and is activated in plants by amino acid deprivation conditions (Zhang *et al.*, 2008), as well other stress stimuli, such as purine deprivation, UV light, cold shock and wounding (Lageix *et al.*, 2008). To this date, there are no published studies on the role of *GCN2* on Fe uptake in plants growing in Fe deficiency, which makes the study of this gene an important innovation in Fe nutrition in plants. However, its regulation is still not well known (Liu *et al.*, 2015), which makes it relevant to study this gene, in order to understand how its expression if affected by Fe deficiency and which mechanisms it may be associated with.

The present study describes the common mechanisms underlying the response to Fe deficiency at a physiological and molecular level, in *G. max* and *M. truncatula* grown hydroponically under Fe deficiency and Fe sufficiency. It also describes further analysis on the role of a novel candidate gene, *GCN2*, on Fe metabolism.

MATERIALS AND METHODS

Plant material and growth conditions

Medicago truncatula cultivar "Luzerna revilheira" and *Glycine max* cultivar "Williams 82" were grown in a growth chamber (Aralab Fitoclima 10000EHF) with 16 h day / 8 h night photoperiod. The temperature was kept at 20 ºC during the light period, with 350 µmol s^{-1} m^{-2} of photon flux density, and at 18 ºC during the dark period, with 75 % of relative humidity. Seeds of *M. truncatula* and of *G. max* were germinated for seven days in the dark and then transferred to hydroponic solutions with 20 µM FeEDDHA (Fe+) or with no FeEDDHA (Fe-) supply. The standard solution for hydroponic growth of *M. truncatula* contained as macronutrients: 3 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4.7H_2O$, 0.5 mM $NH_4H_2PO_4$, 0.75 mM K_2SO_4, 25 µM $CaCl_2$; and as micronutrients: 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4.H_2O$, 0.5 µM $CuSO_4.H_2O$, 0.5 µM MoO_3, 0.5 µM $NiSO_4$. The conditions used for *G. max* included as macronutrients: 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 $CaCl_2$; and as micronutrients: 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 $NiSO_4$. Both hydroponic solutions were buffered by the addition of 1mM MES, pH 5.5. The assay ended at the 14th day of hydroponic growth.

Morphological and biochemical evaluations

At the end of the experimental time period, five plants of each species and treatment were harvested and the length and fresh weight of shoots and roots was measured. Also, the number of secondary roots was counted and the chlorophyll concentration was quantified accordingly to Abadía *et al.* (1984).

Fe reduction was measured in the roots of five intact plants via the spectrophotometric measurement of Fe^{2+} chelated to BPDS, as described in Vasconcelos and Grusak (2006). Rates of reduction were determined using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}. Roots and shoots were dried at 70 ºC and 200 mg of each sample was analyzed for the determination of Fe content using the ICP-OES Optima 7000 DV (PerkinElmer, Massachusetss, USA) with radial configuration, according to Roriz *et al.* (2014).

Gene expression analysis

Additional five replicates of each species and treatments were pooled and the RNA from leaves and roots was extracted following manufacturer's instructions, using the Qiagen RNeasy Plant Mini Kit (USA, #74904). cDNA was synthesized using First Strand cDNA Synthesis Kit (Fermentas).

Candidate genes were selected according to their established (*FRO2*-like, *IRT1*-like, *NRAMP3*-like, *VIT1*-like, *YSL1*-like, *ferritin*) or possible (*GCN2*-like) role on Fe metabolism. In order to identify orthologs for these genes, known sequences from *Arabidopsis* were blasted, and the most homologous sequence (E_{value} < 10^{-20}) was selected (Table 1). Quantitative Real-Time PCR (qPCR) reactions were performed on a Chromo4 thermocycler (Bio-Rad). Amplifications were carried out using 1.25 µM of the specific primers and mixed to 12.5 µL of 2xPCR iQ SYBR Green Supermix (Bio-Rad) and 100 ng of cDNA in a final volume of 25 µl. Three technical replicates were performed for each gene tested in qPCR reactions, as well as for controls.

Table 1 - Gene accession numbers and forward and reverse primer sequences used in quantitative Real-Time PCR analysis

Gene	Species	Accession numbers	Primer sequences
18S rRNA	-	X75080.1	F 5'- TTAGGCCATGGAGGTTTGAG -3' R 5'- GAGTTGATGACACGCGCTTA -3'
FRO2-like	*G. max*	XM_003548612.1	F 5'- TGCTTGGACTCACACCAGAG -3' R 5'- AGAGGTAGAAACCGGGGAGA -3'
	M. truncatula	XM_003622457.1	F 5'- CACTTGTGATGGTGAGTGGA -3' R 5'- GATGGTGTGCCAGAAATAGG -3'
IRT1-like	*G. max*	XM_003520096.2	F 5'- GATTGCACCTGTGACACAAA -3' R 5'- CAGCAAAGGCCTTAACCATA -3'
	M. truncatula	XM_003630873.1	F 5'- GACAAAGGAACCGGAACAAA -3' R 5'- TTGATGGAAGCAAAGTGCAG -3'
YSL1-like	*G. max*	XM_003536126.2	F 5'- GCTTTTGGAGCAGGTCTCAC -3' R 5'- AGACCACAACCCACAAGTCC -3'
	M. truncatula	XM_003602267.1	F 5'- GATCTTGGCCCACAACAAGT -3' R 5'- ACTGCAGGAACCATCAAACC -3'
VIT1-like	*G. max*	XM_003525172.2	F 5'- TTGTTAGCTTGGCGTGACAG -3' R 5'- TGCAACCAAGGTAACCACAA -3'
	M. truncatula	XM_003630932.1	F 5'- GGGTGGAATTGTTCCTCTCA -3' R 5'- AGCACTCCTGATTGGCTTGT -3'
ferritin	*G. max*	U31648.1	F 5'- CCCCTTATGCCTCTTTCCTC -3' R 5'- GCTTTTCAGCGTGCTCTCTT -3'
	M. truncatula	XM_00362331.1	F 5'- GTAAGAAATGGGGTGGTGGA -3' R 5'- CGAGCCAAAGAAACTTGAGG -3'
NRAMP3-like	*G. max*	XM_003524624.2	F 5'- TGTTCAGTCAAGGCAGGTTG -3' R 5'- CCAGCATTTACAAGGCCAAT -3'
	M. truncatula	XM_003611600.1	F 5'- TTTGGATCCTGGAAACTTGG -3' R 5'- GCTGAATCAAAAGCCCCATA -3'
GCN2-like	*G. max*	XM_006592086.1	F 5'- ATCCTTGCCTCATCACCAAC -3' R 5'- ATGGGGAACTGTGTTTGAGC -3'
	M. truncatula	XM_003636896.1	F 5'- GTAACCGAGGTCCGAGATGA -3' R 5'- CTCCACCATGGGTCAGAAGT -3'

The amplification of all genes was performed accordingly to Han *et al*. (2013). The comparative CT method (ΔΔCT) (Livak and Schmittgen, 2001) was utilized for the relative quantification of gene expression value of Fe stress related genes using the 18S rRNA gene as the housekeeping gene (Opticon Monitor 3 Software, Bio-Rad).

RESULTS AND DISCUSSION

For several organisms, Fe represents a cofactor in vital metabolic pathways such as the electron transport chain of respiration. Plants have an additional need for Fe because photosynthesis and chlorophyll biosynthesis both require this micronutrient (Jeong and Guerinot, 2009). Thus, how plants maintain Fe homeostasis and the anatomical modifications concerning Fe absence is a biologically relevant question. In the current work, when Fe was absent, both *G. max* and *M. truncatula* behaved similarly, developing characteristic IDC symptoms, such as impaired growth, observed by the reduction in plant weight and length (Table 2). More specifically, *G. max* had 2.2- and 2.1-fold lower fresh weight in shoots and roots, respectively, under Fe deficiency, which was more pronounced than *M. truncatula*, that had a reduction of 1.5- and 1.8-fold (Table 2).

Another important characteristic associated with the absence of Fe is the development of secondary structures. Here, plants submitted to -Fe conditions showed swelling of root tips and increased number of secondary structures, namely, an average of 60 % more for *G. max* and 69 % more for *M. truncatula* (Table 2). The increased number of secondary structures helps the plant in augmenting the absorbable area for Fe uptake, and the scavenging of Fe in the rhizosphere (Schmidt,

Table 2 - Fresh weight (FW) (g), length (cm) and number (#) of secondary roots of G. max and M. truncatula grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means ± SE of five independent replicates. For each parameter analyzed, different letters represent significant differences between samples (p < 0.05)

		G. max		*M. truncatula*	
		Fe+	Fe-	Fe+	Fe-
Shoot	FW	6.44 ± 0.53 a	2.92 ± 0.32 b	0.82 ± 0.09 c	0.55 ± 0.07 d
	Length	30.5 ± 0.70 a	16.92 ± 0.65 b	10.75 ± 0.49 c	7.33 ± 0.50 d
Root	FW	5.38 ± 0.46 a	2.61 ± 0.24 b	1.03 ± 0.11 c	0.57 ± 0.09 d
	Length	49.75 ± 1.16 a	27.08 ± 0.78 b	35.42 ± 1.51 c	28.75 ± 1.84 d
# Secondary Roots		34.2 ± 3.09 a	57.00 ± 5.14 b	17.4 ± 1.62 c	25.2 ± 2.95 d

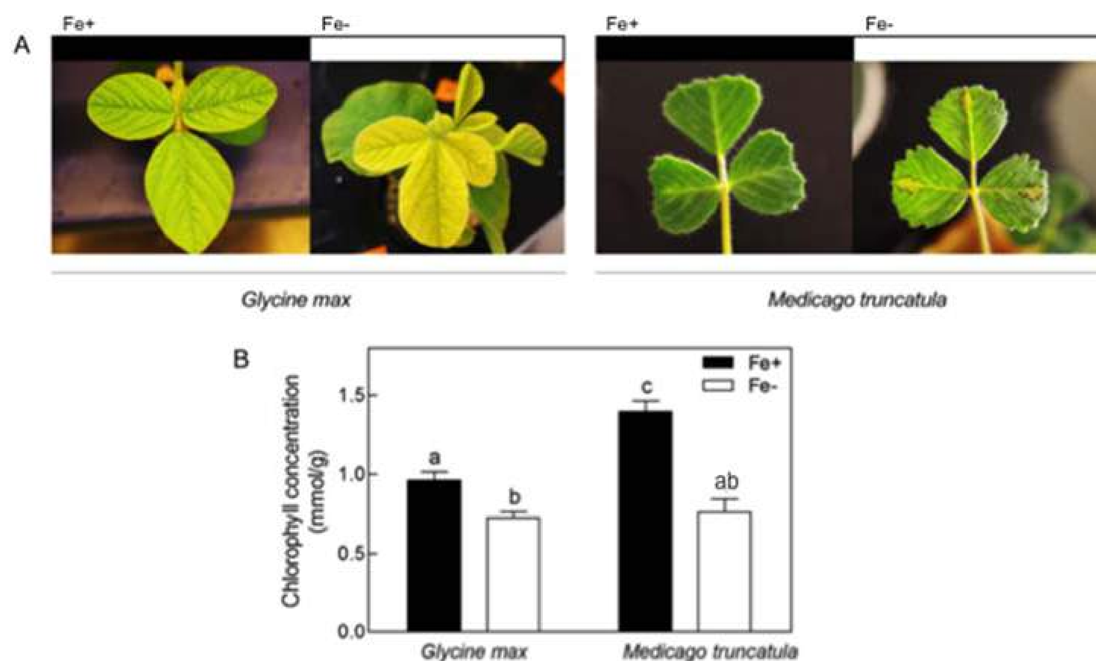

Figure 1 - Visible chlorosis symptoms (A) and chlorophyll concentration (B) of G. max and M. truncatula plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means +SE of five independent replicates. Different letters represent significant differences between samples (P < 0.05).

1999). Since the surface of root hairs can represent up to 70% of the total root surface area (López-Bucio *et al*., 2003), the relevance of root hairs in nutrient uptake is crucial.

At the shoot level, the absence of Fe is known to inhibit chloroplast biogenesis and chlorophyll biosynthesis, leading to the development of chlorosis, especially in younger leaves (Henriques *et al*., 2002). Also, Fe starved plants may be more prone to oxidative damage (Kumar *et al*., 2010), leading to the accumulation of ROS, to oxidative stress, and to lower chlorophyll levels and increased chlorosis symptoms (as seen in Figure 1).

In this work, chlorosis symptoms appear to be more severe in *G. max* plants when compared to *M. truncatula* plants (Figure 1A), but the absolute values of chlorophyll concentration in Figure 1B seem to be contradictory. However, this is due to the fact that *G. max* plants, even under Fe sufficiency, weren't as green as *M. truncatula* plants under the

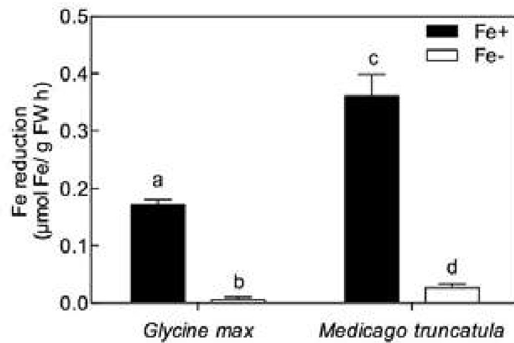

Figure 2 - Root Fe reductase activity of G. max and M. truncatula plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means +SE of five independent replicates. Different letters represent significant differences between samples (P < 0.05).

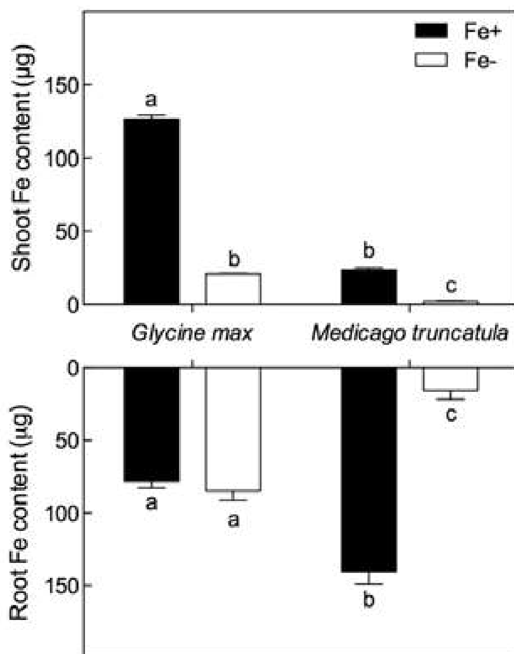

Figure 3 - Fe content of shoots and roots of G. max and M. truncatula grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. Data are means +SE of five independent replicates. Different letters represent significant differences between samples (P < 0.05).

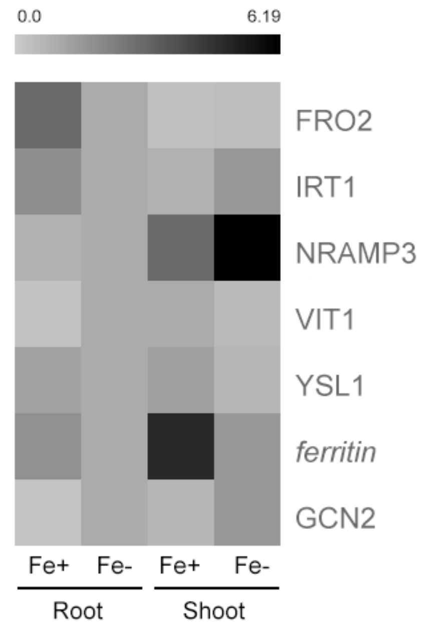

Figure 4 - HeatMap of the expression patterns of FRO2-, IRT1-, NRAMP3-, VIT1- and YSL1-like genes and ferritin and GCN2-like genes in root and shoot tissues of G. max plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. "Fe- Root" was the reference sample; expression was normalized with 18S rRNA housekeeping gene. In black: increased gene expression; in light grey: lower gene expression. Total RNA was extracted from a pool of five independent replicates. Corresponding values are presented in Table 3.

Table 3 - Fe deficiency-related genes relative expression values of G. max plants grown under Fe-sufficient (Fe+) and Fe-deficient (Fe-) hydroponic conditions. Total RNA was extracted from a pool of five independent replicates

	Root		Shoot	
	Fe+	Fe-	Fe+	Fe-
FRO2-like	2.98	1	0.37	0.41
IRT1-like	1.87	1	0.81	1.60
NRAMP3-like	0.84	1	2.98	6.19
VIT1-like	0.28	1	0.98	0.55
YSL1-like	1.28	1	1.32	0.68
ferritin	1.81	1	4.95	1.62
GCN2-like	0.23	1	0.64	1.57

same treatment, leading to an acuter decrease in chlorophyll concentration.

Root Fe uptake capacity is linked with the solubilisation of Fe in the rhizosphere by the plant's root Fe reductase activity, which is necessary to convert the less soluble Fe^{3+} to the more soluble Fe^{2+} (García *et al*., 2013). Here, for both species, the enzyme was more active in Fe+ conditions and was higher in *M. truncatula* plants (Figure 2). It has been hypothesized that, for some genotypes, Fe is necessary for the functioning of the reductase enzyme itself (Blair *et al*., 2010). Although most studies imply that Fe reduction is induced under Fe deficiency (Wang *et al*., 2013; Zha *et al*., 2014), it has already been described that this is not always this way (Vasconcelos and Grusak., 2006; Santos *et al*., 2015).

In order to understand how Fe deficiency affects the mineral composition of Fe in *G. max* and *M. truncatula*, root and shoot tissues were analyzed by ICP-OES. When *G. max* was faced with the lack of Fe, it appeared to accumulate its internal Fe storage in the roots and the shoot Fe content decreased six-fold (Figure 3). It has been seen before that in response to shortage in mineral nutrition plants usually allocate more resources to the roots (Hermans *et al*., 2006; Santos *et al*., 2015). On the other hand, *M. truncatula* plants had a general reduction in Fe content in both tissues under Fe deficiency.

To further understand the mechanisms triggered by Fe shortage, it is crucial to comprehend the key conserved molecular players involved in nutrient uptake (e.g. *FRO2* and *IRT1*), transport (e.g. *NRAMP3*, *VIT1* and *YSL1*) and storage (e.g. *ferritin*), as well as identify novel candidate genes, that could have important roles in Fe metabolism (*GCN2*). When plants are faced with stress situations, the rate of nutrient uptake needs to increase, in order to compensate the lack of Fe. Thus, root Fe uptake related genes *FRO2* and *IRT1* are extremely important since they participate in this critical step concerning the plant response to Fe deficiency, and which control the efficiency of Fe uptake.

The results obtained for *G. max* plants show that in Fe- the expression of *FRO2*-like was decreased by three-fold (Figure 4), accordingly to the Fe reductase activity previously described (Figure 2). On the contrary, *M. truncatula* roots over-expressed *FRO2*-like gene under Fe deficiency (Figure 5), as previously obtained in *A. thaliana* (Robinson *et al*., 1999), tomato (Li *et al*., 2004) and soybean (Santos *et al*., 2016). When Fe was present in sufficient amounts, *M. truncatula* had almost null *FRO2*-like expression (Figure 5); since from the beginning of the trial, plants were in optimal conditions, they captured sufficient Fe to meet their daily requirements, thus inhibiting *FRO2*-like expression in order to avoid Fe toxicity. However, the Fe reductase activity was higher under Fe+ conditions (like in *G. max*). The Fe reduction is thought to be the rate-limiting step for Fe transport since Fe transporters, such as *IRT1*, do not reach saturation at normally achieved concentrations of Fe^{2+} (Grusak *et al*., 1990). If there is no Fe being reduced, *IRT1*-like should consequently present lower activity, which was clearly observed in *G. max* Fe- roots (Figure 4). In both species, the levels of *IRT1*-like expression were very similar to those obtained for *FRO2*-like (Figures 4 and 5), suggesting that *IRT1*-like is co-regulated with this gene, as previously seen in *Arabidopsis thaliana* (Vert, 2002; Kim and Guerinot, 2007).

After Fe is transported into the roots by IRT1, the transport of this nutrient across the plant is another crucial step that needs to be well known to efficiently develop an IDC mitigation or a biofortification strategy. Fe transporter families, such as VIT, NRAMP and YSL, are extremely important in Fe metabolism, as they assure that Fe is efficiently delivered to shoots, and other plant edible parts and storage organs. *NRAMP3* and *VIT1* have contrasting functions: while the first is responsible for the remobilization from the vacuole (Lanquar *et al*., 2005), the second is responsible for the Fe loading in the vacuole (Kim *et al*., 2006). Studies in *A. thaliana* demonstrate that *NRAMP3* is an H^+ metal symporter responsible for Fe and Mn remobilization from the vacuole, a crucial step during early seedling development (Lanquar *et al*., 2010). Accordingly, under Fe deficiency, as plants need more remobilization of Fe to respond to their needs, *NRAMP3*-like was more expressed (Figures 4 and 5) and *VIT1*-like was repressed, because plants activate *VIT1*-like in Fe sufficient conditions to increase Fe^{2+} uptake into the vacuole for storage

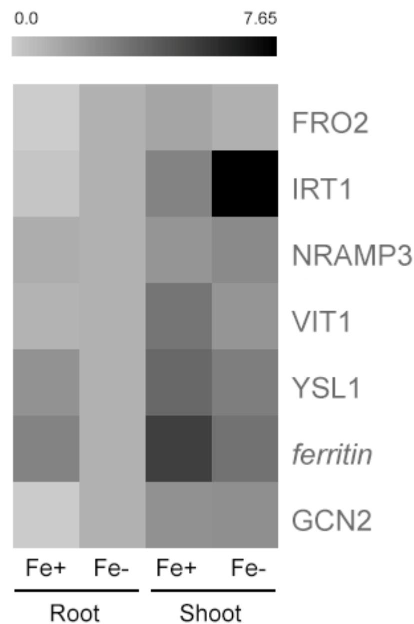

Figure 5 - HeatMap of the expression patterns of FRO2-, IRT1-, NRAMP3-, VIT1- and YSL1-like genes and ferritin and GCN2-like genes in root and shoot tissues of M. truncatula plants grown hydroponically in Fe-sufficient (Fe+) and Fe-deficient (Fe-) conditions. "Fe- Root" was the reference sample; expression was normalized with 18S rRNA housekeeping gene. In light grey: lower gene expression; in black: increased gene expression. Total RNA was extracted from a pool of five independent replicates. Corresponding values are presented in Table 4.

Table 4 - Fe deficiency-related genes relative expression values of M. truncatula plants grown under Fe-sufficient (Fe+) and Fe-deficient (Fe-) hydroponic conditions. Total RNA was extracted from a pool of five independent replicates

	Root		**Shoot**	
	Fe+	**Fe-**	**Fe+**	**Fe-**
FRO2-like	0.01	1	1.45	1.05
IRT1-like	0.25	1	2.75	7.65
NRAMP3-like	1.15	1	2.10	2.46
VIT1-like	0.94	1	3.28	2.10
YSL1-like	2.17	1	3.77	2.91
ferritin	2.73	1	5.25	3.37
GCN2-like	0.05	1	2.19	2.31

purposes (Brear *et al.*, 2013). Studies in *A. thaliana* (Kim and Guerinot, 2007) demonstrated that *AtNRAMP3* and *AtVIT1* mutants present arrested seedling growth when grown on Fe deficient soils. Moreover, Zhang *et al.* (2012) reported that the disruption of the rice *VIT* orthologues (*OsVIT1* and *OsVIT2*) increased Fe and Zn accumulation in rice seeds and decreased Fe and Zn in the leaves.

As well as *NRAMP3*-like and *VIT1*-like, the *YSL1*-like transporter may also play a crucial role in the control of the amount of Fe translocated to the seeds of *G. max* and *M. truncatula*. Both species had similar expression patterns (Figures 4 and 5), where both tissues presented higher levels in Fe+ conditions, suggesting a role in Fe translocation at diverse plant organs, as seen before (Kim *et al.*, 2006). This gene is involved in the transport of the Fe^{2+}-NA complexes (Kim *et al.*, 2006) that are hypothesized as the main transportable Fe form in the phloem (Jean *et al.*, 2005; Waters *et al.*, 2006; Chu *et al.*, 2010). Jean et al. (2005) used *A. thaliana* lines with a knock out mutation in *AtYSL1*, and the levels of NA and Fe in leaves and seeds decreased, as well as germination rates, even when plants were grown in Fe excess, showing that Fe and NA levels in seeds rely in part on *YSL1* function.

Storage proteins such as ferritin play an important role in Fe homeostasis, assuring that Fe in excess is in a bio-available way in case of cellular needs but yet nonreactive with oxygen (Briat *et al.*, 2010). Thus, the higher expression levels of this gene in Fe sufficient soybean and barrel medic plants are understandable (Figures 4 and 5) and are coherent with previous studies (Santos *et al.*, 2016). This protein manages the insolubility and potential toxicity of Fe in the presence of oxygen, being involved in oxidative protection by sequestering free Fe (Lobreaux *et al.*, 1995).

Even though several gene families are known to be involved in the Fe uptake mechanism, transport and storage, there are still many undiscovered genes that may have important roles in these processes. Therefore, it is worthwhile to find candidate genes that could have an important role in Fe metabolism. To this end, a novel gene was studied in the current

work: *GCN2*-like. Both *G. max* and *M. truncatula* plants over-expressed *GCN2*-like under Fe deficient conditions (Figures 4 and 5), particularly at the root level, and it seems to indicate a role for *GCN2*-like in alleviating Fe stress, for both legume species. Lageix *et al*. (2008) showed that *AtGCN2* was strongly activated following wounding and exposure to key hormones, and suggested that this enzyme plays a role in plant defense responses to insect pathogens, representing a key player linking biotic and abiotic stresses. Moreover, no studies have looked at the possible role of *GCN2* and Fe nutrition, which highlights the importance of the current work. Further studies to link its role on Fe metabolism are under way.

The current work compared the responses of two legume species, soybean and barrel medic, to Fe deficiency. Taken together, the results described above suggest a conservation of anatomical and biochemical responses in the two legume species. Also, it is apparent that for genes such as *FRO2*-like and *IRT1*-like the regulation differs between these two legumes and is not conserved with other plants such as *A. thaliana*. It shows that generalizations in Fe uptake processes should not be lightly done. Finally, a novel sequence showing up-regulation under Fe deficiency was identified, opening doors to future studies looking at the role of this gene under Fe deficiency.

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CHAPTER 2

section 2.3

Title: Cultivar variability of iron uptake mechanisms in rice (*Oryza sativa* L.)

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Research article

Cultivar variability of iron uptake mechanisms in rice (*Oryza sativa* L.)

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ABSTRACT

Rice (*Oryza sativa* L.) is the most important staple food in the world. It is rich in genetic diversity and can grow in a wide range of environments. Iron (Fe) deficiency is a major abiotic stress in crop production and in aerobic soils, where Fe forms insoluble complexes, and is not readily available for uptake. To cope with Fe deficiency, plants developed mechanisms for Fe uptake, and although rice was described as a Strategy II plant, recent evidence suggests that it is capable of utilizing mechanisms from both Strategies. The main objective of this work was to compare two cultivars, Bico Branco (*japonica*) and Nipponbare (*tropical japonica*), to understand if the regulation of Fe uptake mechanisms could be cultivar (cv.) dependent. Plants of both cultivars were grown under Fe-deficient and -sufficient conditions and physiological and molecular responses to Fe deficiency were evaluated. Bico Branco cv. developed more leaf chlorosis and was more susceptible to Fe deficiency, retaining more nutrients in roots, than Nipponbare cv., which translocated more nutrients to shoots. Nipponbare cv. presented higher levels of Fe reductase activity, which was significantly up-regulated by Fe deficiency, and had higher expression levels of the Strategy I-*OsFRO2* gene in roots, while Bico Branco cv. induced more genes involved in Strategy II.

These new findings show that rice cultivars have different responses to Fe deficiency and that the induction of Strategy I or II may be rice cultivar-dependent, although the utilization of the reduction mechanisms seems to be an ubiquitous advantage.

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1. Introduction

Rice feeds more than half of the world's population, most of whom in developing countries (FAO, 2004) where it is, at least during certain seasons, their sole source of nutrients (Sautter et al., 2007). Rice, as a very diverse crop, can grow in a wide range of environments, from irrigated soils to upland soils, and where other crops would fail. However, when grown in alkaline soils, which cover approximately 30% of world land, Fe uptake is limited because under these conditions, it forms insoluble complexes and is not readily bioavailable for uptake (Jeong and Guerinot, 2009). Plants require Fe for photosynthesis, mitochondrial respiration, nitrogen assimilation, hormone biosynthesis, pathogen defense, among others. Thus, Fe deficiency results in chlorosis, poor growth and reduced yields (Hansch and Mendel, 2009). Among the grass species, rice is one of the crops most susceptible to Fe deficiency, especially during the early stages of plant development (Mori et al., 1991).

To cope with Fe deficiency, plants developed tightly regulated mechanisms to mobilize Fe from the rhizosphere (Puig et al., 2007). These acquisition strategies are based on two distinct mechanisms, namely, Strategy I and II (for recent reviews please see Hindt and Guerinot, 2012; Ivanov et al., 2012; Kobayashi and Nishizawa, 2012).

The Strategy I response is used by all dicotyledonous species such as *Arabidopsis*, and by non-graminaceous monocotyledonous species (Mukherjee et al., 2006). It involves the release of protons into the rhizosphere to acidify the soil and increase ferric iron (Fe^{3+}) solubility (Fox and Guerinot, 1998). Iron is subsequently reduced to ferrous form (Fe^{2+}) by a ferric reductase-oxidase (FRO) (Robinson et al., 1999) and it is moved across the plasma membrane into root cells by IRT, an Fe-regulated transporter member of the large ZIP family (Vert et al., 2002). The Fe^{3+}-chelate reductases genes, *FROs* (Wu et al., 2005; Mukherjee et al., 2006), and the Fe^{2+} transporters, *IRT1* and *IRT2* (Vert et al., 2002), were first isolated and characterized in *Arabidopsis*. The *FRO2* gene is expressed primarily in the outer layers of roots in response to Fe-deficiency (Grusak et al., 1990). IRT1 is the main Fe-regulated transporter that is induced in response to Fe-deficient conditions and is also capable of transporting Zn, Mn, Co and Cd (Vert et al., 2002).

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The Strategy II Fe-uptake system is used by all the monocotyledonous species (grasses, graminaceous), in which phytosiderophores (PS) are released into the rhizosphere by OsTOM1/OsZIFL4 (Nozoye et al., 2011). The complex Fe^{3+}-PS is taken up into root cells by transmembrane proteins of the yellow-stripe like (YSL) family, such as OsYSL15 (Ishimaru et al., 2006; Inoue et al., 2009). PS are synthesized from methionine and belong to the mugineic acid family (MAs) (Nozoye et al., 2011). Nicotianamine (NA) and 2′-deoxymugineic acid (DMA, product resultant from NA conversion) are biosynthesis precursors of PS and chelate with metals, such as Fe, to transport them through the plant (Mori et al., 1991; Inoue et al., 2003).

Other genes play important roles in this mechanism. For example, a basic helix-loop-helix (bHLH) transcription factor, OsIRO2, was demonstrated to be strongly expressed in roots and shoots under Fe-deficiency. It is involved in the regulation of several genes responsible for DMA biosynthesis, including OsNAS1, OsNAS2, OsDMAS1 and OsNAAT1, as well as OsYSL15 (Ogo et al., 2007). OsIRO2 is positively regulated by IDEF1, a transcription factor that also plays a crucial role in regulating other Fe-deficiency-induced genes involved in Fe homeostasis, such as OsTOM1, OsYSL15, OsYSL2, OsIRT1, OsNAS1 and OsNAS2 (Kobayashi et al., 2009). However, although responses to Fe deficiency in graminaceous plants have been described, the mechanisms of gene regulation related to these responses are largely unknown (Ogo et al., 2007).

There has been some controversy about the mechanisms used by rice for Fe uptake from the rhizosphere (Ricachenevsky and Sperotto, 2014). Until recently, Strategy II plants were thought to only use the above-described response to obtain Fe from the soil (Ishimaru et al., 2006). These studies suggested that rice does not have the ability to reduce Fe^{3+}, a limiting-step of Strategy I plants (Grusak et al., 1990). Moreover, rice expressing the *AtFRO2* gene did not have enhanced reductase activity (Vasconcelos et al., 2004). However, the evidences of Fe^{2+} uptake in rice, suggests that it could benefit from an increased activity of the ferric chelate reductase to generate more available Fe when the plants are grown in upland conditions (aerobic soils), where Fe is often less available and insufficient to sustain proper development of the plant (Vasconcelos et al., 2004). However, an ortholog of the major root Fe transporter in *Arabidopsis*, IRT1, was identified in rice, and unlike other grasses, rice seems to have an efficient Fe^{2+} uptake mechanism (Ishimaru et al., 2006; Cheng et al., 2007), supporting the hypothesis that rice has combined features of both strategies.

Most studies on Fe responses in rice have been conducted in Nipponbare and Taipei 309 (Lucca et al., 2002; Nozoye et al., 2011; Kakei et al., 2012; Masuda et al., 2013; Nozoye et al., 2014) and studies have often been conducted in one or another cultivar, and seldom in two cultivars in parallel. Moreover, few studies have looked at the variability in these responses between different rice cultivars. Here, we analyzed the expression of well-described genes involved in Strategy I and II of Fe uptake, in roots and shoots of two different rice cultivars, to understand if the capacity of rice plants to up-regulate Strategy I or II mechanisms for Fe uptake is cultivar-dependent. We also analyzed the effect of Fe deficiency on the accumulation of Fe and other micronutrients in roots and shoots, on photosynthetic pigment accumulation in rice shoots and on the induction of the Fe reductase enzyme in roots (a typical mechanism of Strategy I plants).

2. Materials and methods

2.1. Plant growth

A screening with 21 cultivars of seven different ecotypes (provided by the International Rice Research Institute – IRRI) was performed in order to select the final two cultivars for this study. Two major parameters were considered for cultivar selection: germination rate and seed Fe concentration. As Bico Branco (*tropical japonica*) and Nipponbare (*japonica*) were the cultivars with higher germination rate and higher seed Fe concentration, these were chosen for the following treatments.

Rice (*Oryza sativa* L.) seeds were germinated on filter paper moistened with deionized water, wrapped in silver paper and incubated in a greenhouse at 25 °C in the dark. They were watered with 250 mM $CaCl_2$ every three days.

After three weeks of germination, a total of ten seeds of each variety were transferred to a nutrient solution. The composition of the nutrient solution was 3 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.75 mM K_2SO_4, 0.5 mM $MgSO_4$, 25 mM $CaCl_2$, 25 mM H_3BO_3, 2 mM $MnSO_4$, 2 mM $ZnSO_4$, 0.5 mM $CuSO_4$, 0.5 mM H_2MoO_4, 0.1 mM $NiSO_4$ and 0.1 mM K_2SiO_3. All nutrients were buffered with 1 mM MES, pH 5.5.

Of the ten germinated seedlings, five were transferred to an Fe deficient nutrient solution (no Fe provided) and another five seedlings were transferred to a nutrient solution containing 20 μM Fe(III)-EDDHA (Fe sufficiency) as control, for three additional weeks. The hydroponic experiments were carried out in an environmental growth chamber (Aralab Fitoclima 10000EHF), with relative humidity of 75% and with a photoperiod of 16 h day (with photosynthetic active radiation of 490 μmol m^{-2} s^{-1} and temperature of 24–26 °C) and 8 h night (with temperatures of 19–20 °C). Growth solutions were changed weekly.

2.2. Photosynthetic pigment extraction

Anthocyanin, chlorophyll and carotenoid concentrations were measured in plants grown in Fe deficient ($n = 5$) and Fe sufficient conditions ($n = 5$), as described previously. The referred compounds were extracted and quantified according to a modified protocol of Sims and Gamon (2002). The absorbances were measured at 470, 537, 647 and 663 nm with a NanoPhotometer™ (Implen, Isaza, Portugal). The amount of anthocyanins, chlorophyll *a* and *b* and carotenoids were determined through the equations referred by Sims and Gamon (2002).

2.3. Elemental analysis

Bico Branco and Nipponbare cultivars grown under Fe deficient ($n = 5$) and Fe sufficient conditions ($n = 5$) for three weeks. Roots and shoots were separately harvested, washed to exclude the contamination of Fe from the hydroponic solution and then dried at 65 °C to determine mineral concentrations.

Two hundred milligram of each variety were digested with five mL of 65% HNO_3 in five steps: 1–130 °C/10 min; 2–160 °C/15 min; 3–170 °C/12 min; 4–100 °C/7 min; and 5–100 °C/3 min in Teflon reaction vessels and heated in a Speedwave™ MWS-3+ (Berghof, Germany) microwave system. After digestion, the resulting clear solutions were diluted to 20 mL with ultrapure water. Mineral concentration determination for molybdenum (Mo), boron (B), zinc (Zn), phosphorus (P), cobalt (Co), nickel (Ni), manganese (Mn), iron (Fe), magnesium (Mg), copper (Cu) and sodium (Na) was performed using the Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES) Optima 7000 DV (PerkinElmer, USA). The elements were quantified using the axial alternate method.

2.4. Root Fe-reductase activity assay

Ten plants were grown under the same conditions as before (five under Fe-deficiency and five under Fe-sufficiency) and were

used for root Fe reductase activity measurements as described by Vasconcelos et al. (2006).

The contribution of root-released soluble reductants to overall root Fe reduction was determined by conducting additional assays with plants grown in the same conditions described before. Roots were placed for 45 min in buffered nutrient solution with no Fe source or BPDS. An aliquot of the solution from each root system was added to a solution containing 100 μM Fe(III)-EDTA and 100 μM BPDS and left for 30 min; absorbance was then read at 535 nm as described above.

2.5. Quantitative RT-PCR

Additional plants were grown in the same conditions and shoots and roots of Bico Branco and Nipponbare cultivars were collected after three weeks growing under Fe sufficient and Fe deficient conditions and immediately frozen in liquid nitrogen. A pool of three plants from each treatment were grinded thoroughly with a mortar and pestle until a fine powder was obtained and total RNA was extracted using a Qiagen RNeasy Plant Mini Kit (USA, Nr. #74904), according to the manufacturer's instructions, and treated with RNase-free DNase I to remove contaminating genomic DNA. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal). Samples were stored at −80 °C for further analyses.

Single-stranded cDNA was then synthesized using the First Strand cDNA Synthesis Kit (Fermentas UAB, Cat. Nr. #K1612) in a Thermal cycler (VWR, Doppio, Belgium), according to manufacturer's instructions.

Accession numbers of genes identified in Fe nutrition in rice plants were chosen using NCBI databases. Accession orthologs to AtTOM1 were identified using the TBLASTN tool against the GenBank databases with search specifications for *O. sativa* [Organism]. The sequences were named *O. sativa* TOM1 (*OsTOM1*). Only sequences that showed an $e^{\text{-value}} < 6e^{-14}$ were considered significant (Table A.1).

Primer sequences were designed for 9 genes, using Primer-BLAST software (Ye et al., 2012) with the following criteria: primer size between 18 and 20 base pairs and primer annealing temperatures between 57 °C and 60 °C. Accession numbers and the respective sequences are presented in Table A.2.

Quantitative Real-Time PCR amplifications were carried out in a Chromo4 Thermocycler (Bio-Rad, CA, USA) using 100 ng of cDNA, 1.25 μL of each primer, 1.5 μL of molecular biology grade water and mixed to 12.5 μL of 2 × PCR iQ SYBR Green Supermix (Bio-Rad) in a final volume of 25 μl. Three technical replicates were performed for each gene tested in qPCR reactions, as well as for controls. Thermal cycling conditions were: initial 2 min denaturation at 50 °C and then 10 min at 95 °C, followed by 39 cycles of 15 s at 95 °C and 1 min at 57 °C, and a final dissociation step of 1 min at 72 °C.

Melting curve from 50.0 °C to 95 °C was read every 0.1 °C holding 1 s. Then, melt curves profiles were analyzed for each gene tested. The comparative CT method ($\Delta\Delta^{CT}$) (Livak and Schmittgen, 2001) for the relative quantification of gene expression was used for assessing the normalized expression value using the 18S rRNA as the housekeeping gene and for normalization of expression of each gene (Opticon Monitor 3 Software, Bio-Rad). Data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.

2.6. Statistical analysis

Data processing and statistical analysis of anthocyanins, chlorophyll a and b, total chlorophylls and carotenoids data, root Fe reductase activity assay and ICP-OES data were performed using Microsoft Excel and GraphPad Software (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences between treatments were tested with an unpaired *t*-test, using the Holm-Sidak method.

3. Results and discussion

3.1. Photosynthetic pigments accumulation

One of the major abiotic challenges for plants is to thrive in Fe deficient conditions, and plants have developed a range of mechanisms to cope with Fe deficiency, such as storage and remobilization of mineral nutrients and changes in morphology and physiology (Marschner, 1995).

The earliest symptom observed in the leaves of plants growing in soils with low Fe availability is chlorosis, usually called “Fe deficiency chlorosis” (IDC) (Curie and Briat, 2003). Shoots of Fe deficient plants showed more chlorosis symptoms than Fe sufficient ones, that remained green throughout the assay (data not shown), and Bico Branco shoots were more chlorotic than the Nipponbare ones (Fig. 1). Anthocyanin, chlorophyll and carotenoid concentrations were measured in Bico Branco and Nipponbare shoots (Fig. 2). After three weeks under Fe deficient conditions, Bico Branco cultivar (cv.) had significantly lower anthocyanin, chlorophyll b and carotenoid values when compared to Nipponbare cv. Since Fe plays a role in the biosynthesis of photosynthetic pigments, IDC has been associated with decreased photosynthetic rate and inhibition of chlorophyll biosynthesis (Pushnika et al., 1984; Belkhodja et al., 1998). If severe, it can lead to a reduction of plant growth and yield or even complete crop failure (Guerinot and Yi, 1994). Thus, under Fe deficiency, the loss of chlorophylls and carotenoids are the primary responses associated with the unavailability of this element (Hendry and Price, 1993; Belkhodja et al., 1998). In rice, Sperotto et al. (2007) also visualized the first symptoms of chlorosis after 11–13 days of Fe deficiency treatment, with consequent significant decreases in chlorophyll concentration. A difference in the size of shoots between treatments was also visually observed in the current experiment (data not shown), as plants were smaller under Fe deficiency, as described by Abbott (1967).

In rice, the effects of Fe deficiency on chlorophyll concentration have been previously reported. Wu et al. (2001) evaluated leaf chlorophyll concentration in Nipponbare cv. during 14 days of Fe deprivation, and found that after five days a significant decline of chlorophyll concentration was already detected and chlorotic symptoms were induced in newly developed leaves. Zheng et al. (2009) also studied the chlorophyll concentration of Nipponbare cv. under Fe and P deficiency, and showed that chlorophyll concentration decreased in Fe deficient plants.

Anthocyanins can accumulate in leaves of plants that grow under diverse environmental and anthropogenic stresses (Neill, 2002; Hodges and Nozzolillo, 1995). Under Fe deficiency, anthocyanin synthase, one of the main enzymes in the biosynthetic pathway, is prone to lose its activity, since it requires Fe for proper functioning (Le Jean et al., 2005). This process leads to a decrease of anthocyanin levels, which could explain the reduction in anthocyanin levels observed in Bico Branco cv. under Fe deficiency (Fig. 2). Carotenoid concentrations were also significantly lower in Bico Branco cv. under Fe deficiency but were not affected in Nipponbare cv. It has been suggested that β-carotene and chlorophyll concentration in *Beta vulgaris* L. leaves also decreases under limited Fe supply (Morales et al., 1990).

In summary, photosynthetic pigment accumulation, in general, seems to be less affected in Nipponbare than in Bico Branco cultivars.

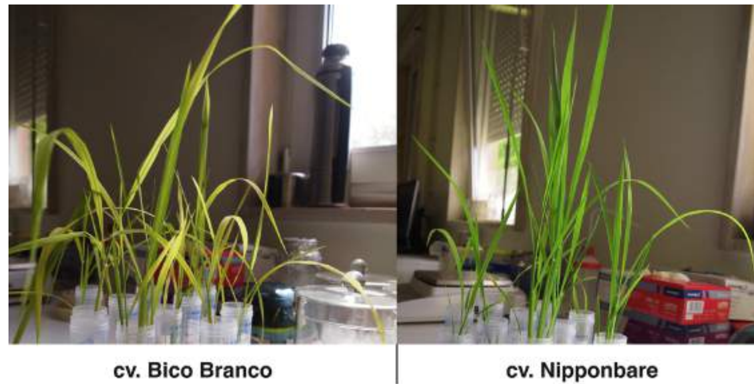

Fig. 1. Visual chlorosis symptoms of Bico Branco cv. and Nipponbare cv. grown in Fe-deficient hydroponic conditions for three weeks.

3.2. Mineral accumulation in shoots and roots

To test if the impact on plant mineral accumulation caused by Fe deficiency is cultivar dependent, mineral concentrations in shoots and roots of Fe-deficient and Fe-sufficient Nipponbare and Bico Branco cultivars were determined by ICP-OES.

Results showed that Bico Branco shoots had 68 μg/g DW of Fe under Fe deficiency and 79 μg/g DW under Fe sufficiency (Fig. 3). Also, roots accumulated more Fe than shoots, as previously reported (Sperotto et al., 2012), namely 1078 μg/g DW under Fe deficiency and 2711 μg/g DW under Fe sufficiency (Fig. 3). Nipponbare cv. also had lower Fe concentrations in Fe-deficient tissues when compared to Fe-sufficient ones: 18 μg/g DW under Fe deficiency and 36 μg/g DW under Fe sufficiency in shoots and 718 μg/g DW under Fe deficiency and 1828 μg/g DW under Fe sufficiency in roots (Fig. 3).

Rice has been shown to accumulate lower Fe concentrations in both shoots and roots of plants grown under Fe deficient conditions (Sperotto et al., 2012), but to accumulate more in roots than in shoots (Silveira et al., 2007), and our results were in accordance to these observations. Sperotto et al. (2012) characterized mineral accumulation in rice (Kitaake cv.) tissues under different Fe supplies, namely 5, 20 and 200 μM. Under intermediate Fe supply, Fe concentration ranged from 50 to 70 μg/g DW in shoots, and from 1000 to 2000 μg/g DW in roots, which is consistent with the results obtained here.

Amongst the other minerals, Cu was the only mineral that showed a tendency for higher accumulation under low Fe supply when compared to Fe-sufficient conditions in Nipponbare roots (Fig. 3). Furthermore, a significantly lower accumulation of Zn, Co and Ni in roots was detected under Fe deficiency compared with the plants grown under Fe sufficiency (Fig. 3). One may hypothesize that this was due to the lower induction of Fe transporter genes in roots of this cultivar under Fe deficiency (as will be seen later in Section 3.4), because it has been shown that IRT1 can also transport other nutrients. In the Nipponbare shoots, higher levels of Mn and Cu and lower amounts of Na, Mo, B, Co and Ni were detected (Fig. 3), probably because, besides Fe, other micronutrients are affected by Fe deficiency in rice, especially in the early stages of rice development (Silveira et al., 2007; Sperotto et al., 2012).

The Bico Branco cv., under Fe deficiency, had an augment (although not statistically significant) of Zn, Cu and Mn values in roots, but not in shoots. Zn, Cu, and Ni were reported to accumulate more in roots and Mn, Ca, Mg and K in leaves, when under low Fe concentrations, and that Fe, Mn and Ca were at lower concentrations in roots and Zn and Ni in leaves (Sperotto et al., 2012). Furthermore, under low Fe concentrations, there was a higher accumulation of Ni and Mo in Bico Branco roots (Fig. 3), which was also obtained by Sperotto et al. (2012).

3.3. Root Fe-reductase activity

In the present study, membrane-bound reductase activity and the contribution from root soluble reductants release were measured in roots of plants grown in Fe-deficient and Fe-sufficient

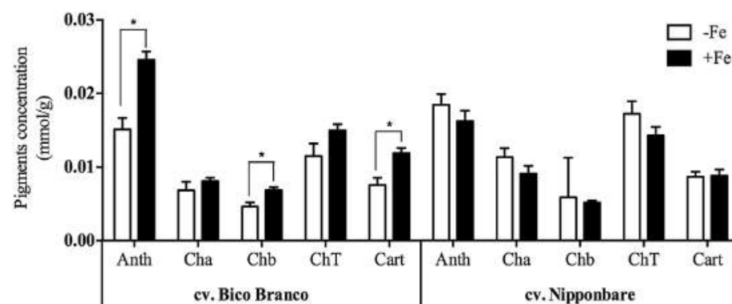

Fig. 2. Anthocyanin (Anth), chlorophyll a (Cha) and b (Chb), total chlorophyll (ChT) and carotenoid (Cart) concentrations in shoots of Bico Branco and Nipponbare cultivars. Plants were grown in Fe-deficient (−Fe) and Fe-sufficient (+Fe) hydroponic conditions for three weeks. Results show the mean + SEM of five independent biological replicates. Significant differences between Fe treatments are indicated by asterisk ($p < 0.05$).

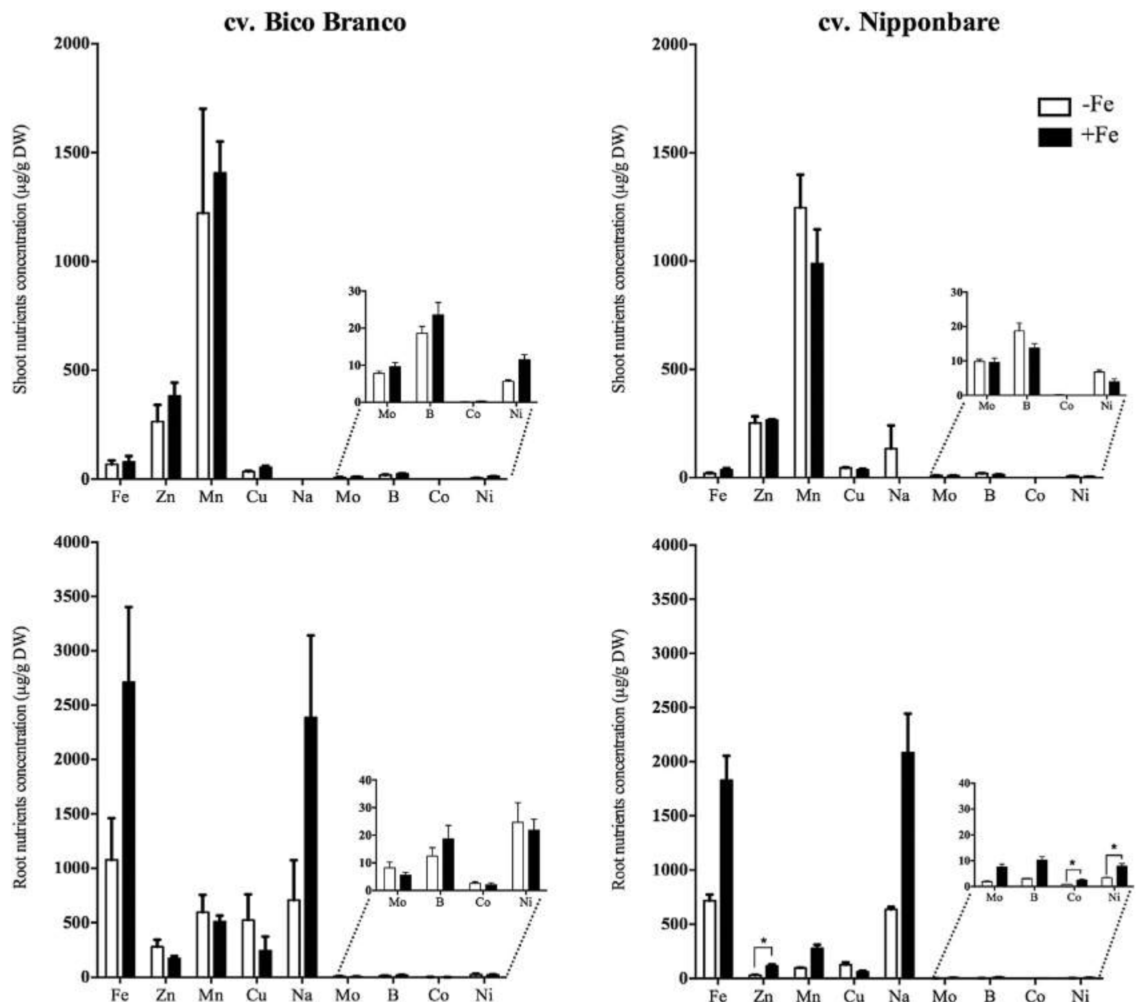

Fig. 3. Micronutrient concentrations (µg/g dry weight) of shoots and roots of Bico Branco and Nipponbare cultivars, using ICP-OES. Plants were grown in Fe-deficient (−Fe) and Fe-sufficient (+Fe) hydroponic conditions for three weeks. Results show the mean + SEM of three independent biological replicates. Significant differences between Fe treatments are indicated by an asterisk ($p < 0.05$).

conditions (Fig. 4). Rice plants have been described to not reduce Fe^{3+} actively to Fe^{2+} because their Fe^{3+} chelate reductase activity is very low (Ishimaru et al., 2006) or most attributable to soluble reductant release (Vasconcelos et al., 2004). Here, root Fe-reductase activity significantly increased under Fe starvation, especially in the Nipponbare cv. (more than two-fold higher when compared to the Bico Branco counterpart) (Fig. 4). Accordingly, the majority of studies report that plants have higher reductase activity under Fe deficiency than under Fe sufficiency (Kochian and Lucas, 1991; Romera et al., 1992; Cinelli et al., 1995), but this is not always so (Santos et al., 2013) as the reductase activity is dependent on many factors. Most root reductase activity assays do not account for Fe reduction due to soluble reductant release. In the study by Vasconcelos et al. (2004), it was shown that most of the reductase activity in rice cultivar IR68144 was in fact attributable to soluble reductant release. In the current study, the contribution to Fe reduction from soluble compounds had maximum values of 0.464 µmol Fe (II) g^{-1} FW h^{-1} for the Nipponbare cv. and 0.141 µmol Fe (II) g^{-1} FW h^{-1} for the Bico Branco cv. (Fig. 4), whereas the majority of reductase activity was membrane associated.

Ishimaru et al. (2006) reported lower values of reductase activity in Nipponbare cv., and it changed over time, ranging from 0.035 to 0.020 µmol Fe (II) g^{-1} FW h^{-1} for plants between zero to five days after the transfer to Fe deficiency. However, these plants were grown for three weeks under optimal conditions and only then were transferred to Fe deficiency, while in our study plants were maintained exclusively under Fe-deficiency, probably eliciting the root reductase system in a more acute way, as the plants could be more stressed. Also they did not report the contribution from root soluble reductants, which could have lowered their values of reductase activity even further. Another report on rice showing that plants possess the strategy I mechanisms of Fe reduction is that of Ishimaru et al. (2007), however these authors also do not refer

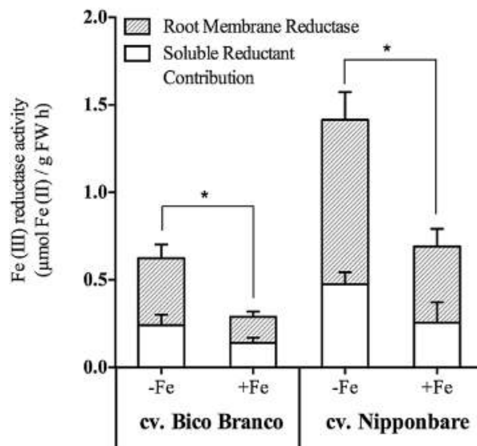

Fig. 4. Root Fe reductase activity of Bico Branco and Nipponbare cultivars. Plants were grown in Fe-deficient (−Fe) and Fe-sufficient (+Fe) hydroponic conditions for three weeks. Results show the mean + SEM of five independent biological replicates. For 'Root Membrane Reductase' results, significant differences between Fe treatments are indicated by asterisk ($p < 0.05$).

soluble reductant reductase capacity. As seen in Fig. 3, the rice cultivars analyzed in the current study presented values of reduction comparable to the ones described by dicotyledonous plants, which supports the hypothesis that rice can reduce Fe^{3+}. Certain Fe deficient bean populations were reported to have reduction values around 0.2 μmol Fe (II) g^{-1} FW h^{-1} and, *Mallus xiaojinensis* reached a maximum of 0.480 μmol Fe (II) g^{-1} FW h^{-1} of reductase activity (Wu et al., 2012). Our values reached similar levels, which may support the latter hypothesis that rice can adopt a combined mechanism of Strategy I and II (Walker and Connolly, 2008; Ishimaru et al., 2006), mainly in anaerobic soils, where Fe^{2+} is present in higher amounts. On the other hand, in aerobic soils, where Fe^{3+} is abundant, its reduction to Fe^{2+} on the root surface is an obligatory process for Fe acquisition in Strategy I plants (Yi and Guerinot, 1996). Rice, despite absorbing Fe^{3+}-PS through OsYSL15 (Inoue et al., 2009; Lee et al., 2009a), secretes PS at lower amounts compared to other grasses (Mori et al., 1991), and for this reason, it suffers from severe problems of Fe deficiency. Thus, our data confirms that rice may benefit from the capacity to reduce Fe, to compensate the lack of Fe in upland soils.

3.4. Molecular responses to Fe deficiency

The response of genes involved in Strategy I for Fe uptake, *OsFRO2* and *OsIRT1*, was studied in both cultivars, grown under Fe-deficient and -sufficient conditions. Under Fe deficiency, the expression of *OsFRO2* was low in roots and shoots of Bico Branco plants, whereas in Nipponbare plants, roots up-regulated *OsFRO2* under Fe starvation and shoots supplied with Fe had a strong induction of expression (Fig. 5).

OsFRO2 is thought to be exclusively expressed in rice shoots (Ishimaru et al., 2006) but in *Arabidopsis*, under limiting Fe availability, the expression of *AtFRO2* in roots is increased (Mukherjee et al., 2006). *FRO* genes encode the Fe^{3+}-chelate reductase enzymes, and our expression results appear to be in accordance with the ones obtained for root Fe-reductase activity, where Bico Branco cv. presented lower root Fe-reductase activity than Nipponbare cv. (Fig. 4) and a concomitant higher expression of *OsFRO2*. Although this general relationship between FRO2 expression and reductase activity can be observed, a direct proportion can not be inferred from gene expression to protein levels, since protein abundances are a reflection of a dynamic balance between RNA transcription, localization and modification (Vogel and Marcotte, 2013).

After Fe reduction by FRO, Strategy I plants transport Fe across the plasma membrane of the root epidermal cells by IRT1 (Grotz and Guerinot, 2006). The expression of *OsIRT1* was higher in roots of Fe sufficient plants in both cultivars (Fig. 5). *IRT1* is usually up-regulated in Fe-deficient conditions, but there are studies showing that its regulation is dependent both on the root Fe pool and on the shoot Fe demand (Vert et al., 2003), so the high levels we detected here can't be exclusively interpreted as IDC stress dependent. Also, in the Fe deficiency treatment, shoots of both cultivars up-regulated this gene, as was also previously described (Ishimaru et al., 2006), where the expression of the *OsIRT1* promoter–GUS fusion showed higher activity levels in the phloem under Fe deficiency, supporting the hypothesis of a possible function in the long-distance Fe transport in rice plants. On the other hand, it has been shown that some members of the *ZIP* family (as it is IRT1 gene) could be associated not only with Fe uptake, but also with detoxification and storage of excessive Fe (Yang et al., 2009; Li et al., 2013) thus putatively explaining the higher levels of expression obtained under Fe sufficiency.

There are several genes known to be related to Fe uptake in Strategy II in which PSs are released into the rhizosphere (Römheld and Marschner, 1990). Here, the expression of *OsTOM1*, a gene known to be related to PS secretion, was studied. In Bico Branco cv. its expression was lower under Fe deficiency when compared to Fe sufficiency, in both shoots and roots. In the Nipponbare cv. this transporter was 3.5 fold more expressed in shoots than in roots, under Fe deficient conditions (Fig. 6). These results suggest that, although *OsTOM1* seems to not be particularly involved in Fe acquisition, it is implicated in Fe transport, as described by others (Nozoye et al., 2011). However, in the aforementioned work, rice plants were transferred to Fe deficiency medium four weeks after germination, staying in this condition for only 5–7 days, whereas our plants were maintained under Fe deficiency for three weeks after germination. It is possible that *OsTOM1* could be mostly implicated in an early response to Fe deficiency.

OsYSL15 gene had higher expression in roots (and null in shoots) (Fig. 6). Moreover, Bico Branco roots had almost two-fold higher expression in Fe sufficiency than in Fe deficiency, whilst Nipponbare plants presented an inverse pattern (Fig. 6). *OsYSL15* was the first characterized YS1 ortholog from rice (Inoue et al., 2009) and functions as a transporter of Fe(III)-NA or Fe(II)-NA complexes (Lee et al., 2009a). Therefore, the higher expression levels of *OsYSL15* under Fe deficiency in Bico Branco cv. corroborates that this cultivar appears to be more susceptible to Fe deficiency than Nipponbare cv., as it is signaling a higher demand for Fe.

The nicotianamine synthase (NAS) enzyme catalyzes the biosynthesis of NA, and the genes encoding NAS are known to be differentially regulated by Fe status in a variety of Strategy I and Strategy II plant species (Higuchi et al., 1999; Inoue et al., 2003; Mizuno et al., 2003; Klatte et al., 2009). In rice, *NAS1*, *NAS2* and *DMAS1* genes are biosynthetic precursors of PSs and their overexpression causes an increase in transport of Fe from roots to shoots. Here, Bico Branco cv. roots had a seven- and four-fold overexpression of *OsNAS1* and *OsNAS2*, respectively, in response to Fe deficiency, when compared to the Fe sufficient plants (Fig. 6). Under Fe sufficiency, *OsNAS1* expression was increased in the shoots of this cultivar. *NAS1* is thought to be involved in Fe long-distance transport, and NA synthesis is required for xylem loading and also for loading and unloading to the phloem (Schmidke et al., 1999). Regarding *OsDMAS1*, its pattern of expression was also higher in Bico Branco roots and shoots under Fe

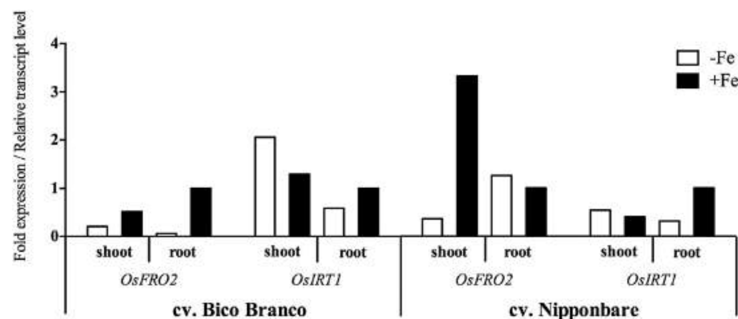

Fig. 5. Quantitative RT-PCR analysis of Strategy I-related genes, *OsFRO2* and *OsIRT1*, in Bico Branco and Nipponbare cultivars. Total RNA was extracted from a pool of three independent biological replicates from shoots and roots of plants grown in Fe-deficient (−Fe) and Fe-sufficient (+Fe) hydroponic conditions for three weeks. The results were normalized using the housekeeping gene 18S rRNA.

starvation, and this gene has been previously reported as being up-regulated by Fe starved plants (Inoue et al., 2003; Bashir and Nishizawa, 2006).

Although all plants can synthesize NA, only grasses convert NA to PSs (Lee et al., 2009b; Conte and Walker, 2011). The augmented expression of these genes (Fig. 6) could have been triggered to increase NA/DMA synthesis and consequently produce and secrete increased amounts of MAs, to help in Fe uptake (Inoue et al., 2003). Additionally, it is also known that these genes participate in Fe long-distance transport, being overexpressed in rice shoots under Fe starvation (Mori et al., 1991; Bashir and Nishizawa, 2006; Bashir et al., 2006). In the Nipponbare cv., under Fe deficiency, these genes were slightly up-regulated in shoots and no drastic changes in root expression were observed, independently of the Fe treatment. As the tolerance of rice plants to low Fe availability is thought to increase with the production and secretion of MAs, the Nipponbare cv. showed less stress signals when compared with Bico Branco, as previously seen with the photosynthetic pigments accumulation (Fig. 6). This corroborates that the Bico Branco cv. is more susceptible to low Fe conditions than the Nipponbare cv., increasing the need to synthesize PS synthesis related genes.

The expression of the transcription factor *OsIRO2* in Bico Branco cv., was two- and five-fold higher in roots and shoots, in Fe-deficient compared to Fe-sufficient conditions respectively (Fig. 6). It showed similar expression levels to the genes that it regulates, namely *OsNAS2* and *OsDMAS1*. Indeed, *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1* and *OsYSL15*, have been found to be under the regulation of *OsIRO2* (Ogo et al., 2006) and this transcription factor was described to regulate the PS-mediated Fe uptake system of rice, but not the Fe^{2+} uptake mechanism (Ogo et al., 2007). The

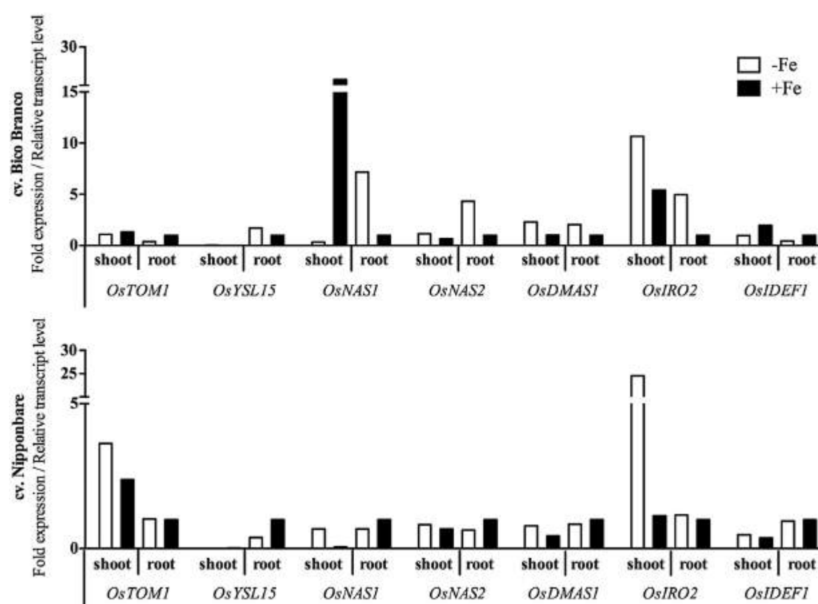

Fig. 6. Quantitative RT-PCR analysis of Strategy II-related genes, *OsTOM1*, *OsYSL15*, *OsNAS1*, *OsNAS2*, *OsDMAS1*, *OsIRO2* and *OsIDEF1*, in Bico Branco and Nipponbare cultivars. Total RNA was extracted from a pool of three independent biological replicates from shoots and roots of plants grown in Fe-deficient (−Fe) and Fe-sufficient (+Fe) hydroponic conditions for three weeks. The results were normalized using the housekeeping gene 18S rRNA.

Nipponbare cv. had also a strong induction of *OsIRO2* in Fe-deficient shoots, but not in associated roots (Fig. 6).

Results reported here show that three weeks after exposure to Fe deficiency, *OsIDEF1* was down-regulated in roots and shoots of Bico Branco cv. (Fig. 6) whereas its expression did not seem to be affected by Fe treatments in the Nipponbare cv. Usually described to be expressed in roots and shoots under Fe deficient conditions, *OsIDEF1* positively regulates the induction of several known Fe related genes in rice, such as *OsYSL2*, *OsYSL15*, *OsIRT1*, *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsNAS3* and *OsDMAS1* (Kobayashi et al., 2007, 2009). OsIDEF1 was described as a sensor of the cellular Fe status in the first days of exposure to Fe deficiency, but to lose its activity after a few days (Kobayashi et al., 2009). This could explain the lower expression of this gene by our Fe deficient plants.

4. Conclusions

Rice is a very diverse species accounting for about 120,000 rice cultivars existing in the world and most studies on Fe deficiency mechanisms in rice usually focus on a single rice cultivar (with Nipponbare, Taipei 309 and, more recently, Kitaake). Here, we compared Nipponbare cv. with an unstudied rice cultivar, Bico Branco, and given the reported high degree of variability in molecular and physiological responses between cultivars, it seems that generalizations of Fe responses cannot be taken lightly.

Bico Branco and Nipponbare cultivars showed contrasting responses to Fe deficiency, where the former was more susceptible to Fe deficiency, as it showed lower concentrations of photosynthetic pigments, had more chlorosis symptoms, and retained more nutrients in roots than the latter cultivar, which translocated more minerals to shoots even under Fe starvation.

Differences in gene expression of Strategy I and Strategy II genes were detected, with a variable pattern of expression of *OsFRO2* and *OsIRT1* in both rice cultivars (Fig. 7). Genes of Strategy I and Strategy II were typically up-regulated by the roots of the more Fe-susceptible cultivar Bico Branco, and were not differentially expressed in the roots of Nipponbare cv. (Fig. 7). Importantly, both cultivars showed membrane-bound Fe reductase activity, a typical

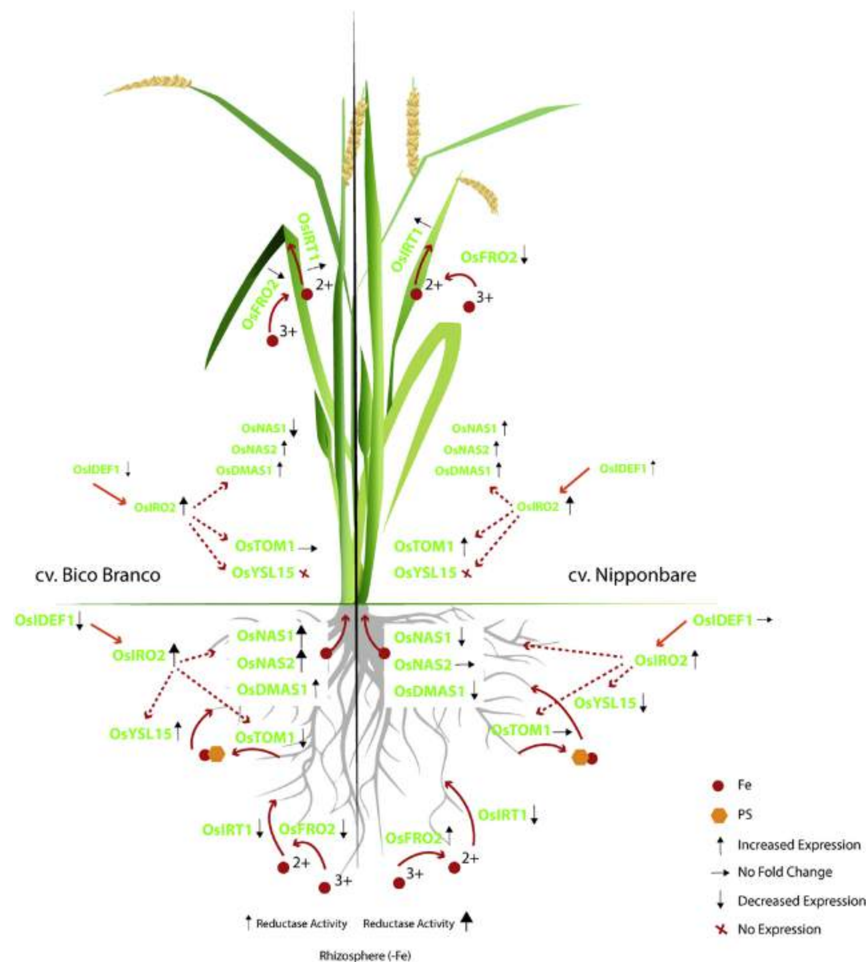

Fig. 7. Schematic representation of the regulation of Fe uptake mechanisms in Bico Branco cv. and Nipponbare cv. grown under Fe-deficient conditions for three weeks. Expression of Strategy I and Strategy II related genes, as well as Fe reductase activity is represented. Bigger arrows represent higher fold changes.

response of Strategy I-type plants, which was significantly enhanced under Fe deficiency (Fig. 7).

These data provide novel insights into Fe regulation by rice plants, showing that these can activate Fe uptake mechanisms used by dicotyledonous and that this capacity seems to be cultivar-dependent, possibly emerging from a need to adapt to different growing conditions.

Contributions

MPP and CS conducted the experimental work; AMG and MWV designed the experiment; all authors contributed for data analysis, interpretation, and manuscript writing.

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CHAPTER 3

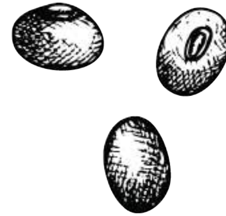
Physiological mechanisms associated with IDC

In this chapter the physiological mechanisms associated with IDC will be studied, using *Glycine max* lines with contrasting Fe-efficiencies. Firstly, in section **3.1**, partitioning study will be presented, where the unifoliate leaves (the first leaves to develop after seedling germination) were cut in the early stages of plants' growth. Several parameters will be used to analyse the impact of this removal and important conclusions on potential markers for IDC-tolerance will be identified.

In section **3.2** section, an innovative approach was undertaken and the correlation results of a Principal Component Analysis comprising the effect of Fe deficiency and the antioxidant and tetrapyrrole systems will be presented. Besides confirming the physiological traits associated with IDC-tolerance in section 3.1, in 3.2 section the role of a tetrapyrrole molecule – heme - will be highlighted, particularly when in its oxidized form (hemin).

CHAPTER 3

section 3.1



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Iron partitioning at an early growth stage impacts iron deficiency responses in soybean plants (*Glycine max* L.)

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Iron (Fe) deficiency chlorosis (IDC) leads to leaf yellowing, stunted growth and drastic yield losses. Plants have been differentiated into 'Fe-efficient' (EF) if they resist to IDC and 'Fe-inefficient' (IN) if they do not, but the reasons for this contrasting efficiency remain elusive. We grew EF and IN soybean plants under Fe deficient and Fe sufficient conditions and evaluated if gene expression and the ability to partition Fe could be related to IDC efficiency. At an early growth stage, Fe-efficiency was associated with higher chlorophyll content, but Fe reductase activity was low under Fe-deficiency for EF and IN plants. The removal of the unifoliate leaves alleviated IDC symptoms, increased shoot:root ratio, and trifoliate leaf area. EF plants were able to translocate Fe to the aboveground plant organs, whereas the IN plants accumulated more Fe in the roots. *FRO2*-like gene expression was low in the roots; *IRT1*-like expression was higher in the shoots; and *ferritin* was highly expressed in the roots of the IN plants. The efficiency trait is linked to Fe partitioning and the up-regulation of Fe-storage related genes could interfere with this key process. This work provides new insights into the importance of mineral partitioning among different plant organs at an early growth stage.

Keywords: soybean, partitioning, iron deficiency chlorosis (IDC), IRT1, FRO2, ferritin

Introduction

Soybean (*Glycine max* L.) is the highest produced legume crop, reaching production levels of about 230 million metric tons per year, across the world (Vasconcelos and Grusak, 2014). In many agricultural areas, where calcareous soils are predominant, iron (Fe) availability becomes a yield-limiting factor with major economic implications for field crop production (Rodríguez-Lucena et al., 2010). Since Fe is an essential element that has a key role in fundamental biological processes, such as photosynthesis and chlorophyll biosynthesis, when this micronutrient is unavailable to the plants, they frequently exhibit yellowing of the upper leaves, interveinal chlorosis, and stunted growth (Jeong and Connolly, 2009). This problem underpins the urgency to develop cultivars that can be more efficient in Fe uptake and further mineral translocation from the roots to the shoots, thus increasing plant nutritional value (Carvalho and Vasconcelos, 2013).

Abbreviations: DW, dry weight; EF, Fe-efficient; ICP-OES, inductively coupled plasma optical emission spectrometer; IDC, iron deficiency chlorosis; IN, Fe-inefficient; PQ, partition quotient; SEM, standard error of mean.

For a long time, soybean plants have been differentiated between EF, if they respond to Fe-deficiency stress by inducing biochemical reactions that make Fe available in a useful form, and IN if they do not (Brown, 1978; García-Mina et al., 2013). However, there is scarce information about the physiological and molecular mechanisms behind tolerance to iron deficiency and about the mechanisms that govern the partitioning of captured mineral nutrients between different plant organs (Vasconcelos et al., 2006; Lemoine et al., 2013; Roriz et al., 2014).

Plants have been divided between Strategy I and Strategy II, depending on their mechanism for Fe uptake. Dicoteledonous and non-grass monocoteledonous plants depend on an Fe reduction mechanism that allows them to reduce Fe (III) to Fe (II) in the rhizosphere (Abadía et al., 2011). Whilst the first Fe form is the most abundant in soils, it is poorly soluble at neutral or basic pH and, therefore, unavailable for uptake, causing IDC. A plasma-membrane Fe(III)-reductase, encoded by the *FRO* gene family, favors inorganic Fe solubilisation and consequent uptake by an Fe(II)-transporter, IRT1, of the ZIP family (Moog and Brüggemann, 1994). On the other hand, when in need for Fe accumulation and storage, plants augment the expression of ferritin, which plays a role in buffering excess Fe in plants (Roschzttardtz et al., 2013). However, excess accumulation in the form of ferritin can impair Fe remobilization from one plant organ to another (Vasconcelos and Grusak, 2014).

The regulation of sink-source relations is a complex process (Fester et al., 2013). It is well-known that mineral nutrient deficiencies may substantially influence dry matter partitioning between plant organs (Marschner et al., 1996), as nutrient-deprived plants generally tend to invest in their root system (Lemoine et al., 2013). Moreover, the shoot to root communication may act as an important feedback control signal for nutrient uptake and partitioning. For instance, sufficient Fe content in the leaves can modulate the synthesis of the ferric chelate reduction system and the capacity of the phloem to carry Fe from the roots, regulating the 'EF reaction,' acting as a negative feedback control (Maas et al., 1988).

Source leaves export photoassimilates to sink tissues when the demand exceeds the production via photosynthesis (Ludewig and Flügge, 2013) and nutrient movement to sink tissues could be controlled by the dynamics of source-sink carbohydrate partitioning (Grusak, 2002). Besides, the sink–sink competition also influences these regulatory processes, usually with one plant organ having a negative effect upon another by consuming or controlling access to a resource that is limited in its availability (Sadras and Denison, 2009). Hence, nutrient deficiency may not only affect the provision of photosynthates by decreasing source capacity, but also by altering partitioning between the source organs and various sinks (Marschner et al., 1996). Therefore, studies on Fe deficiency have utilized leaf excision to better understand the mechanisms of long-distance signaling. For example, the removal of leaves gave positive insights about the regulation of the *NtIRT1* and *NtFRO1* expression in roots of Fe-deficient tobacco plants (Enomoto et al., 2007). In another study, the shoot-tip was removed from apple plants, to understand the role of hormones in the regulation of Fe deficiency responses (Wu et al., 2012). Both studies found that shoots play a critical role in regulating Fe uptake in roots. To the best of our knowledge, few studies have focused on the role of nutrient competition between sink organs in the Fe deficiency responses of contrasting cultivars. So far, studies on efficiency have focused on identifying genetic markers for use in breeding programs, solely explaining the efficiency mechanism using genetic models (Lin et al., 1997; O'Rourke et al., 2007; Peiffer et al., 2012) and recent findings show that efficient genotypes induce energy controlling pathways to promote IDC resistance responses (Atwood et al., 2014). However, these studies only correlate the molecular results with the activity of the ferric chelate reductase or with chlorosis development. Therefore, there is a need for a study that integrates several possible traits that contribute for the efficiency mechanism in soybean plants.

The aim of this work was to understand if the ability to partition Fe could be related to IDC efficiency and to investigate the role of the expression of Fe uptake and storage related genes in this process. Given the fundamental importance of source/sink relations for plant growth and development, and that sink organs compete with each other for the carbohydrates and nutrients provided by source organs, we hypothesize that the ability to manage nutrient partitioning among different organs is an important trait contributing to an EF response. To verify this hypothesis, we removed the unifoliate leaves – strong sink organs in the early stages of plant development that have previously been shown to be correlated with IDC tolerance (Vasconcelos and Grusak, 2014) – and analyzed morphological, physiological, and molecular indicators in two *G. max* accessions with contrasting efficiencies for Fe-deficiency.

Materials and Methods

Plant Material, Growth Conditions, and Treatments

An efficient (EF – PI437929/VIR 316) and an inefficient (IN – PI378676A/Primorskaja 500) *G. max* accession for Fe deficiency (Vasconcelos and Grusak, 2014), with identical phenology, were selected from the USDA (United States Department of Agriculture) germplasm collection via GRIN (Germplasm Resources Information Network)[1]. Seeds were rolled in filter paper and placed vertically in a solution of 250 mM $CaCl_2$, for 7 days in the dark, at 25°C. In the current work, plants were grown hydroponically mimicking Fe deficient soil. Studies have shown that similar QTLs associated with IDC are identified in nutrient solution and field tests and, therefore, both systems identify similar genetic mechanisms of iron uptake and/or utilization (Lin et al., 1998).

In Experiment 1 germinated seedlings were transferred to 20 L vessels containing hydroponic solution with different Fe treatments. Each vessel contained five plants of one accession grown in Fe sufficient (+Fe, 20 μM Fe(III)-EDDHA [ethylenediamine-*N*,N′bis(*o*-hydroxyphenyl)acetic acid]) or in Fe deficient [−Fe, 0 μM Fe(III)-EDDHA] conditions.

[1] http://www.ars-grin.gov/

The vessels were placed in a climate chamber (Aralab Fitoclima 10000EHF) with 16 h day photoperiod providing 325 μmol s^{-1} m^{-2} of photosynthetic photon flux density at plant level supplied by a mixture of incandescent bulbs and fluorescent lights. Temperatures were set to 25°C during the light period and to 20°C during the dark period, whereas relative humidity was maintained at 75% throughout day and night. The standard solution for hydroponic growth of *G. max* 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 $NiSO_4$. Hydroponic solution was buffered with the addition of 1 mM MES [2-(*N*-morpholino)ethanesulfonic acid], pH 5.5 and, during the experimental time, pH was measured and solutions were changed weekly. The experiment ended 10 days after transferring the plants to the climate chamber.

To infer if the removal of the unifoliate leaves could alleviate IDC stress symptoms, a separate experiment was conducted (Experiment 2). In this experiment, plants were grown under the same conditions as described above, but unifoliate leaves were removed 3 days after the transfer to the hydroponic solutions (corresponding to about 10 mm length) and in the control plants the unifoliate leaves were kept on the plant. Please see **Figure 1** for the *G. max* anatomy visualization.

When the first unfolded trifoliate leaves of the inefficient accession showed signs of chlorosis, the experiments were terminated and plants were sampled for further analysis, which corresponded to 10 days after transferring the plants to the climate chamber.

Morphological Parameters

Chlorosis scoring was conducted using a visual scale according to Wang et al. (2008): (1) no chlorosis, plants normal and green; (2) slight yellowing of the upper leaves, no differentiation in color between the leaf veins and interveinal areas; (3) interveinal chlorosis (green veins and chlorotic interveinal areas) in the upper leaves, but no obvious stunting of growth or death of leaf tissue (necrosis); (4) interveinal chlorosis of the upper leaves with some apparent stunting of growth or necrosis of plant tissue; and (5) severe chlorosis with stunted growth and necrosis in the youngest leaves. Also, Soil and Plant Analyzer Development (SPAD) readings were conducted with a portable chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan) at the end of 10 days, using the first expanded trifoliate leaf from the top of the plant.

Sampled roots, stems and leaves were separated, weighed, and measured for length. The material was then dried at 70°C until constant weight and stored for ICP-OES analysis. Foliar area of the trifoliate leaves was measured using a leaf area meter AM300 (ADC BioScientific Ltd., UK).

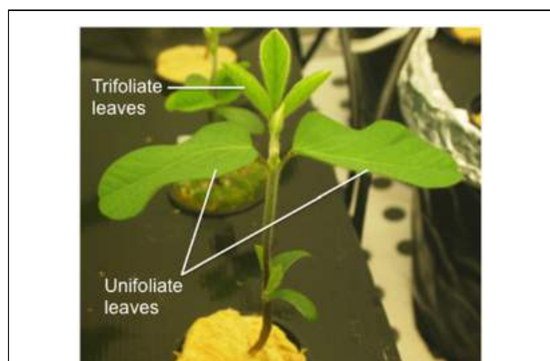

FIGURE 1 | Efficient accession soybean plant 437929 at V1 stage of development (as described by Fehr and Caviness, 1977) showing fully expanded unifoliate leaves and one unfolded trifoliate.

Root Iron Reductase Activity Measurements

Root iron reductase was quantified as described by Vasconcelos et al. (2006). The measurements were carried out in roots of intact plants via the spectrophotometric determination of Fe^{2+} chelated to BPDS (bathophenanthroline disulfonic acid). Roots of each plant were submerged in 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 (ethylenediaminetetraacetic acid) and 100 μM BPDS. All nutrients were buffered with 1 mM MES, pH 5.5. The assays were conducted under dim light conditions at 20°C and were terminated after 45 min by removal of the roots from the assay solution. Absorbance values were obtained spectrophotometrically at 535 nm, and an aliquot of the solution that had no roots during the assay was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 $mM^{-1}cm^{-1}$.

Total Fe Determination by ICP-OES

One hundred mg of the dried plant tissues (root, stem, cotyledon, unifoliate, and trifoliate leaves) of the two *G. max* accessions grown as described above were mixed with 5 mL of 65% HNO_3 in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Each plant organ from all the treatments ($n = 5$) was ground and five independent digestions were carried out.

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 20 mL with ultrapure water for further analysis. Mineral concentration determination was performed using the ICP-OES Optima 7000 DV (PerkinElmer, USA) with radial configuration.

Gene Expression Analysis

Additional plants were grown under the conditions described above, collected at the end of the assay and immediately frozen in liquid nitrogen. A pool of five biological replicates from each treatment were grinded thoroughly with a mortar and pestle until a fine powder was obtained and total RNA was

extracted using a Qiagen RNeasy Plant Mini Kit (USA, Nr. #74904), according to the manufacturer's instructions. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal). Single-stranded cDNA was then synthesized using the First Strand cDNA Synthesis Kit (Fermentas UAB, Cat. Nr. #K1612) in a Thermal cycler (VWR, Doppio, Belgium), according to manufacturer's instructions.

Sequence homologs to *AtFRO2* and *AtIRT1* in *G. max* were queried in NCBI database and the sequences with highest homology were selected (Supplementary Table S1). Primers for *FRO2*-like, *IRT1*-like, and *ferritin* were designed using Primer3[2], specifying an expected PCR product of 100–200 bp and primer annealing temperatures between 56 and 58°C (Supplementary Table S2). qPCR reactions were performed on a Chromo4 thermocycler (Bio-Rad, Hercules, CA, USA) with the following reaction conditions: 10 min at 95°C and 40 cycles with 15 s at 95°C, 15 s at 58°C, and 15 s at 68°C. Amplifications were carried out using 1.25 μM of the specific primers and mixed to 12.5 μM of 2xPCR iQ SYBR Green Supermix (Bio-Rad) and 100 ng of cDNA in a final volume of 25 μl. Three technical replicates were performed for each gene tested in qPCR reactions, as well as for controls. Melt curve profiles were analyzed for each tested gene. The comparative CT method (ΔΔCT; Livak and Schmittgen, 2001) was used for the relative quantification of gene expression values of Fe related genes using the 18S rRNA gene as the control transcript (Opticon Monitor 3 Software, Bio-Rad) and the EF plants, grown under Fe sufficiency, with unifoliate leaves as the reference sample. Data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.

A heatmap with folds of expression was designed using R software (R Development Core Team, 2013).

Statistical Analysis

Data were analyzed with GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla, CA, USA[3]). Differences between treatments were tested with unpaired Student's *t*-test corrected for multiple comparisons using Holm-Sidak method. Statistical significance was considered at $P < 0.05$.

Results

IDC Symptom Evaluation and Reductase Activity Quantification

The clearest symptom of IDC in plants is the interveinal yellowing of the younger leaves that can be assessed by using a visual chlorosis score in which 5 represents full chlorosis and 1, no chlorosis. EF plants grown in Fe sufficiency remained green throughout the experiment, while the IN ones presented some signs of chlorosis. The IDC visual scores of plants grown under Fe shortage were 4.8 ± 0.2 for the IN accession and 2.5 ± 0.5 for the EF one (**Figure 2A**). IDC was also evaluated measuring the chlorophyll content in the younger trifoliate leaves using a SPAD meter (**Figure 2B**). Average SPAD values corroborated that, when in −Fe conditions, the IN accession presented lower SPAD values (6.1 ± 0.9) than the EF one (9.3 ± 2.9), and even under Fe sufficiency the IN plants showed signs of chlorosis, with no significant differences to the −Fe plants (10.3 ± 3.0). The EF plants under Fe sufficiency had the highest SPAD values (24.9 ± 1.9).

Reductase activity was measured in roots of both IN and EF *G. max* accessions, grown under Fe shortage and Fe sufficiency. The Fe^{3+} chelate reductase activity was largely enhanced under Fe sufficiency for both accessions (**Figure 3**), and the activity of this enzyme was threefold higher in the EF accession.

Effects of Unifoliate Leaf Removal on IDC Symptoms and Fe Partitioning

The effect of Fe partitioning on IDC responses was assessed by growing the two accessions and removing the unifoliate leaves at an early growth stage, and comparing these to intact plants. Under Fe sufficiency, unifoliate removal did not significantly impact IDC score, SPAD values, plant DW and trifoliate leaf area (data not shown). Under Fe deficiency, intact plants presented accentuated visual symptoms of chlorosis, particularly in the IN accession (**Figure 4**).

The removal of the unifoliate leaves (−UNIF) led to significant improvements in the IDC symptoms, in both accessions (**Figures 4** and **5**). For instance, the IN accession presented a reduction of IDC visual score from 4.8 to 2.5 (**Figure 4B**) and

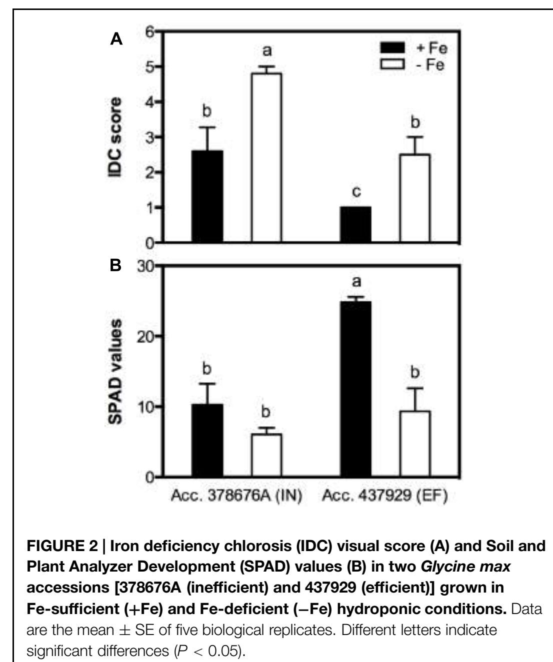

FIGURE 2 | Iron deficiency chlorosis (IDC) visual score (A) and Soil and Plant Analyzer Development (SPAD) values (B) in two *Glycine max* accessions [378676A (inefficient) and 437929 (efficient)] grown in Fe-sufficient (+Fe) and Fe-deficient (−Fe) hydroponic conditions. Data are the mean ± SE of five biological replicates. Different letters indicate significant differences ($P < 0.05$).

[2]Frodo.wi.mit.edu
[3]www.graphpad.com

the SPAD values were increased by 53% (**Figure 4C**). In the EF plants, the IDC visual score was significantly reduced from 2.5 to 1 and the SPAD values increased 62%. Moreover, the removal of the unifoliate leaves led to an increase in the shoot DW in both accessions but this was only significantly higher in the EF plants (**Figure 5A**). On the other hand, root DW was significantly lower in plants without unifoliate leaves, representing a decrease of 27% for the IN plants and 44% for the EF plants (**Figure 5B**). Finally, IN plants grown without unifoliate leaves presented 53% larger trifoliate leaf area and the EF plants had a 40% increase although in the last case this difference was not significant (**Figure 5C**).

In order to study the impact of unifoliate removal on Fe partitioning in each plant organ, IN and EF plants, intact or without unifoliates, were grown under Fe-sufficiency, and Fe-deficiency for 10 days (**Figure 6**, **Table 1**). In Fe sufficient conditions, total Fe content was significantly higher in intact plants (**Figure 6A**). Under Fe deficiency, the removal of the unifoliates had no effect on total Fe content in both accessions, but the EF plants were able to accumulate approximately two times more Fe than the IN plants (**Figure 6A**).

The percentage of Fe content of each organ relative to the total Fe content of the whole plant (Fe content partitioning) was calculated to compare the Fe partitioning between plant organs under Fe sufficiency and deficiency (**Figure 6B**). In this case, content was chosen rather than concentration to have a better idea on the total amount of Fe accumulated in one organ in relation to the whole plant accumulation. Looking firstly at intact plants (+UNIF), for both accessions, the organ that had higher Fe concentrations was the root (**Table 1**) and this was also the organ with higher content partitioning (**Figure 6B**). Under Fe sufficiency, the IN plants accumulated higher amounts of Fe in this organ, having about twofold higher Fe content than the EF plants, but under Fe deficiency no significant differences were detected between accessions (**Figure 6B**). The stem was the organ showing lower Fe concentrations amongst all plant organs (**Table 1**), being highest in Fe supplied EF plants. In **Figure 6B** it is also visible that under Fe sufficiency, the EF plants remobilized more Fe to all above-ground organs (stems, cotyledons, and trifoliates) than the IN ones, whilst under Fe deficiency no significant differences were detected between accessions, except in the cotyledons, where the IN plants had higher Fe content partitioning percentages than

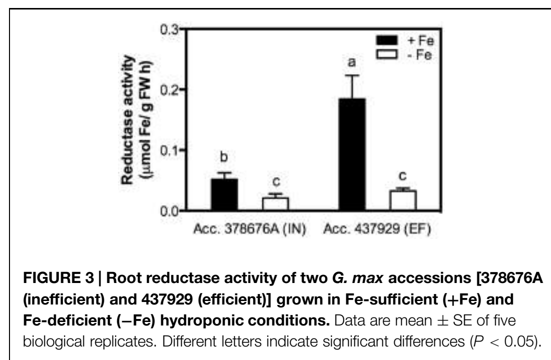

FIGURE 3 | Root reductase activity of two *G. max* accessions [378676A (inefficient) and 437929 (efficient)] grown in Fe-sufficient (+Fe) and Fe-deficient (−Fe) hydroponic conditions. Data are mean ± SE of five biological replicates. Different letters indicate significant differences ($P < 0.05$).

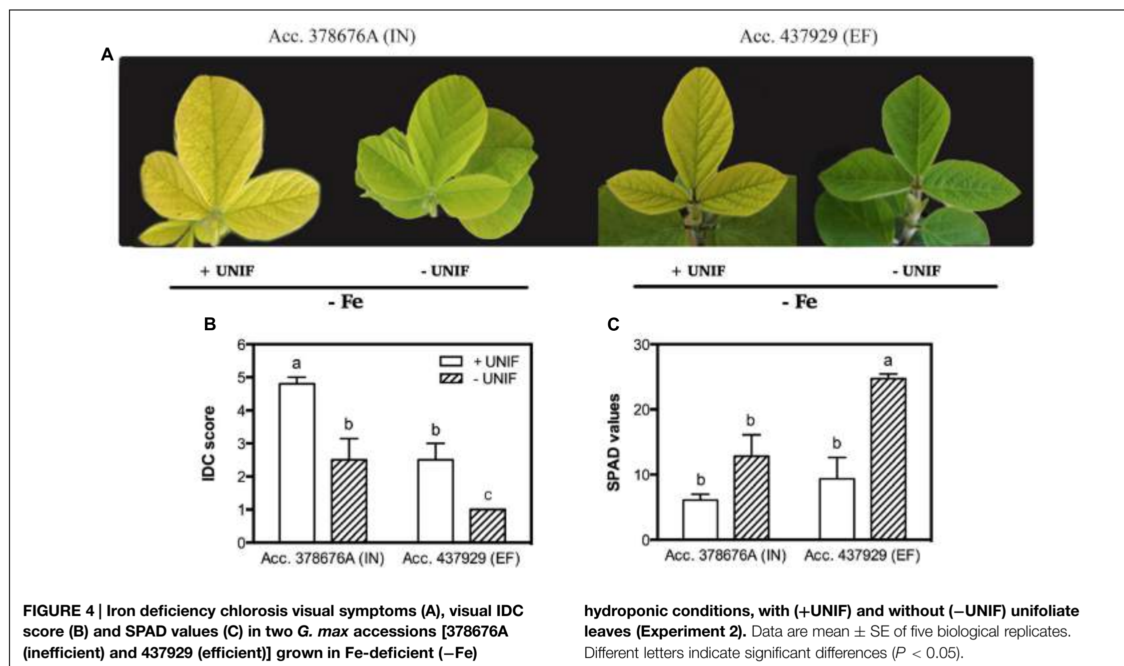

FIGURE 4 | Iron deficiency chlorosis visual symptoms (A), visual IDC score (B) and SPAD values (C) in two *G. max* accessions [378676A (inefficient) and 437929 (efficient)] grown in Fe-deficient (−Fe) hydroponic conditions, with (+UNIF) and without (−UNIF) unifoliate leaves (Experiment 2). Data are mean ± SE of five biological replicates. Different letters indicate significant differences ($P < 0.05$).

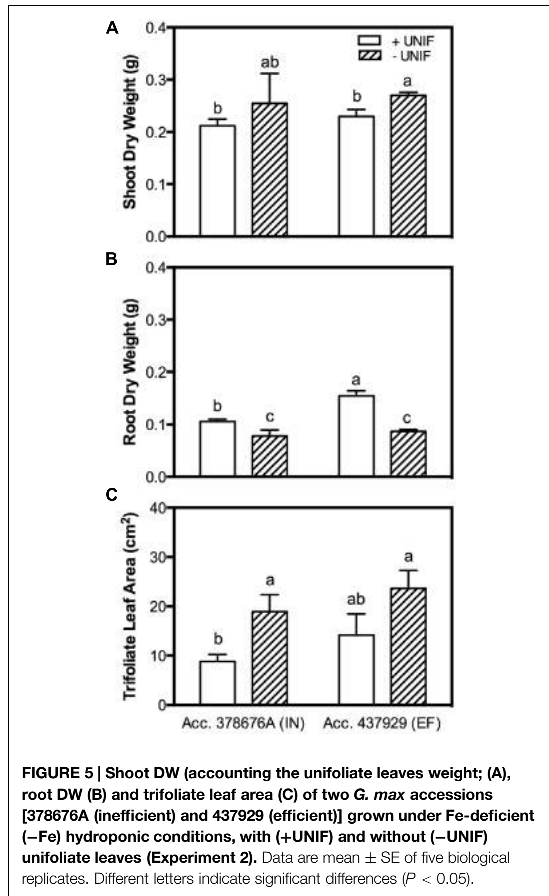

FIGURE 5 | Shoot DW (accounting the unifoliate leaves weight; (A), root DW (B) and trifoliate leaf area (C) of two *G. max* accessions [378676A (inefficient) and 437929 (efficient)] grown under Fe-deficient (−Fe) hydroponic conditions, with (+UNIF) and without (−UNIF) unifoliate leaves (Experiment 2). Data are mean ± SE of five biological replicates. Different letters indicate significant differences ($P < 0.05$).

the EF ones. The trifoliates were the above-ground organ that presented higher Fe concentrations. Under +Fe conditions, the EF plants had a fourfold increase in Fe concentration compared to the IN plants (**Table 1**). As expected, under +Fe conditions, Fe content partitioning was higher in the EF accession (**Figure 6B**).

When looking at the effect of the removal of the unifoliate leaves (−UNIF) it was found that in general unifoliate removal enhanced Fe concentrations in other plant organs (**Table 1**). Under Fe sufficiency, the Fe content partitioning was also enhanced in several instances (**Figure 6B**). Under Fe deficiency, roots of the IN plants accumulated 526 ± 31 ppm Fe and the EF plants 655 ± 57 ppm Fe – whereas by removing the unifoliate leaves, plants accumulated significantly higher amounts of Fe – 816 ± 132 ppm in the IN plants, and 1264 ± 69 ppm for the EF ones (**Table 1**). Also, as plants without unifoliates displayed reduced chlorosis in the trifoliate leaves (**Figures 4A–C**), Fe concentration increased in the trifoliates of IN plants grown under Fe deficiency to similar values of the Fe sufficient intact plants. In the EF plants Fe concentration was two times higher in the trifoliates when unifoliates were removed. Accordingly, the Fe content percentage doubled in the EF trifoliates (**Figure 6B**).

Fe-Deficiency Related Gene Expression Patterns

Three known genes associated with the Fe-deficiency responses – *FRO2*-like, *IRT1*-like, and *ferritin* – were studied using qPCR. These genes were analyzed separately for each plant organ (**Figure 7**, Supplementary Tables S3–S5) and the impact of unifoliate leaf removal was accessed for both accessions. In general, it was found that regardless of Fe supply, the IN plants presented higher gene expression levels than the EF ones, and that when looking at the expression of these genes in the unifoliate leaves, the expression levels were always higher in the EF plants under Fe-deficiency (**Figure 7**).

Firstly, when looking at the expression of *FRO2*-like (**Figure 7A**), the organ with the lowest expression levels was the root, with variable expression patterns in the remaining plant organs and treatments. Intact EF plants presented low basal levels of *FRO2*-like gene expression in all plant organs. The removal of the unifoliates did not augment the expression of *FRO2*-like gene in the roots of both accessions. In fact, the *FRO2*-like gene here studied appeared to have higher expression levels in the shoots than in the roots.

The expression of *IRT1*-like gene (**Figure 7B**) was higher in the IN plants roots than in the EF ones. After removing the unifoliates, the expression levels in the IN roots was even higher, especially noticeable in plants under Fe deficiency. Although levels of *IRT1*-like gene expression were very low in the EF plants, it had higher levels in the shoots than in the roots, and the expression levels were further increased when unifoliate leaves were removed (**Figure 7B**).

With regards to the *ferritin* gene (**Figure 7C**), the EF plants presented low expression in most plant organs, and the removal of the unifoliates had low impact on the levels of *ferritin* expression. Also, *ferritin* expression levels were similar between Fe sufficient and Fe deficient plants. Contrastingly, in the IN plants, the expression was highest in the stems and in the trifoliate leaves of Fe-sufficient plants, and the removal of the unifoliate leaves decreased the expression levels in these organs (**Figure 7C**). Interestingly, *ferritin* expression was higher in the roots of the plants without unifoliate leaves than in the intact plants, and these plants were the ones that accumulated more Fe (**Figure 6**).

Discussion

In the current work the efficiency trait was associated with lower chlorosis development under Fe deficiency (**Figure 2**) according to what has been previously described (Brown, 1978). Fe is essential in redox reactions, often used in electron transport chains, as well as in metabolic processes. Chlorophyll biosynthesis requires Fe, and plants need concentrations of 10^{-9} to 10^{-4} M to achieve optimal growth (Kim and Guerinot, 2007). Also, root Fe reductase is known to be the rate-limiting enzyme for Fe uptake (Wu et al., 2012; García et al., 2013). The ferric

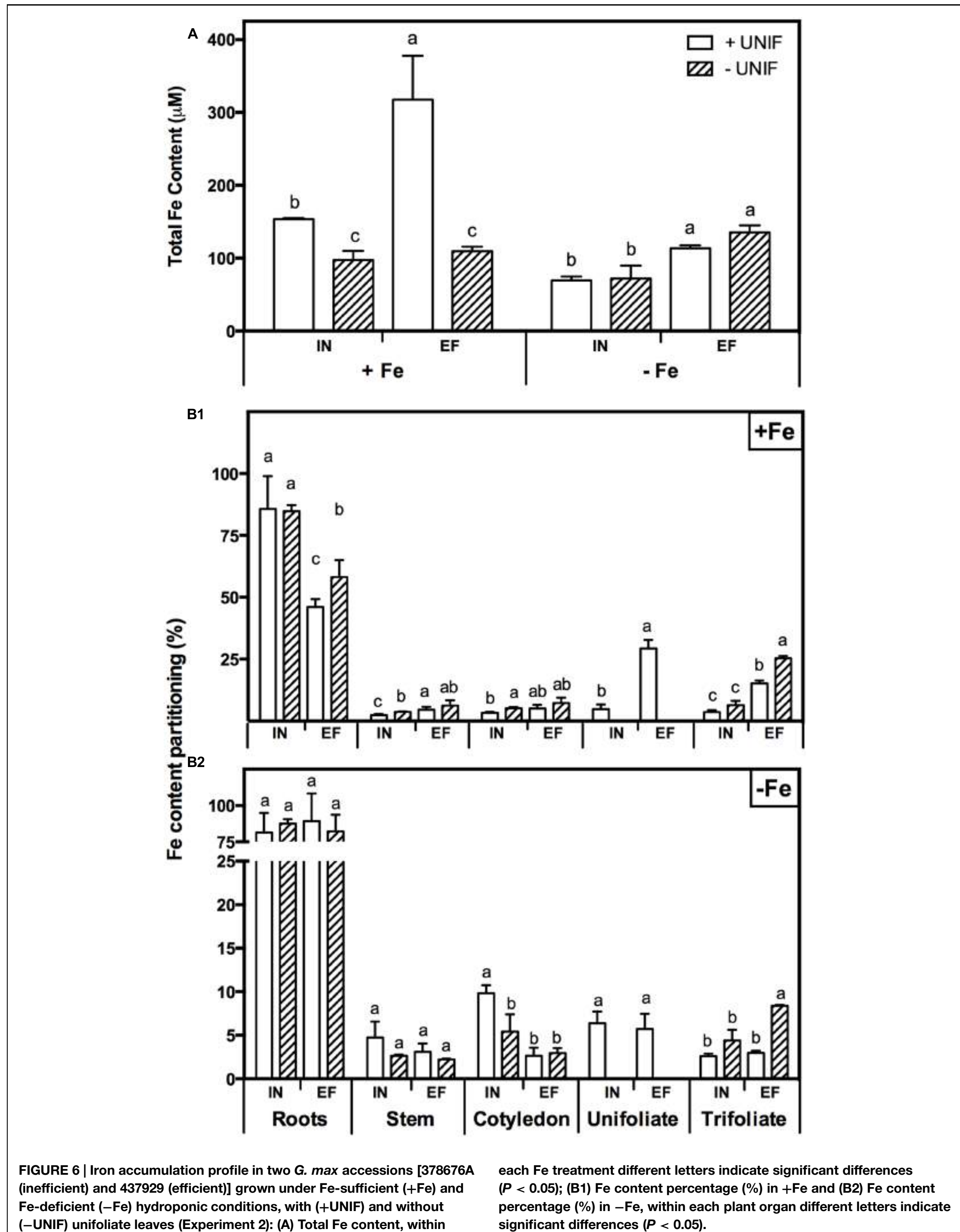

FIGURE 6 | Iron accumulation profile in two *G. max* accessions [378676A (inefficient) and 437929 (efficient)] grown under Fe-sufficient (+Fe) and Fe-deficient (−Fe) hydroponic conditions, with (+UNIF) and without (−UNIF) unifoliate leaves (Experiment 2): (A) Total Fe content, within each Fe treatment different letters indicate significant differences ($P < 0.05$); (B1) Fe content percentage (%) in +Fe and (B2) Fe content percentage (%) in −Fe, within each plant organ different letters indicate significant differences ($P < 0.05$).

TABLE 1 | Fe concentration (μg/g) in root, stem, cotyledon, unifoliate leaves, and trifoliate leaves of inefficient (IN) and efficient (EF) *G. max* accessions grown with (+UNIF) and without (−UNIF) unifoliate leaves, under Fe-deficient (−Fe) and Fe-sufficient (+Fe) hydroponic conditions (Experiment 2).

	Acc. 378676A (IN)				Acc. 437929 (EF)			
	+ Fe		− Fe		+ Fe		− Fe	
	+ UNIF	− UNIF	+ UNIF	− UNIF	+ UNIF	− UNIF	+ UNIF	− UNIF
Root	882 ± 79^{b}	1252 ± 46^{a}	526 ± 31^{c}	816 ± 132^{b}	575 ± 37^{b}	562 ± 27^{b}	655 ± 57^{b}	1264 ± 69^{a}
Stem	44 ± 1^{b}	66 ± 9^{a}	48 ± 11^{b}	45 ± 1^{b}	98 ± 5^{a}	76 ± 11^{a}	44 ± 9^{b}	47 ± 4^{b}
Cotyledon	87 ± 13^{a}	90 ± 4^{a}	90 ± 4^{a}	62 ± 9^{b}	216 ± 2^{a}	108 ± 16^{b}	48 ± 12^{c}	63 ± 16^{bc}
Unifoliate	55 ± 6^{a}	–	41 ± 3^{a}	–	265 ± 33^{a}	–	53 ± 15^{b}	–
Trifoliate	96 ± 6^{ab}	106 ± 10^{a}	61 ± 5^{c}	83 ± 1^{b}	403 ± 22^{a}	243 ± 7^{b}	66 ± 4^{d}	129 ± 7^{c}

Data are the mean of five replicates ± SE. Within each accession and each row different letters indicate significant differences ($P \leq 0.05$).

reductase activity was analyzed in roots of the IN and EF plants, to confirm if under Fe deficiency the EF plants had higher reductase activity as the efficiency trait has been associated with a reductase activity inducible by Fe deficiency (Moog and Brüggemann, 1994). However, here, for both IN and EF plants, the enzyme was more active in Fe-sufficient conditions than in Fe deficiency (**Figure 3**). It is well-known that Fe reductase activity is usually induced under Fe deficiency (Vert et al., 2003; Kong et al., 2013; Wang et al., 2013; Zha et al., 2014). However, in the current work, we observed that under Fe restriction plants had lower reductase activity than under Fe sufficiency. In fact, this is an observation that was already made before in Williams 82 soybean lines (Santos et al., 2013), and was also registered in common bean (Blair et al., 2010). In fact, it seems that reductase induction is not only species dependent (Santos et al., 2013), but also cultivar dependent (Blair et al., 2010; Pereira et al., 2014). These findings could be related to the fact that the Fe reductase enzyme itself has a heme containing Fe group. Thus, having grown the plants in total absence of Fe may have impaired the synthesis or functioning of the enzyme, which could explain the low reduction values in plants grown under Fe deficiency.

Moreover, although the EF plants had higher values of reductase activity under Fe-sufficiency than the IN ones, this alone cannot explain the difference in the efficiency, since under stress conditions none of them were able to activate the enzyme.

Unifoliate Leaf Removal Reduced Chlorosis and Increased Shoot to Root Ratio

As young leaves are one of the major sinks during the early developmental stages of plant growth and the access to photoassimilates and nutrients must be balanced between sinks (Wardlaw, 1990), we hypothesized that the removal of the unifoliate leaves could alleviate chlorosis and other IDC symptoms, since the competition between sinks would decrease. To this end, the unifoliate leaves were removed at a very young stage (in **Figure 1** the morphology of a soybean plant at an early growth stage is depicted) to understand if Fe would be directed to other sinks, diminishing the stress from Fe deprivation. This hypothesis seems to be supported by the observation that soybean plants with bigger unifoliates are more IDC susceptible (Vasconcelos and Grusak, 2014). In fact, under Fe-deficiency, unifoliate removal led to a visual reduction of chlorosis (**Figure 4A**) and a concomitant increase in SPAD values (**Figure 4C**), particularly in the EF plants. The fact that the IN plants when grown without unifoliate leaves (a strong sink for Fe) still showed a certain degree of chlorosis reveals that other processes are limiting Fe availability at leaf level (e.g., the level of remobilization, the storage form of Fe in source tissues, the amount of chelators, the type and amount of organic acid release or the expression of specific transporters).

Our results show that under Fe-deficiency the EF plants had higher root DW than the IN ones (**Figure 5B**), while the shoot DW did not vary among accessions (**Figure 5A**). This increased root to shoot DW ratio reflects the ability of the EF plants to allocate more resources to the organs involved in mineral acquisition when under shortage of mineral nutrients (Marschner et al., 1996; Hermans et al., 2006; Lemoine et al., 2013). Additionally, in plants without unifoliate leaves, root DW decreased whereas shoot DW increased, and they did not differ between accessions. This investment in the aerial organs rather than on the roots is possibly due to a lower sink demand and therefore a diminished Fe-deprivation stress.

Efficient Plants were able to Better Translocate Fe to the Trifoliate Leaves

The EF and IN plants responded differently in terms of Fe accumulation and distribution. When under Fe-deficiency (with or without unifoliate leaves), the EF plants had higher total Fe content (**Figure 6A**). Roots were the organs that accumulated more Fe (**Figure 6B**, **Table 1**). Moreover, under Fe-deficiency, roots of plants without unifoliates accumulated more Fe than roots of the intact plants and no differences were detected in Fe content percentage between accessions. Under optimal, Fe sufficient conditions, the EF plants had lower Fe content in the roots than the IN plants, indicating that less Fe was retained in this organ and was possibly distributed along the plant aerial organs. Unifoliate leaf removal led to an increase in the Fe concentration in the roots of the IN plants, but not in the EF plants (**Table 1**), suggesting that the IN plants have an impairment of Fe re-distribution.

The trifoliate leaves of plants without unifoliates, which were the ones with higher SPAD values and lower IDC visual scores (**Figure 4**), had higher Fe concentrations and Fe content percentages, especially in the EF plants. Studies show that higher Fe

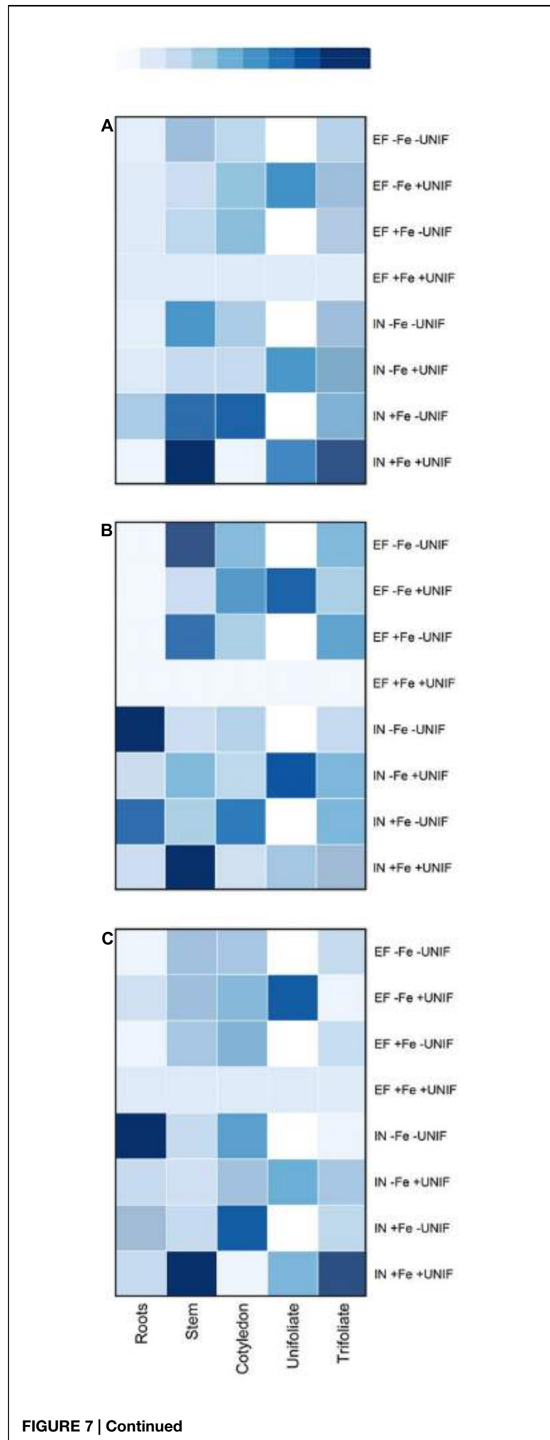

FIGURE 7 | Continued

FIGURE 7 | Continued
HeatMap of the expression patterns of *FRO2*-like (A), *IRT1*-like (B) and *ferritin* (C) genes in roots, stem, cotyledon, unifoliate, and trifoliate leaves of two *G. max* accessions [378676A (IN) and 437929 (EF)] grown under Fe-sufficient (+Fe) and Fe-deficient (−Fe) hydroponic conditions, with (+UNIF) and without (−UNIF) unifoliate leaves (Experiment 2). "EF +Fe +UNIF" was the reference sample. In dark blue: increased gene expression; in light blue: lower gene expression; in white: unifoliate leaf removed. Total RNA was extracted from a pool of five independent replicates. Corresponding values are presented in Supplementary Tables S3–S5.

concentrations can be found in young chlorotic leaves, when compared to green leaves, the so called "chlorosis paradox" that can result from an Fe inactivation in the plant under alkaline conditions (Römheld, 2000). However, in more recent works plants under Fe deficiency have lower Fe concentration in the leaves, showing that this is not an ubiquitous phenomenon, both in hydroponic (Ramírez et al., 2013; Kong et al., 2014), and soil conditions (Chakraborty et al., 2013; El-Jendoubi et al., 2014). Additionally, studies on Fe partitioning show that under Fe-deficiency and sufficiency the senescence of older leaves with a reduction of their sink capacity results in Fe retranslocation to younger leaves (Shi et al., 2012). Here, the removal of the unifoliate leaves also led to an Fe translocation toward the young trifoliate leaves of both IN and EF plants, specially under Fe-deficiency (**Table 1**, **Figure 6B**).

The EF plants grown under Fe-sufficiency, regardless of having unifoliates or not, were the ones with lower Fe concentration in the roots and had a more balanced distribution of the Fe pools throughout all organs, resulting in higher concentrations accumulating in the trifoliate leaves (**Table 1**, **Figure 6B**). There are several theories behind Fe deficiency sensing, but no consensus was yet achieved (for a recent review please see García-Mina et al., 2013). Roots were firstly proposed as the main organ for Fe-deficiency sensing (Bienfait et al., 1987) but more recent research shows that shoots have an important role in the regulation of the Fe-stress signaling (Enomoto et al., 2007; Wu et al., 2012). Our data suggests that the ability to translocate mineral resources from root to shoot contributes directly to the plants' efficiency trait.

The IN Accession Presented Enhanced Gene Expression Levels

In the current work, the IN plants had higher values of gene expression. These results were also obtained in other studies, where soybean inefficient lines responded to Fe stress by increasing the transcripts of genes involved in, for example, signaling and hormonal regulation (O'Rourke et al., 2007). The EF accession, not having suffered as severely as the IN one to the Fe shortage, it did not have the necessity to trigger Fe-uptake related genes. Besides the generally higher levels of gene expression by the IN accession, differences in the expression of individual genes were also apparent.

As previously discussed, the activity of the root reductase was very low under Fe-deficient conditions (**Figure 3**). At a molecular level, the expression of *FRO2*-like gene was also very low in

the roots, independent of the growth conditions. A time-course study in tomato showed that the activation of Fe deficiency stress response occurs in a progressive way, reaching a peak 5 days after Fe depletion and decreasing afterward; also, it showed that the variation in FRO1 transcript level is directly proportional to the root ferric chelate reductase activity (Paolacci et al., 2014).

Additionally, a strong induction of this gene was detected in the shoots, and the removal of the unifoliate leaves led to an increase of the expression under Fe-deficiency. As aforementioned, Fe must be reduced from Fe(III) to Fe(II) at the root surface for the uptake process. However, after entering across the rhizodermal plasma membrane barrier, it is again oxidized and transported through the xylem as an Fe(III) citrate complex, and for assimilation in leaves Fe must be again reduced (Wu et al., 2005). It is possible that the expression of *FRO2*-like in the shoot was increased to free the unavailable Fe (III) and make it more accessible for distribution within the plant. On the other hand, *FRO2* is a member of a gene family that comprises eight members in *Arabidopsis*, each one with tissue specificity (Wu et al., 2005); probably the *FRO* gene here analyzed is not the principal root reductase in soybean, but is functionally more similar to *AtFRO7*, which is known to be more active in the shoots of *Arabidopsis thaliana* (Jeong et al., 2008) than in the roots.

After the reduction step, *IRT1* is necessary for Fe transport, and a strong induction of *IRT1*-like gene expression was detected in the IN plants' roots after the removal of the unifoliates (**Figure 7B**). *IRT1* is usually up-regulated in Fe-deficient conditions, but studies show that its regulation is dependent both on the root Fe pool and on the shoot Fe demand (Vert et al., 2003). Our results also show a strong induction of *IRT1*-like gene in the EF plants shoots (**Figure 7B**). *IRT1* belongs to a family of genes – ZIPs – detected in different tissues (roots, leaves, nodules, and flowers) and it may also be involved in the transport pathways to other plant organs (Grotz et al., 1998). It has been shown that some members of the *ZIP* family could be associated not only with Fe uptake, but also with detoxification and storage of excessive Fe (Yang et al., 2009; Li et al., 2013). This could be the case in the EF plants, since as the unifoliate leaves were removed, more Fe was accumulated in the aboveground organs (**Table 1**, **Figure 6**), and *IRT1*-like gene expression was increased, corroborating its role in Fe homeostasis maintenance.

Ferritins are encoded by nuclear genes regulated by Fe and store Fe in its oxidized form (Harrison and Arosio, 1996). The IN accession had higher induction levels of the *ferritin* gene than the EF one. It has been suggested that Fe, when stored in the form of ferritin, may not be readily available for retranslocation (Vasconcelos and Grusak, 2014). It is possible that the higher accumulation of Fe with ferritin by the IN plants could be responsible for its lower partitioning capacity (**Table 1**, **Figure 6B**). Alternatively, the induction of ferritin synthesis is correlated with the degree of PSI degradation during Fe deficiency (Briat et al., 2010), which is in accordance to the results presented here: IN plants, that had acuter chlorosis symptoms and, therefore, higher degradation of PSI, had a strong induction of the expression in the trifoliate leaves (**Figure 7C**). With the removal of the unifoliate leaves, the levels of *ferritin* gene expression were lowered in the trifoliates, and so did the symptoms of chlorosis (**Figure 4**), again confirming that Fe bound to ferritin in the roots could have been hampering its partitioning to the aerial parts.

Conclusion

Although it is known that Fe deficiency induces both morphological and physiological responses in plants (Wu et al., 2012), how these responses are triggered is still unclear. Moreover, the partitioning of Fe between different plant organs could be a key mechanism for plant adaptation to this type of stress and could be related to the expression of specific genes.

Our results corroborated our initial hypothesis that the ability to remobilize Fe could be related to IDC susceptibility. The removal of the unifoliate leaves increased total Fe content in Fe-deficient conditions but Fe was mainly accumulated in the roots. Nonetheless, in the EF accession without unifoliate leaves, Fe concentration also significantly augmented in the trifoliate leaves and IDC symptoms were alleviated almost to full correction. Moreover, the EF plants under optimal conditions were able to distribute Fe in a more balanced way throughout all organs, fact that was not verified in the IN plants, suggesting that the ability to translocate Fe from the roots to the aboveground organs could explain the different IDC susceptibility between accessions.

Moreover, the IN plants induced higher expression levels of Fe uptake related genes, which may be an indicator of the higher susceptibility of this accession to Fe deficiency and shows that low gene expression levels cannot be responsible for the plant's low efficiency, as previously suggested (O'Rourke et al., 2007). The high level of ferritin expression by the roots of the IN plants could be responsible for the accumulation of Fe with ferritin in this plant organ and, consequently, making Fe partitioning to the shoots more difficult.

The enhanced overall growth of the plant and the reduced chlorosis and Fe accumulation in the trifoliate leaves here obtained by the removal of the unifoliate leaves appears to be due to a reduction in source–sink imbalance that reduced IDC symptoms. These findings suggest a key role of shoots in Fe-stress response signaling and identified possible factors that could influence plant IDC susceptibility. This comprehensive analysis helped to better understand some of the mechanisms behind mineral partitioning and resource allocation in soybean, and our conclusions can possibly be extrapolated to other agricultural crops suffering from IDC. Still, nutrient solutions cannot fully mimic agricultural, alkaline soil conditions, and as such further studies are necessary to extrapolate our findings to natural settings.

Author Contributions

CS carried out the sample preparation and analysis, gene confirmation experiments and drafted the manuscript; MR performed the ICP-OES analysis and helped in results interpretation; SC helped conceive the study and its design, and participated in the critical review of the manuscript; MV conceived the study, its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00325/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Electronic Supplementary Material

Table S1. Sequence orthologs of *Arabidopsis thaliana FRO2* and *IRT1* genes in *Glycine max*

Gene	*G. max* Accession nr.	*A. thaliana* Accession nr.	Maxim score	Total score	Query cover	E value	Identity
FRO2-like	XM_003548612.1	NM_100040.2	399	399	91 %	$5e^{-109}$	65 %
IRT1-like	XM_003520096.2	NM_118089.3	105	105	19 %	$9e^{-21}$	69 %

Table S2. Primer sequences and correspondent accession numbers (Acc. No)

Primer	Forward (5'-3')	Reverse (5'-3')	Acc. No
18S	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA	X75080.1
FRO2-like	TGCTTGGACTCACACCAGAG	AGAGGTAGAAACCGGGGAGA	XM_003548612.1
IRT1-like	GATTGCACCTGTGACACAAA	CAGCAAAGGCCTTAACCATA	XM_003520096.2
Ferritin	CCCCTTATGCCTCTTTCCTC	GCTTTTCAGCGTGCTCTCTT	U31648.1

Table S3. *FRO2* gene RNA relative expression values of inefficient (IN) and efficient (EF) *G. max* plants grown under Fe-sufficient (+Fe) and Fe-deficient (-Fe) hydroponic conditions, with (+UNIF) and without (-UNIF) unifoliate leaves

Treatment	Roots	Stem	Cotyledon	Unifoliate	Trifoliate
IN +Fe +UNIF	0.140	3054	0.849	3.41	24.3
IN +Fe -UNIF	1.75	4.40	3.71	--	6.50
IN -Fe +UNIF	0.226	0.977	1.75	2.80	6.45
IN -Fe -UNIF	0.250	3.35	2.93	--	4.39
EF +Fe +UNIF	1.00	1.00	1.00	1.00	1.00
EF +Fe -UNIF	0.258	1.33	2.24	--	3.05
EF -Fe +UNIF	0.863	1.17	2.36	2.83	3.87
EF -Fe -UNIF	0.627	3.38	1.76	--	3.02

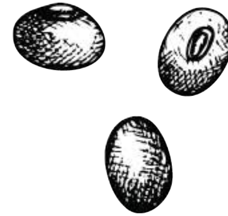
Table S4. *IRT1* gene RNA relative expression values of inefficient (IN) and efficient (EF) *G. max* plants grown under Fe-sufficient (+Fe) and Fe-deficient (-Fe) hydroponic conditions, with (+UNIF) and without (-UNIF) unifoliate leaves

Treatment	Roots	Stem	Cotyledon	Unifoliate	Trifoliate
IN +Fe +UNIF	0.998	835	0.953	3.51	12.0
IN +Fe -UNIF	5.76	2.86	3.07	--	2.95
IN -Fe +UNIF	1.16	2.72	1.95	3.66	2.51
IN -Fe -UNIF	186	1.40	2.30	--	2.68
EF +Fe +UNIF	1.00	1.00	1.00	1.00	1.00
EF +Fe -UNIF	0.538	2.35	1.23	--	1.59
EF -Fe +UNIF	1.03	1.53	2.30	2.53	1.62
EF -Fe -UNIF	0.538	3.60	1.38	--	1.45

Table S5. *Ferritin* gene RNA relative expression values of inefficient (IN) and efficient (EF) *G. max* plants grown under Fe-sufficient (+Fe) and Fe-deficient (-Fe) hydroponic conditions, with (+UNIF) and without (-UNIF) unifoliate leaves

Treatment	Roots	Stem	Cotyledon	Unifoliate	Trifoliate
IN +Fe +UNIF	0.705	663	0.849	1.18	6.23
IN +Fe -UNIF	5.55	0.883	3.71	--	1.49
IN -Fe +UNIF	0.964	0.777	1.75	1.27	1.64
IN -Fe -UNIF	188	1.24	2.93	--	1.82
EF +Fe +UNIF	1.00	1.00	1.00	1.00	1.00
EF +Fe -UNIF	0.582	1.84	2.24	--	1.27
EF -Fe +UNIF	1.35	2.08	2.36	2.189	1.79
EF -Fe -UNIF	0.520	1.97	1.76	--	1.08

CHAPTER 3
section 3.2



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Oxidative stress impairs iron efficiency in soybean plants

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Abstract

The role of oxidative stress and the tetrapyrrole cycle on iron (Fe) deficiency chlorosis (IDC), a serious condition affecting plant growth and crop productivity, is poorly understood. The study of cultivars with contrasting efficiencies to IDC could provide a useful tool to address this important knowledge gap. In this study, two soybean lines with contrasting Fe efficiencies were grown under hydroponic conditions with 20 μM or no additional Fe supply. Under Fe deficiency, the tetrapyrrole precursor ∂-aminolevulinic acid (ALA) concentration was 40% lower in the leaves of 'Fe-inefficient' (INF) plants when compared to 'Fe-efficient' (EF) plants, and the first displayed 45% lower dry weight, 46% lower chlorophyll levels and six (roots) and three-fold (leaves) higher hemin concentrations. INF plants also accumulated 53% more malondialdehyde (MDA) in the roots and had four-fold higher gluthatione reductase activity in the leaves, indicating higher oxidative stress. The activity of the heme-containing enzyme ferric reductase was three times lower in the INF plants, and of catalase was nine-fold higher in the roots and

three-fold higher in the shoots. This study sheds light into novel mechanisms behind IDC susceptibility and proposes how these may be related to the regulation of antioxidant defenses and the tetrapyrrole cycle.

Keywords: ∂-aminolevulenic acid, ascorbate peroxidase, catalase, ferric reductase, glutathione reductase, hemin, oxidative stress

1 Introduction

Iron (Fe) is an essential micronutrient required for proper plant growth, being involved in several metabolic processes, including photosynthesis, respiration, nitrogen fixation, DNA synthesis, hormone production and chlorophyll biosynthesis (Guerinot and Yi, 1994). Although Fe is present in sufficient amount in the soil, under alkaline conditions its bioavailability is limited, resulting in the appearance of a disease called iron deficiency chlorosis (IDC). IDC symptoms are characterized by yellowing of the upper leaves, interveinal chlorosis and reduced growth and yield (Prasad, 2003). When there is a depletion of Fe, it is predictable that chlorophyll and other photosynthetic pigment's content, like anthocyanins and carotenoids, decreases as Fe is essential for their biosynthesis (Prasad, 2003).

Soybean (*Glycine max* L.) is the highest produced legume crop with an estimated world production of more than 300 million tons in 2014 (http://faostat3.fao.org/browse/Q/QC/E). This crop is particularly affected by IDC. Soybean cultivars have been differentiated regarding their IDC susceptibility, where Fe-efficient (EF) plants activate biochemical reactions to make Fe more bioavailable, and Fe-

inefficient (INF) do not (Brown and Jolley, 1989), reinforcing the interest of this crop as a model system for studies regarding Fe uptake (Vasconcelos and Grusak, 2014). The main biochemical reaction induced by dicotyledonous plants to cope with Fe deficiency is a reduction-based strategy for iron absorption, the so-called Strategy I. This strategy is characterized by the acidification of the soil, leading to the reduction of Fe^{3+} to Fe^{2+} by a ferric chelate reductase (like Ferric Reductase Oxidase, FRO), and transport to the cytoplasm via an iron regulated transporters (Jeong and Connolly, 2009; Robinson et al., 1999).

One important characteristic of the FRO enzymes, in the context of Fe nutrition, is that it has a heme group as a constituent, which is essential for its functioning (Jeong and Connolly, 2009). In turn, heme is part of the tetrapyrrole cycle and Fe is essential for its biosynthesis (Mauzerall and Granick, 1956). Briefly, this cycle occurs mainly in the plastids, and after the conversion of glycine and succinyl-CoA into 5-aminolevulinic acid (ALA) by ALA synthase, several common and conserved steps occur, and this molecule is converted to protoporphyrin IX (Tanaka et al., 2011; Larkin, 2016). The cycle is then divided in two branches, the “magnesium-branch” that ends with the synthesis of chlorophyll, and the “iron-branch” that leads to the formation of heme. In the first branch, Mg^{2+} is inserted into the backbone of proto forming Mg-protoporphyrin IX, which, after a series of modifications, forms chlorophyllide *a* that is esterified to synthesize chlorophyll *a*. Chlorophyllide *a* can also be converted into chlorophyllide *b*, forming chlorophyll *b* which can be converted again into chlorophyll *a*, forming the chlorophyll cycle. In the second branch, a ferrochelatase is responsible to insert Fe^{2+} into proto to form heme *b* (protoheme) (Tanaka et al., 2011; Brzezowski et al., 2015). It is known that heme suffers degradation when exposed to oxidative stress, being oxidized into its ferric form hemin (Müllebner et al., 2015), that is also pro-oxidant (Lu et al., 2012).

On the other hand, Fe stress can also result in the accumulation of reactive oxygen species (ROS) leading to the appearance of oxidative stress which, in turn, results in DNA damage, enzyme inactivation and lipid peroxidation (Mittler et al., 2004). The mechanisms behind ROS formation under Fe deficiency are poorly understood, however it has already been shown that Fe deficient plants are ROS producers (Sun et al., 2007). To cope with oxidative stress and regulate ROS levels, plants have evolved the antioxidant system comprised by two levels of regulation, mediated by: (i) enzymes, such as superoxide dismutase, catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR); and (ii) metabolites, like ascorbate (ASC), glutathione (GSH), phenolics and carotenoids (Spirt et al. 2010) among others (Shi et al., 2007; Lee et al., 2001; Mittler et al., 2004). CAT catalyzes the conversion of hydrogen peroxide (H_2O_2) to H_2O (Mai and Bauer, 2016), being an important part of the plant antioxidant system, and, like FRO, it is also a heme-dependent enzyme (Broadley et al., 2012). APX catalyzes the reduction of H_2O_2 to H_2O through the oxidation of ASC and it is highly substrate specific, requiring high energy levels for its functioning, being particularly associated with enhanced tolerance against abiotic stress (Asada, 2006). GR is involved in defense against oxidative stress and regenerates GSH from its oxidized form, allowing the ASC-GSH cycle to proceed (Ramírez et al., 2013). Reports show that GR activity varies depending on the mineral stress to which the plants are subjected (Gill and Tuteja, 2010), but it has already been suggested that under Fe deficiency the activity of this enzyme may be increased (Bashir et al., 2007).

There are few studies that have evaluated the relationship between the tolerance to Fe deficiency and the triggering of the antioxidant defense mechanism in plants. In the present study, the responses of two soybean lines with different susceptibilities to Fe stress were evaluated by analyzing morphological, physiological and biochemical parameters.

The constituents of the tetrapyrrole cycle were evaluated (ALA, chlorophyll and heme in its oxidized form), as well as the photosynthetic pigments anthocyanins and carotenoids. In order to evaluate the oxidative stress of the plant tissues, lipid peroxidation was measured as the amount of malondialdehyde (MDA); also, the activity of several heme and non-heme containing enzymes related to Fe nutrition, such as ferric reductase, CAT, APX and GR, was accessed. A PCA analysis was performed to integrate the obtained data set in order to extract the most important information. At the end of this work, we propose a model that putatively explains how INF plants regulate the antioxidant metabolism and its role on IDC development.

2 Materials and methods

2.1 Plant material and growth conditions

An efficient (EF - PI437929 / VIR 316) and an inefficient (INF - PI378676A / Primorskaja 500) *G. max* accession for Fe deficiency (Vasconcelos and Grusak, 2014), with identical phenology, were selected from the USDA (United States Department of Agriculture) germplasm collection via GRIN (Germplasm Resources Information Network) (http://www.ars-grin.gov/). Seeds were germinated for seven days in the dark, at 25 ºC. Germinated seedlings were transferred to 5 L vessels containing hydroponic solution with different Fe treatments. Each vessel contained five plants of one accession grown under Fe sufficiency (+Fe, 20 μM Fe(III)-EDDHA [ethylenediamine-N,N'bis(o-hydroxyphenyl)acetic acid]) or Fe deficiency (-Fe, no additional Fe).

The vessels were placed in a climate chamber (Aralab Fitoclima 10000EHF) with 16 h day photoperiod providing 325 μmol s^{-1} m^{-2} of photosynthetic photon flux density at plant level, supplied by a mixture of incandescent bulbs and fluorescent lights.

Temperatures were set to 25 ºC during the light period and to 20 ºC during the dark period, whereas relative humidity was maintained at 75% throughout day and night. The standard solution for hydroponic growth of *G. max* 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 $NiSO_4$. Hydroponic solution was buffered with the addition of 1mM MES [2-(N-morpholino)ethanesulfonic acid], pH 5.5 and, during the experimental time, pH was measured and solutions were changed every three days. The experiment ended 14 days after transferring the plants to the climate chamber.

2.2 Fe determination by ICP-OES

One hundred mg of dried plant tissue (root and trifoliate leaves) was mixed with 5 mL of 65% HNO_3 in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Each plant organ from all the treatments (n=5) was ground and five independent digestions were carried out.

The digestion procedure was conducted in five steps, consisting of different temperature and time sets: 130°C/10min, 160°C/15min, 170°C/12min, 100°C/7min, and 100°C/3min. The resulting solutions of the digestion procedure were then brought to 20 mL with ultrapure water and filtered for further analysis. Mineral concentration determination was performed using inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 7000 DV (PerkinElmer, USA) with radial configuration.

2.3 ALA and hemin quantification

Protocols for ALA quantification in the leaves were optimized based on (Mauzerall and Granick, 1956). In short, 200 mg of ground sample were suspended in 1.5 mL of 20 mM

potassium phosphate buffer (pH 6.8). After centrifuging for 10 min at 16000 *g*, 400 µL of the supernatant were mixed with 100 µL of acetylacetone. The mixture was incubated for 10 mins at 100 ºC and then transferred to RT, until cool. At this point, 500 µL of modified Ehrlich's reagent were added to each sample, the mixture was let to stand for 5 min, and then centrifuged for another 5 min at 16000 *g*. Absorbance was read at 553 nm and ALA concentration was calculated according to a standard (Sigma-Aldrich, #A3785) calibration curve.

Heme protein content quantification in leaves and roots was performed by measuring the oxidized version of this protein, hemin, using an enzymatic assay kit (Hemin Assay Kit; Sigma-Aldrich) following the manufacturer instructions.

2.4 Photosynthetic pigments quantification

Chlorophyll, anthocyanin and carotenoid concentrations were measured on the last fully expanded trifoliate leaf of plants grown in the previously described conditions (n=5). The referred compounds were extracted and quantified according to a modified protocol of (Sims and Gamon, 2002). Absorbance was measured at 470, 537, 647 and 663 nm. The amount of anthocyanins, chlorophyll *a* and *b* and carotenoids were determined through the equations referred by (Sims and Gamon, 2002).

2.5 Lipid peroxidation assay

MDA was measured using a colorimetric method adapted from (Li, 2000). In short, 0.1 g of roots or trifoliate leaf samples (n=5) were homogenized in 10 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v) and incubated at 100 ºC for 30 mins. The reaction was stopped on ice and samples were centrifuged at 5000 rpm for 10 mins. The supernatant

was filtered, absorption was read at 450, 532 and 600 nm and MDA concentration (µmol g^{-1}) was calculated from: 6.45 x (A_{532} – A_{600}) – 0.56A_{450}.

2.6 Enzymatic analysis

Root iron reductase activity was quantified as described by (Vasconcelos et al., 2006). The measurements were carried out in roots of intact plants via the spectrophotometric determination of Fe^{2+} chelated to BPDS (bathophenanthroline disulfonic acid). Roots of each plant were submerged in 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 (ethylenediaminetetraacetic acid) and 300 µM BPDS. All nutrients were buffered with 1 mM MES, pH 5.5. The assays were conducted under dim light conditions at 20 ºC and were terminated after 45 min by removal of the roots from the assay solution. Absorbance values were obtained spectrophotometrically at 535 nm, and an aliquot of the solution that had no roots during the assay was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 $mM^{-1}cm^{-1}$.

For the evaluation of CAT and APX activity, an enzymatic extraction was performed according to (Ruley et al., 2004). Roots and trifoliate leaf samples were analyzed separately (n=5) and 100 mg of ground tissue were homogenized with 1.5 mL of extraction buffer composed of 0.1 M potassium phosphate buffer (pH 7.0), 0.1 mM EDTA and 1% polyvinylpyrrolidone. Samples were vortexed for 2 min and then centrifuged for 10 min at 5000 rpm at 4 ºC. The supernatant was collected and diluted 3-fold. CAT was measured using 666 µL of the diluted supernatant, to which 334 µL of 73 mM H_2O_2 in 0.5 M Tris-HCl buffer (pH 7.0) was added. Absorbance was read for 3 min at 240 nm and calculated according to (Aebi 1983). APX was measured using 100 µL of the initial

supernatant, to which 450 μL of 25 mM ascorbic acid and 450 μL of 17 mM H_2O_2 in 0.5 M Tris-HCl buffer (pH 7.0) were added. Absorbance was measured for 3 min at 290 nm and calculated according to (United States Environmental Protection Agency, 1994).

For GR, 100 mg of ground roots and trifoliate leaf tissue (n=5) was homogenized with 1.5 mL of extraction buffer containing 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The mixture was vortexed for 2 min and centrifuged for 10 min at 5000 rpm at 4 ºC. To 100 μL of the previous mixture, 1 mL of a solution containing 1 mM EDTA, 0.5 mM GSSSG, 0.15 mM NADPH, 50 mM Tris-HCl buffer (pH 7.5) and 3 mM $MgCl_2$ was added to each sample. Absorbances was read for 1 min at 340 nm and calculated according to (Groppa et al., 2001).

2.7 Statistical analysis

Data were analyzed with GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences between treatments were tested with ANOVA corrected for multiple comparisons using *Tukey* method. Statistical significance was considered at $P < 0.05$.

Principal component analysis (PCA) was performed to establish the relationships among the different variables. The data set included 16 continuous variables, namely, the concentration of anthocyanins, total chlorophylls, carotenoids, leaf ∂-aminolevulinic acid, root ∂-aminolevulinic acid, leaf hemin, root hemin, leaf MDA and root MDA; and the activity of leaf APX, root APX, leaf GR, root GR, root reductase, leaf CAT and root CAT. This analysis was performed using Tanagra data mining software, version 1.4.5 (Lyon, France) (Rakotomalala, 2005).

3 Results

3.1 Growth and chlorosis evaluation

The main symptoms of IDC consist on stunted growth, interveinal chlorosis on the youngest leaves and reduced Fe concentration in plant organs (Prasad, 2003), and these factors were evaluated in both EF and INF lines (Figure 1). INF plants under Fe deficiency had the lowest total plant DW (0.90 ± 0.08 g), which corresponded to about half of the DW observed in INF plants under Fe sufficiency and in EF plants under Fe deficiency (Figure 1A). Visible interveinal chlorosis with remaining green veins was apparent in both lines under Fe deficiency, but was more acute in INF plants, confirming their initial classification (Figure 1B).

Figure 1: Morpho-physiological effects of Fe deficiency in efficient (EF) and inefficient (INF) soybean lines. (A) Total dry weight (DW); (B) Chlorosis symptoms; (C) Fe concentration in roots and trifoliate leaves. Plants were grown under Fe sufficiency (+Fe, 20 μM) or Fe deficiency (-Fe, no additional Fe) for 14 days under hydroponic conditions. Data are means ± SE; different letters indicate significant differences ($P < 0.05$) by ANOVA with *Tukey* correction test.

As expected, Fe concentration was about two times lower in Fe deficient roots of both EF and INF plants, when compared to the Fe-sufficient plants (Figure 1C). In INF

plants, Fe was mostly accumulated in the root tissues, with very low levels of leaf Fe concentration. In contrast, the EF plants had higher concentrations of Fe in the trifoliate leaves and no significant differences were found between this organ and the roots.

3.2 ALA as the precursor for chlorophyll and heme

The compound ALA is the precursor of the tetrapyrrole cycle, which ends with the biosynthesis of chlorophyll and heme and has a role in antioxidant metabolism and metabolite accumulation (Hotta et al., 1997).

Although Fe stress did not cause a significant effect on ALA concentrations, under Fe deficiency, the INF plants accumulated 40% less ALA than the EF ones in the trifoliate leaves (Figure 2A). This decreased leaf ALA concentration was reflected in about 50% lower chlorophyll *a* and *b* levels in the INF plants under Fe deficiency (Figure 2B). On the other hand, the concentration of the oxidized form of heme – hemin – was always higher in INF tissues when compared to the EF counterparts (Figure 2C).

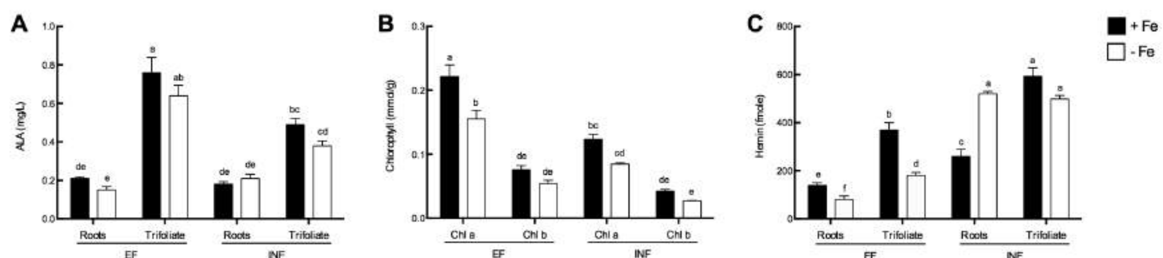

Figure 2: ∂-aminolevulenic acid (ALA), leaf chlorophyll and hemin concentrations in roots or trifoliate leaves of efficient (EF) and inefficient (INF) soybean lines. (A) ALA, (B) leaf chlorophyll *a* (Chl a) and *b* (Chl b), and (C) hemin concentrations. Plants were grown under Fe sufficiency (+Fe, 20 µM) or Fe deficiency (-Fe, no additional Fe) for 14 days under hydroponic conditions. Data are means ± SE; different letters indicate significant differences ($P < 0.05$) by ANOVA with *Tukey* correction test.

Anthocyanins and carotenoids are associated to chlorophyll as photosynthetic pigments, and have activity as antioxidant molecules. Their concentrations were evaluated (Table 1). The accumulation of these pigments in INF plants was not significantly affected by Fe availability, but it was always lower when compared to the EF plants. More specifically, under Fe deficiency, INF plants accumulated 32% less anthocyanins and 50% less carotenoids than EF plants (Table 1).

Table 1. Photosynthetic pigment concentrations (mmol/g) in the trifoliate leaves of efficient (EF) and inefficient (INF) soybean lines grown under Fe sufficiency (+Fe, 20 μM) or Fe deficiency (-Fe, no additional Fe) for 14 days under hydroponic conditions

Photosynthetic pigments	**EF**		**INF**	
	+ Fe	**- Fe**	**+ Fe**	**- Fe**
Total chlorophyll	0.298 ± 0.024^{a}	0.209 ± 0.018^{b}	0.166 ± 0.011^{bc}	0.112 ± 0.002^{c}
Anthocyanins	0.089 ± 0.008^{a}	0.056 ± 0.001^{b}	0.040 ± 0.004^{b}	$0.038 \pm 2.0e^{-4\ b}$
Carotenoids	0.128 ± 0.009^{a}	0.090 ± 0.006^{b}	0.058 ± 0.007^{c}	$0.045 \pm 1.9e^{-4\ c}$

* Data are means ± SE. In each row different letters indicate significant differences ($P < 0.05$) by ANOVA with *Tukey* correction test.

3.3 Enzymatic activity

As a first approach to the analysis of the oxidative stress in the tissues, lipid peroxidation, measured as the amount of MDA, was evaluated (Table 2). A significant effect of the soybean line on the lipid peroxidation levels in the roots was found, where the average of the MDA values of both Fe treatments of INF plants was 55% higher than the average of the Fe treatments of the EF plants. In contrast, in the trifoliate the opposite trend was

found, with a higher MDA concentration (20% increase) in the EF line. Moreover, there was no significant effect of the Fe availability on MDA accumulation.

Table 2. Malondialdehyde (MDA) concentration (nmol/g) in the roots and trifoliate leaves of efficient (EF) and inefficient (INF) soybean lines grown under Fe sufficiency (+Fe, 20 μM) or Fe deficiency (-Fe, no additional Fe) for 14 days under hydroponic conditions

	EF		**INF**	
MDA	**+ Fe**	**- Fe**	**+ Fe**	**- Fe**
Roots	28 ± 1.1^{b}	32 ± 2.7^{b}	44 ± 1.4^{a}	49 ± 1.5^{a}
Trifoliate	28 ± 1.2^{a}	23 ± 1.0^{ab}	19 ± 1.6^{b}	21 ± 3.0^{ab}

* Data are means ± SE. In each row different letters indicate significant differences ($P < 0.05$) by ANOVA with *Tukey* correction test.

Ferric reductase is a heme-containing enzyme (Jeong and Connolly, 2009) and its activity is often described as a limiting factor for Fe uptake in dicotyledonous plants, especially under stress conditions (Jain et al., 2014). INF plants presented significantly lower levels of reductase induction when compared to the EF plants (Table 3). Under Fe stress, root reductase activity of INF plants was of 0.007 ± 0.001 μmol Fe / g FW h, which was three times lower than that of the EF plants (0.021 ± 0.005 μmol Fe / g FW h).

Table 3. Root ferric reductase activity (µmol Fe/g FW h) of efficient (EF) and inefficient (INF) soybean lines grown under Fe sufficiency (+Fe, 20 µM) or Fe deficiency (-Fe, no additional Fe) for 14 days under hydroponic conditions

	EF		**INF**	
	+ Fe	**- Fe**	**+ Fe**	**- Fe**
Root reductase activity	0.0384 ± 0.052[a]	0.0210 ± 0.006[b]	0.0142 ± 0.0029[bc]	0.007 ± 0.001[c]

* Data are means ± SE. Different letters indicate significant differences ($P < 0.05$) by ANOVA with *Tukey* correction test.

CAT levels were highly increased in the INF plants when compared to the EF ones (Figure 3A). In general, Fe availability did not have an effect on CAT activity, with the exception of the roots of Fe sufficient INF plants, that showed significantly 30% lower activity than the Fe deficient roots.

APX presented an opposite activity pattern to CAT, where it was lower in the INF plants when compared to the EF ones, with no significant changes registered between Fe treatments and tissues (Figure 3B).

Finally, in the INF plants GR activity was lowest in the roots, but was highly induced in the trifoliate leaves, with Fe deficiency leading to a 30% increase when compared to Fe sufficiency (Figure 3C). Concerning the activity of this enzyme in the EF plants no significant changes were registered between Fe treatments both in roots and shoots.

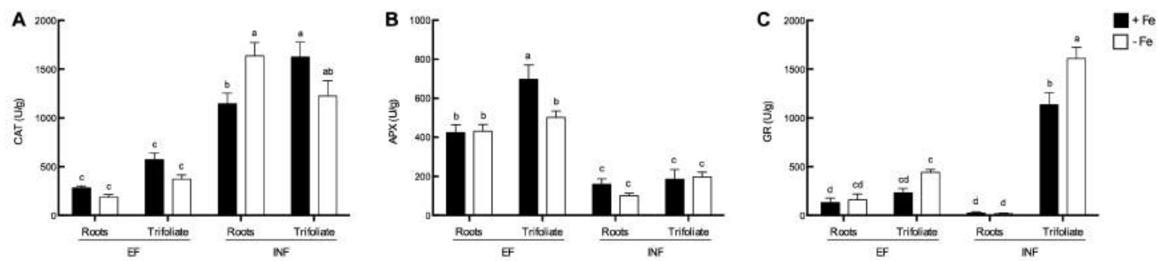

Figure 3: Enzyme activity in the roots and trifoliate leaves of efficient (EF) and inefficient (INF) soybean lines. (A) catalase activity (CAT); (B) ascorbate peroxidase activity (APX); (C) glutathione reductase activity (GR). Plants were grown under Fe sufficiency (+Fe, 20 μM) or Fe deficiency (-Fe, no additional Fe) for 14 days under hydroponic conditions. Data are means ± SE; different letters indicate significant differences ($P < 0.05$) by ANOVA with *Tukey* correction test.

3.4 Principal component analysis

A PCA model was performed to extract the most important information from the current data set. The resulting components explained 75% of the variance (Figure 4).

When analyzing the score plot of PC1 vs PC2 (Figure 4) it was found that samples were grouped in four clusters: two of them, corresponding to EF or INF plants, were separated along the PC1 (60% of total variance) and the other two, corresponding to Fe deficient and Fe supplied plants, were separated along the PC2 (15% of total variance).

Moreover, a high correlation between the photosynthetic pigments, leaf ALA concentration, APX activity (leaves and roots), leaf MDA concentration, GR activity in the roots and root reductase activity was observed. These factors were also highly correlated to the EF plants. On the other hand, root ALA concentration, hemin concentration (leaves and roots), CAT activity (leaves and roots), leaf GR activity and root MDA concentration were grouped, being correlated to the INF plants.

Additionally, it was possible to further correlate: photosynthetic pigments, leaf MDA concentration, leaf ALA concentration and leaf activity of APX with Fe sufficient EF plants; root APX activity, root GR activity and root reductase activity with Fe deficient EF plants; root ALA concentration, hemin concentration and CAT activity with Fe sufficient INF plants; and leaf GR activity and root MDA concentration with Fe deficient INF.

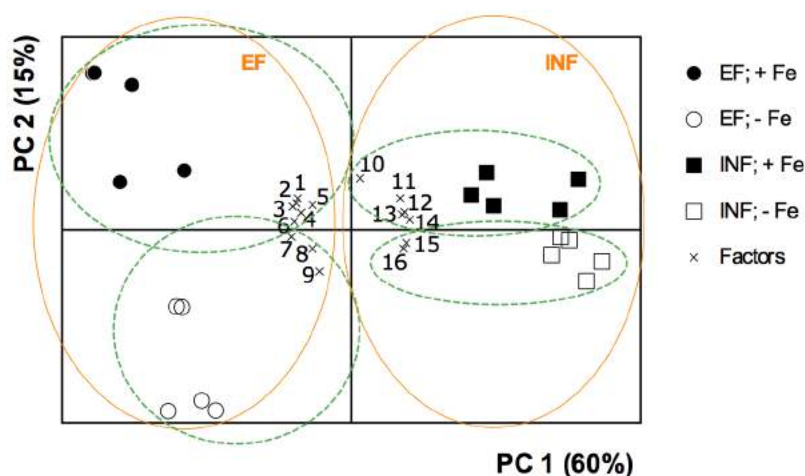

Figure 4: Biplot of score and loading factors of the Principal Component Analysis (PCA). Efficient (circles) and inefficient (squares) soybean lines, grown under Fe sufficiency (+Fe, 20 μM; solid symbols) or Fe deficiency (-Fe, no additional Fe; open symbols) for 14 days under hydroponic conditions and associated factors: 1-anthocyanin concentration; 2- total chlorophyll concentration; 3-carotenoid concentration; 4-leaf ∂-aminolevulinic acid concentration; 5-leaf MDA concentration; 6-leaf ascorbate peroxidase activity; 7-root ascorbate peroxidase activity; 8-root gluthatione reductase; 9- root reductase activity; 10-root ∂-aminolevulinic acid concentration; 11-leaf hemin concentration; 12-root hemin concentration; 13-leaf activity of catalase; 14-root activity of catalase; 15- leaf activity of gluthatione reductase; 16- root MDA concentration.

4 Discussion

In calcareous soils, Fe uptake is impaired causing severe yield losses in different crops worldwide. One of the possible strategies to reduce this problem is to select tolerant or efficient cultivars that are able to sustain Fe deprivation stress (Carvalho and Vasconcelos, 2013). As aforementioned, the definition for this Fe efficiency comprises the ability to induce biochemical reactions that make Fe available in a useful form (Brown and Jolley, 1989). However, this definition still lacks information on other factors that could contribute to this trait and recent studies have shown the importance of physiological (Vasconcelos and Grusak, 2014; Roriz et al., 2014) and molecular (Santos et al., 2015) mechanisms in the Fe efficiency trait of soybean plants. Meanwhile, recent studies reported an induction of oxidative stress related reactions when Fe is unavailable for plant uptake and mobilization, since this nutrient is essential for a vast number of biological processes (Li et al., 2015; Mbonankira et al., 2015).

Taking into account that the ability to induce the antioxidant machinery could have an important role in the Fe efficiency trait and that, to the best of our knowledge, no studies have focused on the tetrapyrrole cycle regulation in plants under Fe deficiency, in this study, an integrative overview was adopted to understand the differences between two soybean lines with contrasting susceptibilities to Fe limitation. Firstly, the difference in susceptibility to Fe stress was evaluated looking at the main symptoms associated to IDC, namely, stunted growth and interveinal chlorosis. As seen in Figure 2, INF plants were smaller (Figure 1A) and displayed more noticeable visual IDC symptoms than the EF plants (Figure 1B), which confirmed previous studies using these accessions (Santos et al., 2015). Previous works have also shown that INF soybean lines have less Fe translocation ability and tend to accumulate most of the absorbed Fe in the root tissue (Roriz et al., 2014;

Santos et al., 2015). This was also true in the present study (Figure 1C), showing that this factor could be one of the major contributors for Fe stress tolerance.

In higher plants, the tetrapyrrole cycle begins with glutamate being converted on the universal tetrapyrrole precursor – ALA – forming its final products, the porphyrins chlorophyll and heme (Tanaka et al., 2011). Lately, this cycle has gained especial attention in the Fe metabolism research area, not only because Fe is essential for chlorophyll and heme biosynthesis (Jeong et al., 2008), but also due to the fact that the heme prosthetic group could work as a 'sensor' in plants, in particular, for Fe deficiency (Kobayashi and Nishizawa, 2014). Here, Fe deficiency did not have a significant effect on ALA accumulation, but the INF plants had lower leaf ALA concentrations when compared to the EF plants (Figure 2A). It is known that the exogenous application of ALA has plant growth promoting properties and induces higher chlorophyll accumulation, therefore, the positive correlation between these two products is well described (Hotta et al., 1997; Yang et al., 2014). As expected, the chlorophyll concentrations shared similar patterns of accumulation as the leaf levels of ALA, as depicted in Figure 2B, and mirrored the visual symptoms presented in Figure 2b. Under oxidative stress conditions, heme is released from hemoproteins and forms hemin, its oxidized form (Chiabrando et al., 2014). Here, hemin accumulation did not show a pattern similar to ALA in either root or leaf tissue, but had a significant increase in the leaves, particularly in the INF plants (Figure 2C). This fact is important since the tetrapyrrole cycle is mainly located in photosynthetic tissues (Tanaka et al., 2011), which is in agreement with the higher leaf accumulation levels here obtained. Also, chlorophyll and hemin results did not share similar patterns and although a putative negative correlation has been proposed for the two branches, the interplay between them is still largely unknown (Zhang et al., 2015). Furthermore, hemin is a form of protoporphyrin IX containing ferric Fe (Müllebner et al., 2015) and, when present, it also acts as a strong

pro-oxidant in cells due to its participation in H_2O_2-dependent redox reactions and to the release of ferric Fe upon its degradation (Müllebner et al., 2015; Lu et al., 2012). These reactions cause the reduction of molecular oxygen and form reactive oxygen species (Pospíšil, 2014) thus, intracellular accumulation of hemin is highly toxic for cells.

Given the fact that Fe is an essential constituent of chlorophyll, under Fe limitation, these other photosynthetic pigments are expected to decrease under Fe stress (Prasad, 2003). Anthocyanin and carotenoid levels were lower in INF plants, but the Fe treatment did not affect their accumulation in the leaf tissue (Table 1). As seen in Figure 2C, INF plants had higher levels of hemin, very likely inducing higher levels of photooxidative damage. When chloroplasts of the mesophyll cells cease to function or are damaged, both anthocyanins and carotenoids have an important photoprotective role, acting as powerful antioxidants (Landi et al., 2014; Spirt et al., 2010). Thus, since INF plants showed lower levels of these molecules, their capability to manage photooxidation could be hampered. Furthermore, in Table 2 is shown that MDA levels were higher in the roots of INF plants than in EF plants, corroborating that the former plants were under higher oxidative damage, since this is an often used oxidative stress indicator (Gill and Tuteja, 2010; Santos et al., 2016).

The membrane-bound ferric chelate reductase enzyme contains the heme-group as a constituent, and it is responsible for the reduction of extracellular Fe with its activity being necessary for Fe uptake (Robinson et al., 1999). In this study, Fe deficiency did not induce higher levels of reductase activity (Table 3), although this would be the expected phenotype (Robinson et al., 1999). It is not the first time that plants of this soybean line express this behavior, however, the INF plants consistently show lower levels of this enzymes' activity (Santos et al., 2015; Vasconcelos and Grusak, 2014), which is in agreement with the classical definition that states that INF plants are not able to induce the

biochemical reactions necessary for Fe uptake (Brown and Jolley, 1989). Interestingly, INF plants, that showed lower capability to reduce Fe, had higher levels of hemin (Table 3 and Figure 2C).

Alike ferric reductase, there are other heme enzymes that are susceptible to low Fe supply, namely catalases and peroxidases (Broadley et al., 2012). Again, as increased levels of hemin promotes oxidative damage (Lu et al., 2012), this could elicit the activity of these enzymes. This was true for CAT enzyme (Figure 3A), which showed enhanced activity in both roots and leaves of INF plants. In fact, the accumulation pattern for both catalase and hemin was very similar (Figs 3A and 2C, respectively). On the other hand, APX activity was lower in the INF plants and higher in the EF ones (Figure 3B). Besides being known that APX activity is drastically reduced in Fe deficient conditions (Jelali et al., 2014), the inverse regulation between this enzyme and CAT is also well documented both in response to Fe stress (Mbonankira et al., 2015) and to other metals (Kayıhan et al., 2016). Both enzymes are responsible for the conversion of H_2O_2 into water, however, while CAT is able to directly reduce H_2O_2 into water with no energy consumption, APX requires ascorbate as a reducing equivalent (Gill and Tuteja, 2010). Since the latter is a more energy demanding reaction, inefficient genotypes have been reported to decrease APX activity under stress conditions (Broadley et al., 2012) as registered in the present study.

Also involved in the ROS detoxification is GR, which is responsible for the reduction of the oxidized form of glutathione (glutathione disulfide, GSSG) to glutathione, that is able to scavenge H_2O_2 through the ascorbate-glutathione cycle (Gill et al., 2013). Here, INF roots, which had the lowest GR activity, had the highest MDA accumulation; INF trifoliate leaves, that showed an abrupt increase in GR activity, had the lowest MDA accumulation (although not significant when compared to EF trifoliate leaves); and EF

roots and trifoliate leaves, did not present any significant change in GR activity, as also seen in MDA results (Figure 3C and Table 2). As described by others, the up-regulation of the antioxidant systems has a direct effect on peroxidative conditions, particularly GR that, as shown here, contributes directly for MDA accumulation decrease (Agarwal and Shaheen, 2007).

The correlation analysis performed here (Figure 4) shows that the efficient and inefficient lines have distinct behaviors and are clearly separated in what concerns oxidative stress response. Moreover, while in the INF plants group there was almost no separation between +Fe and –Fe treatments, the EF plants group is divided in two sub-groups correspondent to the Fe treatment. It is evident that hemin levels are highly correlated to the INF plants, which could be key to explain the trait of inefficiency: as these plants are unable to reduce the oxidative stress caused by Fe deficiency, heme molecules are oxidized and, consequently, unavailable to integrate in the Fe metabolism related enzymes. This could explain the lower levels of ferric reductase enzyme induction by INF plants, observed here (Table 3) and in other studies (Santos et al., 2015). The presence of enhanced hemin levels could have caused more oxidative stress, particularly on the root tissue (Table 2), and INF plants only seem to trigger the low substrate affinity enzyme CAT. Also, INF plants were correlated to GR activity at the leaf level, possibly due to the high chlorosis developed by these plants (Figure 1). Additionally, Figure 4 displays a high correlation of EF plants with the antioxidant pigments, leaf MDA accumulation and APX accumulation.

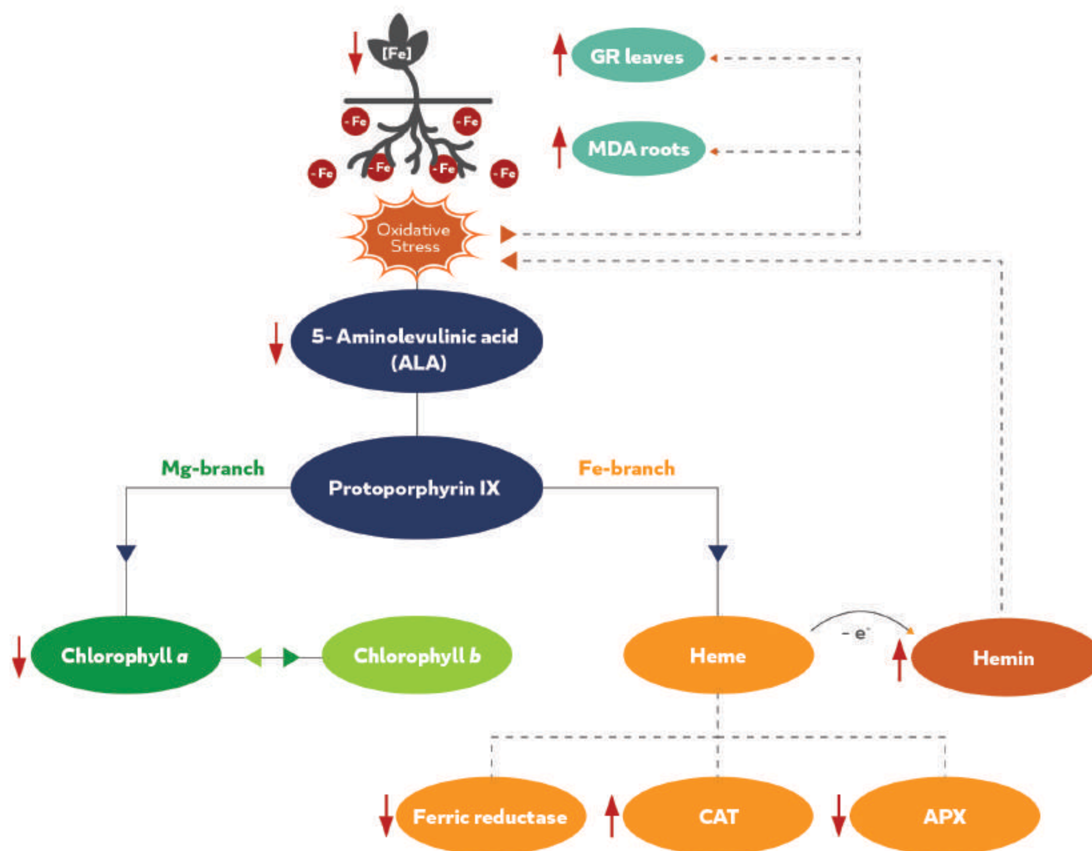

Figure 5: Proposed scheme for antioxidant system regulation of inefficient (INF) plants under Fe deficiency (- Fe) stress. Full lines connect the main components of the tetrapyrrole cycle; dashed lines represent the influence of one product on another; red arrows represent increased (up) or decreased (down) concentration of a certain product.

5 Conclusions

We propose a possible schematic model to explain the antioxidant responses that characterize a plant as INF when exposed to Fe-stress (Figure 5). In this model, we suggest that, as a consequence of Fe deficiency stress, oxidative stress levels increase and ALA levels decrease in the trifoliate leaves, inducing decreased chlorophyll content and heme oxidation into hemin. Since hemin is a strong pro-oxidant, it contributes to greater

accumulation of oxidative stress. This is reflected in higher MDA accumulation in the roots and higher GR activity in the leaf tissue, where Fe concentration is severely decreased. Given this heme/hemin pool imbalance, heme does not seem to be available for enzyme integration, and the activity of heme-containing enzymes, such as root ferric reductase and APX, is decreased. On the other hand, the available heme seems to be channeled into the antioxidant enzyme CAT, which is the less energy-requiring enzyme to trigger.

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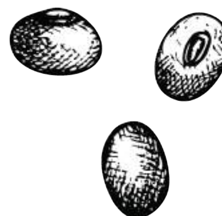
CHAPTER 4

Finding an alternative for IDC prevention

In this chapter, after gathering the molecular and physiological tools developed in Chapters 2 and 3, a new Fe chelating agent will be tested for its efficacy in IDC prevention in soybean plants.

CHAPTER 4



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Research article

Effect of *tris*(3-hydroxy-4-pyridinonate) iron(III) complexes on iron uptake and storage in soybean (*Glycine max* L.)

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ABSTRACT

Iron deficiency chlorosis (IDC) is a serious environmental problem affecting the growth of several crops in the world. The application of synthetic Fe(III) chelates is still one of the most common measures to correct IDC and the search for more effective Fe chelates remains an important issue. Herein, we propose a *tris*(3-hydroxy-4-pyridinonate) iron(III) complex, $Fe(mpp)_3$, as an IDC corrector. Different morphological, biochemical and molecular parameters were assessed as a first step towards understanding its mode of action, compared with that of the commercial fertilizer FeEDDHA. Plants treated with the pyridinone iron(III) complexes were significantly greener and had increased biomass. The total Fe content was measured using ICP-OES and plants treated with pyridinone complexes accumulated about 50% more Fe than those treated with the commercial chelate. In particular, plants supplied with compound $Fe(mpp)_3$ were able to translocate iron from the roots to the shoots and did not elicit the expression of the Fe-stress related genes *FRO2* and *IRT1*. These results suggest that 3,4-HPO iron(III) chelates could be a potential new class of plant fertilizing agents.

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1. Introduction

Soybean (*Glycine max* L.) production reaches levels of about 230 million metric tons per year across the world (Vasconcelos and Grusak, 2014). This legume is a highly nutritious crop, containing more protein (40%) and oil (20%) than any other ordinary food source, including meat, cheese and fish (Krishnan, 2005; Bolon et al., 2010).

Iron (Fe) deficiency chlorosis (IDC) is a severe problem affecting crops mainly in areas of alkaline soils (Chaney, 1985), which correspond to approximately 30% of the world's arable land. Under such conditions, and despite its abundance in the earth's crust, Fe becomes insoluble and poorly bioavailable for uptake (Chaney, 1985; Marschner et al., 1996). Iron is necessary for various physiological processes such as chlorophyll synthesis, respiration, nitrogen fixation, enzyme activation and electron transfer (Taylor et al., 1982; Engels et al., 2012). Fe-deficient plants develop yellowing of the younger leaves, exhibit reduced leaf areas and shoot and root dry weight (Roriz et al., 2014), leading to reduced crop yield and serious economic losses. In order to overcome this mineral deficiency, plants induce tightly regulated mechanisms to maximize iron uptake from the soil (Hindt and Guerinot, 2012; Sperotto et al., 2012). Dicotyledonous plants, like soybean, utilize strategy I type-mechanisms for Fe uptake. Root H^+-ATPases acidify the rhizosphere so that Fe(III) solubility is increased, allowing Fe(III) reduction by membrane-bound ferric reductases, like Ferric Reductase Oxidase 2 (FRO2). The reduction step has been shown to be a crucial step in Fe acquisition, since plants suffering from Fe deficiency often increase this genes' activity (Grusak et al., 1990).

Abbreviations: FeEDDHA, iron (III) complex of ethylenediamine-*N,N'*-*bis*(o-hydroxyphenyl)acetic acid; $Fe(mpp)_3$, *tris*(2-methyl-3-hydroxy-4-pyridinonate) iron(III); FRO, ferric reductase oxidase; IDC, iron deficiency chlorosis; IRT, iron-regulated transporter.

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After ferric Fe reduction, Fe(II) is then absorbed into the root epidermal cells by Fe transporters, such as Iron-Regulated Transporter 1 (IRT1) (Fox and Guerinot, 1998). Once inside the plant, to cope with Fe toxicity, most of the Fe fraction can be stored in plastids by ferritins, key proteins in Fe homeostasis and response to environmental stresses (Roschzttardtz et al., 2013). Moreover, Fe storage can also occur in vacuoles, in a process mediated by a vacular iron transporter (Briat et al., 2007). Besides these mechanisms, it is known that phenolic compounds, which are released by the roots, have a role in the reduction of Fe (III) to Fe (II) (Cesco et al., 2010; Mimmo et al., 2014). More recently, it has been shown that flavins and scopoletins are also involved in the solubilisation of apoplasmic Fe (Jin et al., 2007; Fourcroy et al., 2014; Schmid et al., 2014).

Soybean is very susceptible to IDC and it has been used to study physiological and molecular mechanisms related to Fe uptake, transport and accumulation (Vasconcelos et al., 2006; Roriz et al., 2014). Also, cultivars with contrasting susceptibilities to IDC are available, which makes soybean a good crop to study these mechanisms (Vasconcelos and Grusak, 2014). Conventional plant breeding is one of the most well accepted measures to select tolerant lines to IDC, however with only limited success (Carvalho and Vasconcelos, 2013). The application of Fe-fertilizers is still vastly used in an agricultural context to correct Fe chlorosis (Abadia et al., 2011), and the parameters for evaluating the efficacy of Fe chelates have been described: the ligands should be able to maintain large amounts of Fe in solution, to enable plants to use the Fe and, when free, should be able to take more Fe and supply it again to the plant (Lucena, 2003). This implies that the ligand should have affinity for Fe, high solubility in water and bioavailability to the plant.

Three main categories of Fe-fertilizers are known: (i) inorganic Fe compounds, such as Fe salts and insoluble oxides, that have low efficiency in the soil as they rapidly transform into insoluble compounds, being usually applied as foliar fertilizers (Shenker and Chen, 2005); (ii) natural Fe complexes, such as humates, amino acid and citrate complexes, also applied as foliar fertilizations due to their low stability in the soil, and (iii) synthetic Fe-chelates with ligands such as ethylenediamine tetraacetic acid (EDTA), ethylenediamine-*N,N′*-bis(*o*-hydroxyphenylacetic) acid (EDDHA), *N,N′*-bis(2-hydroxybenzyl)ethylenediamine-*N,N′*-diacetic acid (HBED) and N,N′-bis(2-hydroxy-5-methylbenzyl)ethylenediamine-N,N′-diacetic acid (HJB), mainly for soil application (Lopez-Rayo et al., 2009), and ethylenediaminedisuccinic acid (EDDS), for foliar application (Rodríguez-Lucena et al., 2010).

EDTA and EDDHA are the most commonly used Fe chelators in an agricultural context (Abadia et al., 2011). However, when plants are grown in hydroponic conditions, EDTA is not able to maintain the given amount of Fe in the solution, resulting in less available Fe to the plant when compared to FeEDDHA (Lucena, 2003). The compounds HBED and HJB are hexadentate ligands and have been tested for their ability to maintain Fe in soil solution (Lopez-Rayo et al., 2009) and in calcareous soil conditions (Nadal et al., 2012, 2013), having shown effective results in agronomical conditions.

From the chemical point of view, the previously mentioned chelating agents are hexadentate ligands of the polyaminocarboxylate family (Álvarez-Fernández et al., 2005; Gomez-Gallego et al., 2005; Lopez-Rayo et al., 2009).

Although it is recognized that the polyaminocarboxylate chelating agents are efficient in the treatment of IDC, the ligands are under investigation due to their persistence on the environment (Nowack, 2002, 2008). The limited amount of alternative Fe complexes calls for the identification of novel chelators which can be highly soluble, cost effective, highly bioavailable to the plant and environmentally friendly.

The chemistry of 3-hydroxy-4-pyridinone ligands (3,4-HPO) and their complexes (Burgess and Rangel, 2008) as well as their biological (Rangel et al., 2009; Nunes et al., 2010; Moniz et al., 2011, 2013a) and analytical applications (Mesquita et al., 2013; Suárez et al., 2015) have been thoroughly studied. 3-Hydroxy-4-pyridinones are synthetically versatile bidentate oxygen ligands, which allow the synthesis of a variety of chelators of variable denticity and physico-chemical properties (Silva et al., 2010; Leite et al., 2011; Moniz et al., 2013b; Queirós et al., 2014). The ligands have interesting structural and solvation properties and in particular have a strong affinity towards M(III) and M(II) metal ions forming a large variety of complexes (Burgess and Rangel, 2008). Most ligands of the 3,4-HPO family are non-toxic and have been utilized in biomedical applications, namely in the treatment of iron overloaded patients suffering from β-thalassemia (Galanello, 2007). They are hard ligands that bear two oxygen coordinating atoms and consequently show a very high capacity to trap Fe(III) providing an O_6 coordination sphere for Fe(III) through the binding of three ligands originating a complex of the $[FeL_3]$ type. The observed values of stability constants and pFe are of the same magnitude of those observed for the chelates of the hexadentate polyaminocarboxylate ligands (Lopez-Rayo et al., 2009, 2010). Complexation of 3,4-HPO bidentate ligands with Fe in aqueous solution involves formation of three Fe(III) complex species, $[Fe(OH_2)_4L]^{2+}$, $[Fe(OH_2)_2L_2]^{+}$ and $[FeL_3]$ whose relative amount is dependent on the amount of ligand and the pH of the solution (Liu and Hider, 2002; Nurchi et al., 2008; Santos et al., 2012). The different stability constants of the corresponding polyaminocarboxylate and 3,4-HPO Fe(II) complexes is indicative of lower values of redox potentials for the pyridinone complexes (Burgess and Rangel, 2008).

In this work we investigated the potential of an Fe(III) complex of the ligand 2-methyl-3-hydroxy-4-pyridinone (Hmpp) (Fig. 1) as a potential Fe chlorosis corrector. To the best of our knowledge, 3,4-HPO chelates have never been used as Fe chelates for plants, and this work is a first report on their application as a new class of plant fertilizers. Due to its high solubility in water (Burgess and Rangel, 2008), low cost and simplicity, we analysed the potential of $Fe(mpp)_3$ as a chlorosis correcting agent and looked at several parameters in plants at a morphological, physiological, biochemical and molecular level in order to compare its ability to deliver Fe to the plant with that of the commercial fertilizer FeEDDHA.

2. Materials and methods

2.1. Iron(III) chelates

The commonly used ethylenediamine-*N,N′bis* (o-hydroxyphenyl)acetic acid (EDDHA) was used as a comparison term to the new chelate. FeEDDHA was purchased from *Phyto*Technology Laboratories (#16455-61-1). The 3-hydroxy-4-pyridinone ligand, Hmpp, and the corresponding Fe(III) complex, $Fe(mpp)_3$, were synthesized in-house.

2.1.1. Synthesis and characterization of the tris(3-hydroxy-4-pyridinonate)iron(III) complex

Reagents and solvents were purchased from Sigma-Aldrich as reagent-grade and used without further purification unless otherwise stated. The ligand Hmpp was synthesized according to the methods described in the literature (Queiros et al., 2011). Fe(III) complex of general formula, $FeL_3 \cdot x\ H_2O$ (L = 3-hydroxy-4-pyridinone) was prepared as described before (Schlindwein et al., 2006) by dissolving stoichiometric amounts of the iron salt $Fe(NO_3)_3 \cdot 9H_2O$ and the corresponding ligand in aqueous or ethanolic solutions and adjusting the pH to 8 with a diluted solution of

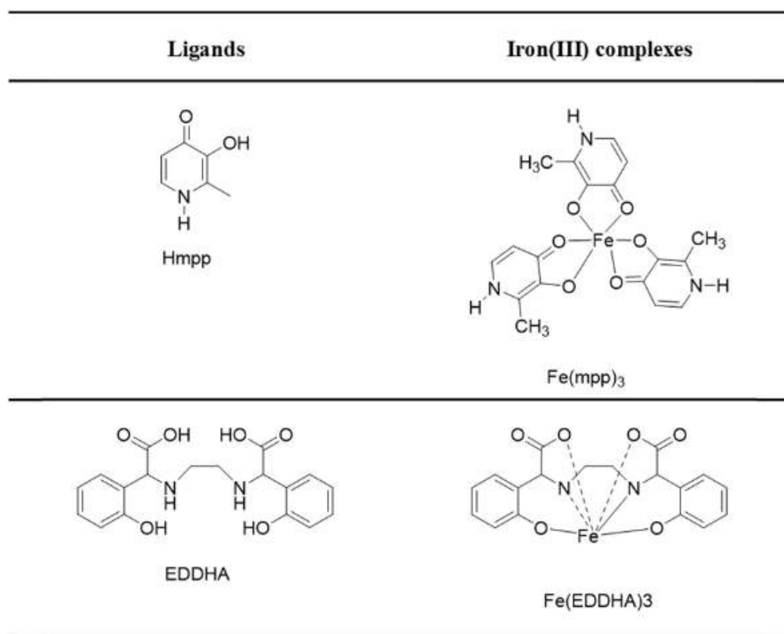

Fig. 1. Formulae and abbreviations of ligands (Hmpp, EDDHA) and Fe(III) chelates used in this work.

NaOH. The reaction mixture was kept with stirring, for one day at room temperature. The red precipitate that formed was collected by filtration and washed with water.

The characterization of the compounds was done according to the results obtained in Elemental Analysis (C, H, N), ^{1}H and ^{13}C NMR and UV–vis spectroscopy. NMR spectra were recorded with a Bruker Avance III 400 spectrometer (400.15 MHz for ^{1}H and 100.63 MHz for ^{13}C) at Laboratório de Análise Estrutural, Centro de Materiais da Universidade do Porto (CEMUP) (Portugal). Elemental analyses were performed at the analytical services of University of Santiago (Spain). The elemental analyses results obtained for the Fe(III) chelate are given below:

Tris (3-hydroxy-1-(H)-2-methyl-4-pyridinonate)iron(III), $Fe(mpp)_3 \cdot 4H_2O$.

Elemental analysis for $C_{18}H_{18}N_3O_6Fe \cdot 4H_2O$% calculated (% Found): C 43.22 (43.60) H 5.24 (5.23) N 8.40 (8.25).

2.1.2. Characterization of the Fe(III) chelates in the hydroponic solution

Electronic spectra were acquired for the aqueous solution of the Fe(III) chelate and for solutions with variable metal:ligand ratios. In order to characterize the Fe(III) chelate species in the conditions of the hydroponic solution, UV–visible spectra were obtained and the results compared with those obtained in aqueous solution and described in the literature (Nurchi et al., 2008).

2.2. Plant material, growth conditions and treatments

Seeds of *G. max* cultivar "Williams 82" were rolled in filter paper and placed vertically in a solution of 250 mM $CaCl_2$, for seven days in the dark, at 25 °C. Germinated seedlings were transferred to 5 L vessels (five seedlings per vessel). The vessels were placed in a climate chamber (Aralab Fitoclima 10000EHF) with 16 h day photoperiod providing 325 μmol s^{-1} m^{-2} of photosynthetic photon flux density at plant level supplied by a mixture of incandescent bulbs and fluorescent lights. Temperatures were set to 25 °C during the light period and to 20 °C during the dark period, whereas relative humidity was maintained at 75% throughout day and night. The standard solution for hydroponic growth of *G. max* included: 1.2 mM KNO_3; 0.8 mM $Ca(NO_3)_2$; 0.3 mM $MgSO_4 \cdot 7H_2O$; 0.2 mM $NH_4H_2PO_4$; 25 μM $CaCl_2$; 25 μM H_3BO_3; 0.5 μM $MnSO_4$; 2 μM $ZnSO_4 \cdot H_2O$; 0.5 μM $CuSO_4 \cdot H_2O$; 0.5 μM MoO_3; 0.1 μM $NiSO_4$. Hydroponic solution was buffered with the addition of 1 mM MES, pH 5.5 as this is the optimum pH for nutrients absorption and to understand plants' physiological and molecular responses (Li and Lan, 2015; Carrasco-Gil et al., 2016; Ziegler et al., 2016). Solutions were changed every three days.

Two different experiments were set. 'Experiment 1' consisted in growing plants with five different compounds at a final concentration of 20 μM. The treatments were: Fe(III) sulfate; Hmpp ligand; FeEDDHA; Fe (III) sulfate and Hmpp, in a 1:3 ratio (Fe + Hmpp); and $Fe(mpp)_3$ chelate. In order to further elucidate the $Fe(mpp)_3$ mode of action, a second experiment was set. 'Experiment 2' consisted in growing plants in three vessels with three different treatments: no added Fe (-Fe); 20 μM of FeEDDHA; and 20 μM of $Fe(mpp)_3$. Both sets of experiments ended 14 days after transferring the plants to the climate chamber.

2.3. Physiological parameters

Leaf chlorosis was assessed with Soil and Plant Analyzer Development (SPAD) readings, measured with a portable chlorophyll meter (Konica Minolta SPAD-502Plus; Minolta, Osaka, Japan), using the youngest trifoliate leaf of five independent biological replicates. Sampled roots, stems and leaves of the five independent biological replicates were separated and weighed. Foliar area of all leaves was measured using a leaf area meter AM300 (ADC BioScientific Ltd., U.K.).

2.4. Root iron reductase activity measurements

Root iron reductase was quantified as described before (Vasconcelos et al., 2006). The measurements were carried out in intact roots of five plants from 'Experiment 2' via the spectrophotometric determination of Fe^{2+} chelated to BPDS (bathophenanthroline disulfonic acid). Roots of each intact plant were submerged in 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 (ethylenediaminotetraacetic acid) and 100 μM BPDS. All nutrients were buffered with 1 mM MES, pH 5.5. The assays were conducted under dim light conditions at 20 °C and were terminated after 45 min by removal of the roots from the assay solution. Absorbance values were obtained at 535 nm, and an aliquot of the solution that had no roots during the assay was used as blank. Rates of reduction were determined using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}.

2.5. Total Fe determination and ionome study

The plant material from 'Experiment 2' was dried at 70 °C until constant weight and 100 mg of dried plant tissue (root, stem, cotyledon, unifoliate and trifoliate leaves) were mixed with 5 mL of 65% HNO_3 in a Teflon reaction vessel and heated in a SpeedwaveTM MWS-3+ (Berghof, Germany) microwave system. Each plant organ from all treatments (n = 5) was pulverized and five independent digestions were carried out. The 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 20 mL with ultrapure water for further analysis. Mineral concentration determination was performed using the inductively coupled plasma optical emission spectrometer (ICP-OES) Optima 7000 DV (PerkinElmer, USA) with radial configuration.

2.6. Lipid peroxidation assay

Malondialdehyde (MDA) was measured using a colorimetric method adapted from Li (2000). In short, 0.1 g of trifoliate leaf or root samples (n = 5) were homogenized in 10 mL of 0.5% thiobarbituric acid in 20% trichloroacetic acid (w/v) and incubated at 100 °C for 30 min. The reaction was stopped in ice and samples were centrifuged at 5000 rpm for 10 min. Supernatant was filtered, absorption was read at 450, 532 and 600 nm and MDA concentration (μmol g^{-1}) was calculated from: $6.45 \times (A_{532} - A_{600}) - 0.56A_{450}$.

2.7. Gene expression analysis

Additional plants were grown under the same conditions described for 'Experiment 2', collected at the end of the assay and immediately frozen in liquid nitrogen. Three biological replicates from each treatment were individually pulverized thoroughly with a mortar and pestle, until a fine powder was obtained, and total RNA was extracted using Qiagen RNeasy Mini Kit (USA, #74904), according to the manufacturer's instructions. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal). Single-stranded cDNA was then synthesized using First Strand cDNA Synthesis Kit (Thermo Scientific, #K1612) in a Thermal cycler (VWR, Doppio, Belgium), according to the manufacturer's instructions. Sequence homologs to *AtFRO2* and *AtIRT1* in *G. max* were queried in NCBI database and the sequences with highest homology were selected. Primers for *FRO2*-like, *IRT1*-like and *ferritin* were designed using Primer3 (Frodo.wi.mit.edu), specifying an expected PCR product of 100–200 bp and primer annealing temperatures between 56 and 58 °C (Table 1). qPCR reactions were performed on a StepOne™ Real-Time PCR Systems (Applied Biosystems, USA) with the following reaction conditions: 2 min at 50 °C, 2 min at 95 °C and 40 cycles with 15 s at 95 °C, 15 s at 58 °C and 1 s at 72 °C. Amplifications were carried out using 200 μM of the specific primers and mixed to 10 μL of 2xSYBR® Select Master Mix and 100 ng of cDNA in a final volume of 20 μl. Melt curve profiles were analysed for each tested gene. The comparative CT method (ΔΔCT) (Livak and Schmittgen, 2001) was used for the relative quantification of gene expression values of Fe related genes using the 18S rRNA gene as the control transcript and the plants grown with FeEDDHA as the reference sample. Two technical replicates were analysed and data were transferred to Excel files and plotted as histograms of normalized fold expression of target genes.

2.8. Statistical analysis

Data were analysed with GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com). Differences between treatments were tested with ANOVA corrected for multiple comparisons using Holm-Sidak method. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Chemistry of the new chelate

Fe-chelates of 3-hydroxy-4-pyridinone ligands, $[FeL_3]$, were obtained as stable hydrated crystalline powders with a high purity and the study of the interaction of 3,4-HPO ligands with Fe(III) in aqueous solution showed that the relative amount of the three possible Fe(III) complex species, $[Fe(OH_2)_4L]^{2+}$, $[Fe(OH_2)_2L_2]^{+}$ and $[FeL_3]$ was dependent on the amount of ligand and the pH of the solution as described in the literature (Liu and Hider, 2002; Nurchi et al., 2008; Santos et al., 2012). In order to characterize the Fe species present in the hydroponic medium at pH 5.5 we analysed the electronic spectra of the Fe^{3+}/ligand in aqueous solution with metal:ligand molar ratios of 1:1, 1:2 and 1:3 as well as the electronic spectra of the Fe-chelates upon dissolution in the hydroponic medium. The results obtained for $Fe(mpp)_3$ are shown in Fig. 2. The comparison of the spectra clearly shows that the predominant species in the hydroponic medium were the *tris* Fe-chelates, $[FeL_3]$.

3.2. Initial screening of $Fe(mpp)_3$ on plant growth ('Experiment 1')

In a first experiment, we tested the effect of plant supplementation with different compounds, including the Hmpp ligand alone, Fe(III) sulfate, a combination of Hmpp + Fe(III) sulfate, FeEDDHA and the chelated form of the complex $[Fe(mpp)_3]$ As expected, supplementation with Fe(III) sulfate or Hmpp alone had the lowest effect ($P < 0.05$) on all analysed parameters (Table 2). No significant differences were registered between these two treatments, with the exception of total leaf area, which was higher in Fe(III) sulfate treated plants. Regarding the plants supplied with Fe(III) sulfate + Hmpp, a significant improvement was registered in SPAD units (25%) and root DW (62%), when compared to the commercial fertilizer FeEDDHA (Table 2). Lastly, plants supplied with $Fe(mpp)_3$ showed the best performance in all studied growth parameters as compared to plants treated with FeEDDHA, resulting in 62% higher SPAD units, whereas the shoot DW, root DW and total leaf area more than doubled, increasing by factor 2.26, 2.48 and 2.35,

Table 1
Primer sequences and correspondent accession numbers (Acc. No).

Primer	Forward (5′–3′)	Reverse (5′–3′)	Acc. No
18S	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA	X75080.1
FRO2-like	CAGAACATGGAAGGGTCAAC	AGCAAGAACTCCCACACTTG	XM_003528793.2
IRT1-like	CTGAGGTTGTTCCTGGTGAG	TGCCAAGTCCTATCACCACT	KF542819.1
Ferritin	CAATGCTTCCTATGCGTACC	CTGAGGGGACATTCTTGATG	NP_001236534

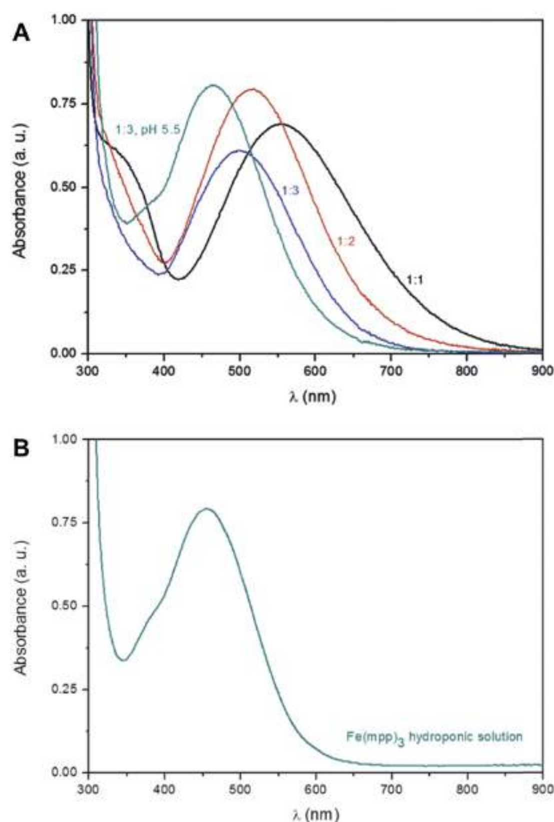

Fig. 2. Electronic spectra of the Fe^{3+}/Hmpp system obtained in aqueous solution with metal:ligand molar ratios of 1:1, 1:2, 1:3 and 1:3 at the pH value of hydroponic solution (from right to left) (A); Electronic spectrum of $Fe(mpp)_3$ complex upon dissolution in the hydroponic solution (B).

3.3. IDC symptoms evaluation ('Experiment 2')

In a second experiment, a mode detailed evaluation was performed in plants grown with no Fe (-Fe), FeEDDHA or with $Fe(mpp)_3$. Plants supplied with FeEDDHA or with $Fe(mpp)_3$ were greener than the Fe deficient ones, as shown in Fig. 3. The relative chlorophyll content was assessed using a SPAD meter. Comparing the effect of the 3,4-HPO type complex with FeEDDHA, plants grown with $Fe(mpp)_3$ had 42% higher SPAD units ($P < 0.01$).

Shoot DW was significantly increased in plants treated with the Fe chelates, however the highest increase, was observed with $Fe(mpp)_3$ which more than tripled when compared to Fe deficiency (increasing by a factor of 3.16), (Table 3). Root DW was lowest in Fe deficient plants (0.13 ± 0.02 g) and, again, $Fe(mpp)_3$ supplied plants had the highest root DW increase (0.42 ± 0.02 g) of more than triple (by a factor of 3.23) (Table 3). Regarding total leaf area, Fe sufficient plants had significantly increased values, with $Fe(mpp)_3$ plants showing a 41% higher leaf area than FeEDDHA (Table 3).

3.4. Root Fe(III) chelate reductase activity

Reductase activity was measured in roots of Fe deficient, FeEDDHA and $Fe(mpp)_3$ treated plants (Table 3). No significant differences were detected amongst treatments, however, there was a trend for an increased activity of this enzyme under Fe deficiency.

3.5. Mineral accumulation analysis

Total Fe content (Fig. 4A) and trifoliate leaf (Fig. 4B) and root (Fig. 4C) Fe concentration were evaluated. In general, Fe sufficient plants had significantly higher Fe accumulation levels than Fe deficient ones ($P < 0.001$). Plants grown under Fe deficiency presented a total Fe content of 44 ± 0.5 μg whereas plants grown with FeEDDHA had values of 272 ± 17 μg. The tested Fe chelate induced the highest Fe accumulation (608 ± 28 μg), with an increase of more than double of FeEDDHA values ($P < 0.001$). Trifoliate leaf and root Fe concentrations showed a similar pattern to total Fe content, as shown in Fig. 4B and C, where $Fe(mpp)_3$ treated plants had increases of 8.7 and 1.8 fold respectively, as compared to Fe deficiency

Table 2
Chlorophyll (SPAD units), shoot and root dry weight (DW) and total leaf area of *G. max* plants supplied with Fe(III) sulfate, Hmpp, FeEDDHA, Fe(III) sulfate + Hmpp and $Fe(mpp)_3$ for 14 days, under hydroponic conditions ('Experiment 1').

	Fe(III) sulfate	Hmpp	FeEDDHA	Fe + Hmpp	$Fe(mpp)_3$
Chlorophyll (SPAD units)	4.86 ± 2.09^d	6.60 ± 0.90^d	23.0 ± 2.0^c	28.8 ± 1.4^b	37.2 ± 0.7^a
Shoot DW (g)	0.18 ± 0.03^c	0.24 ± 0.01^c	0.38 ± 0.03^b	0.56 ± 0.08^b	0.86 ± 0.04^a
Root DW (g)	0.064 ± 0.011^d	0.078 ± 0.009^d	0.140 ± 0.002^c	0.227 ± 0.038^b	0.347 ± 0.026^a
Total Leaf Area (cm^2)	22.1 ± 3.7^c	8.54 ± 0.83^d	60.4 ± 2.4^b	69.6 ± 8.3^b	142.2 ± 7.3^a

Data are means ± SE of five biological replicates.
Different letters indicate significant differences ($P < 0.05$) by ANOVA with Holm-Sidak correction test.

respectively ($P < 0.05$) (Table 2). Even when compared to Fe + Hmpp, $Fe(mpp)_3$ treated plants had significantly improved values.

($P < 0.001$).

Also, the impact of $Fe(mpp)_3$ on the roots' and trifoliate leaves' ionome was studied (Table 4). In the root tissue, all nutrients had

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Fig. 3. Visual symptoms of *G. max* plants supplied with no Fe (-Fe), FeEDDHA and $Fe(mpp)_3$ for 14 days, under hydroponic conditions ('Experiment 2').

Table 3
Chlorophyll (SPAD units), shoot and root dry weight (DW), total leaf area and root reductase activity of *G. max* plants grown without Fe (-Fe) or supplied with FeEDDHA or $Fe(mpp)_3$ for 14 days, under hydroponic conditions ('Experiment 2').

	-Fe	FeEDDHA	$Fe(mpp)_3$
Chlorophyll (SPAD units)	2.41 ± 1.68^c	20.0 ± 2.0^b	28.4 ± 0.9^a
Shoot DW (g)	0.35 ± 0.05^c	0.85 ± 0.07^b	1.10 ± 0.04^a
Root DW (g)	0.13 ± 0.02^c	0.31 ± 0.03^b	0.42 ± 0.02^a
Total Leaf Area (cm^2)	41.9 ± 10.7^c	167.2 ± 7.0^b	235.1 ± 2.4^a
Root reductase activity (μmol Fe g^{-1} FW h^{-1})	0.038 ± 0.02^a	0.007 ± 0.003^a	0.017 ± 0.002^a

Data are means ± SE of five biological replicates.
Different letters indicate significant differences ($P < 0.05$) by ANOVA with Holm-Sidak correction test.

similar concentrations, with the exception of K and Cu, where plants under Fe deficiency presented higher values ($P < 0.05$). No significant differences were found between the Fe chelates treatments in the trifoliate leaves, with the exception for the concentration of K, which was lower in $Fe(mpp)_3$ treated plants (Table 4).

3.6. Lipid peroxidation

Low levels of lipid peroxidation, measured by the MDA concentrations in trifoliate leaves and roots of Fe deficient and sufficient plants showed no significant differences between treatments for each plant organ (Fig. 5).

3.7. Gene expression analysis

The expression levels of *FRO2*-like, *IRT1*-like and *ferritin* genes in plants grown without Fe and with the Fe complexes are represented in Fig. 6. The expression of *FRO2*-like in the roots was highest under Fe deficiency. FRO2-like expression pattern was similar in plants grown with FeEDDHA and $Fe(mpp)_3$, being 27 fold less expressed ($P < 0.001$) when compared to Fe deficient plants (Fig. 6). The root expression of *IRT1*-like gene was similar in Fe deficiency and FeEDDHA-treated plants, but in plants supplemented with $Fe(mpp)_3$ expression of *IRT1*-like was almost null ($P < 0.001$) (Fig. 6). Finally, the leaf expression of *ferritin* was very low in Fe deficient plants and also low for FeEDDHA treated plants. In contrast, plants grown with the 3,4-HPO Fe complex had a strong induction of *ferritin* expression in the trifoliate leaves (Fig. 6).

4. Discussion

To the best of our knowledge, this is the first report on the application of the bidentate ligands 3-hydroxy-4-pyridinone, as vehicles to supply plant iron. Although most of the commercially available Fe chelators used are hexadentate ligands, it is known that the reduction of the Fe(III) polyaminocarboxylate chelates by the ferric chelate reductase requires detaching of a coordinating atom of the N_2O_4 coordination sphere creating a vacant position that is occupied by a water molecule and providing lower redox potentials (Gomez-Gallego et al., 2005; Lopez-Rayo et al., 2009). The results obtained by the group of Mar Gomez-Gallego (Escudero et al., 2012) regarding the activity of aquo complexes of polyaminocarboxylate ligands towards activating ferric chelate reductase brought a new concept of Fe(III) complexes for Fe-chlorosis correction. Considering the chemical properties of 3-hydroxy-4-pyridinone chelators and their complexes, and the fact that these ligands are non-toxic and seem to prefer Fe(III) to other metal ions (Burgess and Rangel, 2008; Santos et al., 2012) offers great possibilities for their use in an agricultural context. Studying *tris*(3-hydroxy-4-pyridinonate) Fe(III) chelates in terms of plant availability, phytotoxicity and IDC symptoms development is a step forward to understand their potential as novel fertilizers. The study of novel chelators is usually performed in hydroponic conditions at high pH in order to mimic the in-field alkaline conditions (López-Rayo et al., 2016). However, given the fact that this is the first study on the use of 3,4-HPO in an agricultural context, this work was conducted under optimal pH (5.5) to understand if this type of ligands could have a positive impact at a physiological level.

4.1. The 3,4-HPO complex reduces chlorosis and improves growth

A first experiment ('Experiment 1') was conducted in order to test the possible toxicity of the Hmpp ligand itself or the $Fe(mpp)_3$ complex, as well as to test their effect on plant growth, when compared to FeEDDHA (Table 1). The selected indicators were SPAD units, since yellowing of the upper leaves and stunted growth are the main symptoms of Fe deficiency in plants (Prasad, 2003); shoot and root dry weight and total leaf area. Firstly, the application of Fe(III) sulfate and the ligand by themselves had no impact on plant development. On the contrary, the application of the Fe complexes led to significant improvement on plant growth. We also aimed at understanding if it would be necessary to synthesize the $Fe(mpp)_3$ complex or if a mixture of Fe(III) sulfate and the Hmpp ligand would have the same effect, hence decreasing the production costs. The

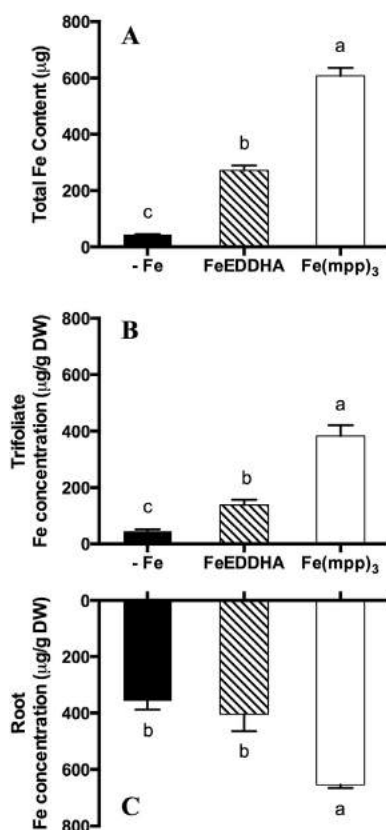

Fig. 4. Total Fe content (A), and trifoliate (B) and root Fe concentration (C) of *G. max* plants supplied with no Fe (-Fe), FeEDDHA and Fe(mpp)$_3$ for 14 days, under hydroponic conditions ('Experiment 2'). Total Fe was determined by ICP-OES analysis. Data are means ± SE of five biological replicates. Different letters indicate significant differences ($P < 0.05$) by ANOVA with Holm-Sidak correction test.

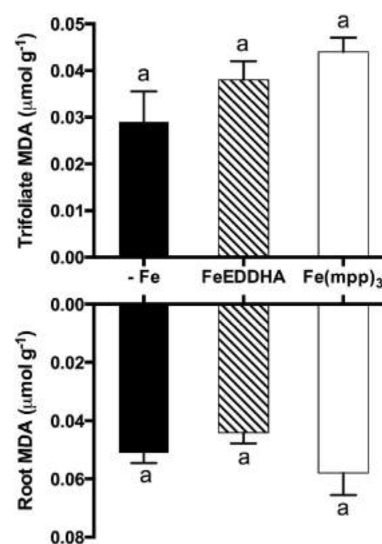

Fig. 5. MDA concentration in trifoliate leaves and roots of *G. max* plants supplied with no Fe (-Fe), FeEDDHA and Fe(mpp)$_3$ for 14 days, under hydroponic conditions. Data are means ± SE of five biological replicates. Different letters indicate significant differences ($P < 0.05$) by ANOVA with Holm-Sidak correction test.

With 'Experiment 2', plants grown with the Fe(mpp)$_3$ complex were compared to plants grown with FeEDDHA and with no added Fe, as it is usually performed when testing the physiological responses to IDC in plants (El-Jendoubi et al., 2014; Paolacci et al., 2014; Santos et al., 2015).

Since the chlorophyll content of the leaves is often utilized to evaluate the success of the fertilization procedure, mainly when a foliar fertilizer is applied (El-Jendoubi et al., 2014), Fe(mpp)$_3$ seemed to be better suited for fertilization than FeEDDHA (Fig. 3, Table 3). In what concerns plant growth, it is known that an increase in root biomass promotes higher soil volume exploration (Marschner et al., 1996; Nenova, 2006), thus allowing a better Fe scavenging from the solution. Moreover, higher leaf area allows an increase in the photosynthetic area (Engels et al., 2012), which explains the higher total dry weight observed in the Fe(mpp)$_3$ treatment (Table 3).

Table 4
The ionome of roots and trifoliate leaves of *G. max* plants grown without Fe (-Fe) or supplied with FeEDDHA or Fe(mpp)$_3$ for 14 days under hydroponic conditions.

Mineral ($\mu g\ g^{-1}$)	Roots			Trifoliate leaves		
	-Fe	FeEDDHA	Fe(mpp)$_3$	-Fe	FeEDDHA	Fe(mpp)$_3$
Mn	54 ± 1.8^a	26 ± 3^a	26 ± 1^a	95 ± 8^a	57 ± 4^a	38 ± 4^a
Zn	505 ± 51^a	258 ± 29^a	202 ± 26^a	215 ± 32^a	166 ± 10^a	110 ± 16^a
Mo	114 ± 19^a	70 ± 2^a	72 ± 31^a	27 ± 2^a	23 ± 3^a	27 ± 4^a
B	31 ± 1^a	27 ± 1^a	31 ± 1^a	43 ± 1^a	33 ± 1^a	31 ± 1^a
Na	3577 ± 1115^a	3767 ± 83^a	3475 ± 389^a	3217 ± 1387^a	4516 ± 772^a	3956 ± 220^a
Mg	3691 ± 849^a	3622 ± 101^a	5401 ± 1404^a	5677 ± 699^a	3849 ± 144^a	3825 ± 56^a
K	56,461 ± 12986^a	39,635 ± 1263^b	39,430 ± 17950^b	44,732 ± 5373^{ab}	48,806 ± 532^a	41,379 ± 1234^b
Ca	3618 ± 832^a	4273 ± 485^a	4714 ± 277^a	12,922 ± 5424^b	18,987 ± 2389^a	18,418 ± 1554^a
P	9516 ± 390^a	9414 ± 166^a	10,015 ± 4985^a	11,924 ± 590^a	11,357 ± 870^a	9949 ± 399^a
Cu	132 ± 30^a	17 ± 1^b	60 ± 1^b	26 ± 0.4^a	9 ± 1^a	38 ± 25^a
Ni	17 ± 5^a	13 ± 1^a	17 ± 1^a	8 ± 1^a	6 ± 1^a	7 ± 0.3^a

Data are means ± SE of five biological replicates.
Different letters indicate significant differences ($P < 0.05$) within tissue types by ANOVA with Holm-Sidak correction test.

mixture had less impacting effects when compared to the complex. Therefore, with these results, the application of 20 µM of Fe(mpp)$_3$ was shown to be non-toxic to the plants and more beneficial than the commercial product FeEDDHA, allowing us to proceed with more detailed tests.

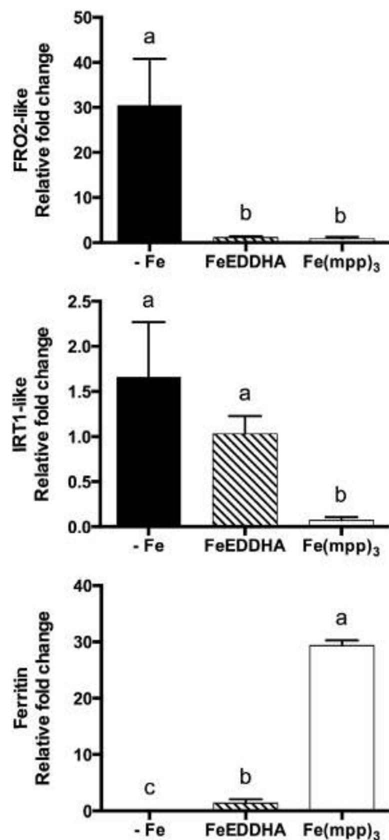

Fig. 6. Gene expression analysis of *FRO2*-like and *IRT1*-like in the roots and of *ferritin* in the shoots of *G. max* plants supplied with no Fe (-Fe), FeEDDHA and $Fe(mpp)_3$ for 14 days, under hydroponic conditions ('Experiment 2'). Data are means of three biological replicates ± SE relative to the housekeeping gene 18S rRNA. Different letters indicate significant differences ($P < 0.05$) by ANOVA with Holm-Sidak correction test.

The membrane-bound ferric chelate reductase enzyme is responsible, in plants, for the acquisition of soluble Fe from the soil. In fact, the reduction step has been proposed as the rate limiting step for Fe absorption (Grusak et al., 1990), but the elicitation of this enzyme is not the only process involved in the reduction of IDC (Klein et al., 2012). Plants grown with FeEDDHA had only half of the reductase activity registered in plants grown with $Fe(mpp)_3$ (Table 3) ($P > 0.05$). Although the general accepted concept is that plants enhance the activity of this enzyme under low Fe conditions, as it is observed in *Arabidopsis* (Robinson et al., 1999; Vert et al., 2003), several studies report on its variability. A study with beans showed that reductase activity can vary with the cultivar under study, the type of Fe chelator utilized and the pH of the hydroponic solutions (Blair et al., 2010). Also, a recent study in rice plants particularly shows the variability in Fe reductase activity and its dependence on the cultivar and ecotype (Pereira et al., 2014). Moreover, studies in soybean show that when no Fe is given to the plants, the enzyme may not be triggered since it needs Fe for its functioning (Krishnan, 2005; Santos et al., 2013).

The lack of differences in reductase activity amongst Fe treatments could be due to the fact that in this study the FCR activity was measured at the end of the assay, which corresponded to 14 days of hydroponic growth. Although this is commonly performed (Zocchi et al., 2007; López-Rayo et al., 2015; Klein et al., 2012), it has been shown that reductase activity varies in time (Andaluz et al., 2009), therefore it would be interesting to conduct a future time course analysis to test this hypothesis.

4.2. Plants grown with $Fe(mpp)_3$ had higher total Fe content

The results of the Fe accumulation analysis (Fig. 4) are in agreement with the ones obtained for the chlorophyll content and plant growth: plants with higher Fe content were greener and had higher biomass production (Table 3).

Plants treated with $Fe(mpp)_3$ accumulated more Fe in the roots than the ones treated with FeEDDHA ($P < 0.001$) (Fig. 4C). As both symplasmic and apoplasmic Fe were measured in this study, part of the root accumulation can represent a ferric Fe pool precipitated in the free space of roots (Zhang et al., 1991; Becker et al., 1992). Nevertheless, when looking at the Fe concentration in trifoliate leaves (Fig. 4B), a significant difference was detected in $Fe(mpp)_3$ treated plants, where these accumulated almost the triple amount of Fe ($P < 0.001$). Such difference may perhaps be explained by the fact that $Fe(mpp)_3$ has higher water solubility than FeEDDHA thus promoting Fe uptake and transport within the plant.

These high Fe accumulation levels could potentially lead to higher oxidative stress (Küpper and Andresen, 2016). Lipid peroxidation, measured as the amount of MDA, is the most often used indicator of oxidative stress, as it is produced when polyunsaturated fatty acids in the membrane undergo oxidation by the accumulation of free oxygen radicals (as reviewed in Gill and Tuteja, 2010). Our data show that, despite a tendency for an increased MDA accumulation shown by $Fe(mpp)_3$, there was no significant differences between treatments (Fig. 5). This is in agreement with other studies where no variation of MDA concentration was found from the control to the Fe deficient conditions on wheat leaves (Iturbe-Ormaetxe et al., 1995; Tewari et al., 2005).

Moreover, with the exception for K concentration in the trifoliate leaves, no significant difference was detected in the mineral accumulation pattern between FeEDDHA and $Fe(mpp)_3$ treated plants (Table 4), which shows the specificity of $Fe(mpp)_3$ to Fe.

4.3. Expression of Fe uptake-related genes was lower in plants grown with $Fe(mpp)_3$

In this study, the expression of three genes involved in major steps of Fe uptake and accumulation (*FRO2*-like, *IRT1*-like and *ferritin*) were analysed, and the results are presented in Fig. 6. FRO2 and IRT1 genes are responsible for the two main steps of Fe absorption and mobilization and are under a coordinate control (Connolly et al., 2003). These two genes are the most frequently studied in what concerns Fe deficiency mechanisms not only in *Arabidopsis* but also in different crops, such as soybean (O'Rourke et al., 2007), potato (Legay et al., 2012), tomato (Paolacci et al., 2014) or cucumber (De Nisi et al., 2012).

Regarding *FRO2*-like gene expression, in the roots of plants without Fe and with the two Fe complexes, while the expression was very low in FeEDDHA and $Fe(mpp)_3$-treated plants, Fe deficient plants had high levels of this transcript. As aforementioned, soybean uses an Fe-reduction based mechanism to absorb Fe in response to Fe deficiency, which is coherent with the results obtained for −Fe plants (Table 3). Once Fe(II) is available to the plant, it is transported by the IRT1 protein, and as such the root expression of the *IRT1* gene was also studied (Fig. 6). *IRT1* is usually upregulated in Fe-deficient conditions, but studies show that its regulation is dependent both on the root Fe pool and on the shoot Fe demand (Vert et al., 2003). Here, all treatments except $Fe(mpp)_3$

induced *IRT1*-like expression. Numerous theories aim to explain the mechanisms behind Fe deficiency sensing and, lately, shoots have been presented as the main responsible organ (Enomoto et al., 2007; Wu et al., 2012). Additionally, the expression of the *ferritin* gene was evaluated in the shoots (Fig. 6) and it was found that plants treated with 3,4-HPO Fe chelate induced high levels of expression of this gene. This increase in *ferritin* expression was expected, since it may be a response of the plant to the high Fe concentrations in the shoot tissues (Fig. 4B) allowing the plant to regulate Fe and maintain homeostasis, storing or releasing the required Fe by the plant as needed, thus avoiding toxic effects (Ting-Bo et al., 2006). Higher rates of *ferritin* expression, particularly in the shoots, have been detected before in plants grown under high Fe concentrations (Vasconcelos et al., 2014).

5. Conclusions

In this work, a *tris* 3-hydroxy-4-pyridinone iron(III) chelate was tested as a possible Fe chelator to combat Fe deficiency chlorosis, in plants grown in hydroponic conditions. To assess the extent of its beneficial effect, FeEDDHA, a standard chelate routinely utilized in chlorosis treatments, was included as control. Fe(mpp)$_3$ proved to be more efficient than FeEDDHA, and plant growth was significantly improved (increased shoot and root dry weight and increased total leaf area). Plants presented no signs of chlorosis, and the analysis of the expression of IDC -related genes, *FRO2*-like and *IRT1*-like, showed that plants with Fe(mpp)$_3$ had low expression levels.

Due to its high solubility, non-toxicity and high affinity for Fe(III), 3-hydroxy-4-pyridinone ligands seem to be a good alternative to produce more efficient iron chelates compared to the current commercial products. In order to fully understand the mode of action of the *tris*(3-hydroxy-4-pyridinonate) Fe(III) chelates and establish structure activity relationships further work is needed, namely looking at extrusion of organic acids, labelled Fe, among others. Furthermore, studies at alkaline pH should be conducted in order to understand the new chelates' behaviour at conditions of low Fe solubility in the soil.

Contributions

CSS conducted the experimental work; AL and TM synthesized Fe(mpp)3; MRoriz conducted the ICP analyses; SMPC, AOSSR, MRangel and MWV designed the experiment; all authors contributed for data analysis, interpretation and manuscript writing.

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CHAPTER 5

General Discussion

In this chapter, a general overview of the results obtained in the previous chapters will be presented. In the Discussion section (**5.1**) the achievement of each goal will be commented, as well as the common theory behind all research works. The main conclusions will be highlighted, as well as future perspectives for the Fe research field and several outstanding questions that have to be address in the near future. Finally, a Critical Reflection on the up-coming difficulties associated to Fe nutrition in plants and its contextualization in the future of agriculture will be presented in the form of an opinion article (section **5.2**).

CHAPTER 5

section 5.1

Discussion

In agricultural context, Iron Deficiency Chlorosis (IDC) is the cause of severe decline in legume crops' production and growers' profits. This is due to the fact that 30% of the world's arable land is calcareous, Fe forms insoluble oxides and becomes unavailable for plants' uptake (Prasad, 2003). Despite being an agricultural problem for a long time, there is still no effective solution to correct IDC and strategies to overcome it include the selection of tolerant cultivars, conventional breeding and the application of Fe fertilizers (Abadía *et al*., 2011; Blair, 2013; Boodi *et al*., 2016).

Several knowledge gaps impair the full understanding of the Fe deficiency response and, in the last decades, studies converge in finding feasible solutions to increase Fe content in the edible part of legume plants (Sperotto *et al*., 2012). This general and common objective aims at decreasing human diseases associated to vitamin or mineral deficiencies, by which two billion people are affected in the world (von Grebmer *et al*., 2014). Hence, the production of more nutritious food, without undervaluing food security protocols, should be key in helping to improve humans' health and nutritional habits (Gupta and Prakash, 2014). As mentioned in Chapter 1, to achieve this goal, several biofortification programs are being developed and (some) successfully established, but a clear method to increase mineral content in the seeds is yet to be defined, since there is still incomplete understanding of the Fe translocation routes from the uptake steps to its unloading into the edible parts (Carvalho and Vasconcelos, 2013).

Gathering molecular information on IDC-response

The ascension of the omics era has contributed with vast information on Fe deficiency in different crops, however to develop plant genotype improvement programs more information is needed (Briat *et al*., 2015; Vasconcelos *et al*., 2017). The first objective of this thesis was to study the molecular mechanisms behind IDC-responses in legume crops. After selecting 223 up-regulated genes by Fe-deficiency from three legume crops – *Phaseolus vulgaris*, *Glycine max* and *Medicago truncatula* – a set of common gene families was identified (**Chapter 2, section 2.1**). It was shown that, in response to Fe stress, all three cultivars up-regulated genes of protein kinase, tranferase and metal and zinc ion binding families (Chapter 2, section 2.1, Fig. 6A). Other studies have also found that protein kinases may have an essential role in Fe deficiency responses, possibly by mediating protein phosphorylation at a post-translational level (Lan *et al*., 2013). Transferase type proteins have also been associated to the phosphorylation process, at a post-transcriptional level (Lan *et al*., 2012). Inserted in the metal ion binding protein group

we can find several of the proteins essential to iron homeostasis, as is example the Fe deficiency responsive element-binding factor 1 (IDEF1) (Kobayashi and Nishizawa, 2014), mentioned in Chapter 1 for its role in Fe accumulation, or the zinc finger genes that have lately been associated to increased tolerance to oxidative stress (Le *et al*., 2016). When looking at specific up-regulated genes, an interesting result was found, where both *G. max* and *M. truncatula* induced genes of the isoflavonoid pathway (Chapter 2, section 2.1, Fig. 7A and 7C). As also described in Chapter 1, in the last few years, attention have been directed to the role of phenolics in Fe deficiency response, and several studies have confirmed their increased production under Fe-stress conditions in *M. truncatula* (Rodríguez-Celma *et al*., 2013), *Arabidopsis* (Fourcroy *et al*., 2014; Schmid *et al*., 2014) and *Beta vulgaris* (Sisó-Terraza *et al*., 2016).

In Chapter 2, section 2.1 (Fig. 6B) we also registered that the oxidoreductase family was amongst the most commonly down-regulated families under Fe-limited conditions. Ferric reductases belong to this family and, as seen in the ferric reductase activity results (Chapter 2, section 2.1, Fig. 4), plants were not able to induce this enzyme under Fe deficiency. Although specific oxidoreductase genes were also found among the five most down-regulated genes in all three species (Chapter 2, section 2.1, Fig. 7), these types of genes are usually up-regulated under Fe deficiency, as shown in Arabidopsis (Salazar-Henao and Schmidt, 2016).

Having into consideration the up-regulated genes identified in Chapter 2, section 2.1 and other literature information, a set of specific genes were chosen for a targeted comparison between the model crops *G. max* and *M. truncatula*, in **Chapter 2, section 2.2** (Fig. 4 and 5). Here, we selected genes that encoded proteins of the Fe uptake mechanism, *FRO2* and *IRT1* (Chapter 1, Fig. 1.3). Both genes appeared to be co-regulated, as they behaved similarly in response to Fe-deficiency, however, the pattern of expression was opposite between species. These results show that the activation of the uptake system might be species-dependent, and support other studies that show diversity in iron reductase activity at the genotype level (Blair *et al*., 2010). Again, in *G. max* roots, *FRO2* gene expression was lower under Fe deficient conditions, corresponding to the enzymatic pattern obtained for ferric reductase activity (Chapter 2, section 2.2, Fig. 2).

We have also looked at transport genes, namely *NRAMP3*, *VIT1* and *YSL1*. While *NRAMP3*, which is responsible for the remobilization of Fe from the vacuole, was up-regulated under Fe deficiency, *VIT1* and *YSL1*, that encode proteins responsible for Fe storage and translocation, were repressed. Our results showed that these genes behaved as

expected under Fe deficiency, accordingly to their functions (Chapter 1, Fig. 1.4), showing a conserved expression in both species. Just like in other studies (Vasconcelos *et al*., 2003; Masuda *et al*., 2013) we observed that the ferritin gene, encoding the main Fe storage protein, is induced in the presence of Fe, confirming that this is a good target for Fe biofortification as it might increase Fe content in plants. In fact, many reported strategies have used ferritin for this goal (Boonyaves *et al*., 2017). Finally, we have also looked at the expression of *GCN2* gene that has not been previously associated with Fe nutrition in plants, and we observed that Fe deficiency modulated its expression. This gene encodes a protein kinase responsible for the phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2α) (Faus *et al*., 2015) and has been characterized to sense and respond to nutrient deprivation by modulating amino acid metabolism in yeast and Arabidopsis (Liu *et al*., 2015; Uluisik *et al*., 2011).

In order to expand our knowledge on the role of some of the aforementioned genes in a broader set of plants and to compare the strategy I dicotyledoneous plants behaviour with that of strategy II grass species, in **Chapter 2, section 2.3**, genes relevant in both Fe uptake strategies (Chapter 1, Fig. 1.3) were evaluated in rice (Fig. 5 and 6). In this chapter we addressed the recently debated concept that rice could use a combined strategy to uptake Fe (Sperotto *et al*., 2012). Utilizing two rice cultivars with different Fe susceptibilities we observed that the more susceptible cultivar had higher rates of ferric reductase enzyme activity and induced the expression of FRO2 gene and that the gene encoding the Fe transporter IRT1 was up-regulated in the shoots of both cultivars. Succeeding reports also emphasize the up-regulation of these strategy I genes in other rice cultivars, alongside with NAS and YSL encoding genes (Wang *et al*., 2015; Paul *et al*., 2016; Chen *et al*., 2017). The transcription factors IRO2 and IDEF1 are among the main regulators of Fe deficiency response in rice (Kobayashi *et al*., 2014) and have been utilized as mineral stress markers in different studies (Feng *et al*., 2016; Kobayashi *et al*., 2016).

A recent study on Fe deficiency transcriptional response in two cultivars of grapevine with different Fe-stress susceptibilities has shown that the abovementioned genes, selected for analysis in Chapter 2, are still the most preponderant for the characterization of the mechanisms associated to Fe deficiency (Vannozzi *et al*., 2017). In that study, and similarly to our findings, *FRO2* and *IRT1* genes were also co-regulated. Moreover, a transferase belonging to the flavonoid branch was up-regulated under Fe-deficiency, as well as a *NRAMP* gene, that was highly induced. In the same study, *VIT*-like genes were down-regulated under Fe deficiency, and it has been suggested that this down-

regulation can be correlated to the control of root-to-shoot Fe translocation at the transcriptional level, highly influencing chlorosis symptoms development (Yan *et al*., 2016). Alike *VIT*-like gene regulation results, *YSL* was down-regulated under Fe-deficiency. The major function attributed to *YSL* is the transport of metal-NA complexes through the phloem to the shoots (Conte *et al*., 2013) thus its expression is expected to increase when Fe is abundant, as shown by others (Feng *et al*., 2017) and observed here (Chapter 2, section 2.2, Figures 4 and 5; and Chapter 2, section 2.3, Figure 6).

Chapter 2 evidenced the intricacy of IDC genetic response. This chapter also contributed to describe molecular markers that can improve breeding programs in legume plants and that can provide information on molecular tools to study the mechanisms behind IDC.

Fe-efficiency trait as a mean to understand IDC-related physiological mechanisms

The second objective of this thesis was to examine the physiological mechanisms underlying IDC and, for that, we have selected soybean cultivars with distinct Fe-efficiencies based on previous studies (Vasconcelos and Grusak, 2014). Foliar chlorosis, growth parameters, Fe tissue accumulation and genetic markers (*FRO2*, *IRT1* and *ferritin*) were evaluated on plants grown under Fe-deficient conditions when compared to Fe-sufficient plants (**Chapter 3, section 3.1**). An additional treatment was added to the analysis of Fe-efficient (EF) and Fe-inefficient (IN) plants, the removal of the unifoliate leaves that are the first leaves to expand after seed germination (Chapter 3, section 3.1, Fig. 1), and are strong sink organs in the early stages of plant development. The unifoliates removal alleviated chlorosis symptoms in the trifoliate leaves, probably due to the decreased sink competition as a previous report showed that the bigger the unifoliate leaves, less Fe is left for remobilization to the trifoliates (Vasconcelos and Grusak, 2014). This excision also helped to understand that IN plants had, in general, lower ability for Fe remobilization to the shoots, mainly accumulating their Fe pool at the root level (Chapter 3, section 3.1, Fig. 6).

We also looked at ferric reductase activity and, although it was not induced under Fe deficiency (as also seen at the molecular level in Chapter 2), EF plants had higher levels of this enzymes' activity. The analysis of the genetic markers showed that *FRO2* gene was more expressed in the trifoliate leaf tissues (Chapter 3, section 3.1, Fig. 7A), putatively revealing that the sequence here analysed could be more homologous to *FRO7*, responsible for Fe(III) reduction at the chloroplast level (Chapter 1, Fig. 1.4). In this case, the higher

induction registered in IN plants could be due to the increased necessity of these plants to avoid the degradation of the photosynthetic machinery due to the lack of Fe. Other studies reporting the difference in Fe-deficiency response between tolerant and susceptible cultivars have shown that *FRO2* gene expression and the enzymes' activity have common patterns of induction and are usually increased in the tolerant cultivars when compared to the susceptible ones (Vannozzi *et al*., 2017). Here, this was true mostly for the root reductase activity and also for *FRO2* at the root level, where its expression was higher in the EF plants.

Besides being an Fe transporter, *IRT1* is a marker gene for Fe metabolism regulation and it is deeply responsive under stress conditions (Guo *et al*., 2017). *IRT1* expression was generally higher in IN plants (Chapter 3, section 3.1, Fig. 7B). A recent study showed that Fe-deficiency susceptible apple plants might adapt to be more tolerant by altering the *IRT1* promoter, in order to increase transcriptional activation of the gene (Zhang *et al*., 2017). This could explain the observed increased levels of *IRT1*, as INF plants could be modelling Fe-uptake associated mechanisms to avoid more damage. Lastly, ferritin gene expression was also increased in INF plants (Chapter 3, section 3.1, Fig. 7C), which could be related to the higher demand for Fe in the shoots, again, to avoid further damage at the photosynthetic level that could be impairing the Fe partitioning to this tissue. In fact, a novel ferritin gene was just identified in *Triticum aestivum*, and shown to be essential for protecting cells against ROS and oxidative stress (Zang *et al*., 2017).

In **Chapter 3, section 3.2** we deepened the understanding of the physiological responses by analysing the role of Fe deficiency on activating the antioxidant and tetrapyrrole systems activation. Although it is known that Fe deficiency triggers oxidative stress in plants (Jelali *et al*., 2013; Le *et al*., 2016), to date, no correlation studies have been performed to understand the influence between these two systems' regulation under Fe stress. The use of lines with contrasting Fe-efficiencies, allowed us to understand that higher levels of oxidative stress (indicated by higher MDA accumulation in the root tissue and by higher GR activity in the leaf tissue) might induce the oxidation of the tetrapyrrole heme into hemin, unbalancing the heme/hemin pool and leading to the triggering of catalase enzyme. Possibly due to this fact, heme prosthetic group is no longer integrated into the heme-containing enzyme ferric reductase, putatively explaining the lower levels of this enzymes' activity in INF plants (Chapter 3, section 3.2, Table 3). Also, possibly to correct this heme/hemin imbalance, the tetrapyrrole cycle (Chapter 1, Fig. 1.2) seems to be more directed to the Fe-branch, neglecting the production of chlorophyll through the Mg-

branch, thus contributing to chlorosis development (Chapter 3, section 3.2, Fig. 5). As recently seen in a study in groundnut cultivars with different Fe-susceptibilities (Boodi *et al*., 2016), increased chlorophyll levels and APX activity was highly associated to the EF line (Chapter 3, section 3.2, Fig. 4).

Taken together, the results of **Chapter 3** indicate that, besides the well known indicator of chlorosis and reduced leaf chlorophyll content (Prasad, 2003), two parameters seem to be associated with Fe-deficiency susceptibility: (i) lower ferric reductase activity; and (ii) higher Fe accumulation in the root tissue. Further studies should be performed to associate the oxidized form of heme –hemin - to IDC susceptibility. These characteristics seem to be major contributors to the inability to respond to IDC and here we suggest their use as IDC physiological markers.

Chelate from the 3,4-HPO family as an effective strategy for IDC prevention

Using the above-mentioned molecular and physiological IDC-markers, the efficacy of a novel Fe-chelating agent as fertilizer was evaluated in soybean plants (**Chapter 4**). This novel ligand, 2-methyl-3-hydroxy-4-pyridinone (Hmpp), belongs to the *tris*(3-hydroxy-4-pyridinonate) (3,4-HPO) family, only applied in biomedical context and never tested in plants. It was our hypothesis that, based on its high affinity for Fe and its high solubility (Burgess and Rangel, 2008), it could be a good Fe fertilizer. On the contrary to the other usually employed synthetic chelates that are hexadentate, these ligands are bidentate, which makes them smaller. Their reduced size could explain these molecules' higher solubility and could help in Fe penetration through the root membrane.

Firstly, the efficacy of $Fe(mpp)_3$ complex was compared, under hydroponic conditions, to: (i) an Fe salt; (ii) to the ligand itself; (iii) to the commercial chelate FeEDDHA; and (iv) to a mixture of Fe and the ligand. As expected, the Fe salt and the ligand had no positive results on plants chlorophyll accumulation, total dry weight and leaf area (Chapter 4, Table 2). From an economical point of view, the use of an Fe salt and ligand mixture, would be less expensive to synthetize, which could result in a more affordable solution for IDC. These results showed that although not as efficient as the complex itself, the mixture could be used as an alternative to FeEDDHA, as it led to higher SPAD values and plant growth.

After this initial assessment, the activity of Fe complex was further evaluated at a physiological level. One of the physiological IDC-indicators was the lower ability to induce ferric reductase enzyme activity under Fe-stress. Usually, under Fe-sufficiency, the

values of the reductase enzyme are repressed (Jeong and Connolly, 2009) and, here, both FeEDDHA and $Fe(mpp)_3$ supplied plants showed a tendency for lower reductase induction than Fe deficient plants (Chapter 4, Table 3).

The plants' capacity for Fe translocation through the root to the aboveground organs was another physiological marker pinpointed in Chapter 3. The analysis of Fe concentration confirmed the potential of the new chelate in plants' nutrition, since $Fe(mpp)_3$ treated plants not only accumulated more Fe in the roots, but also had triple the amount of Fe accumulated in the leaf tissue, when compared to plants grown with the commercial chelate, FeEDDHA (Chapter 4, Fig. 4). A recent study has also concluded that the fast and efficient allocation of Fe to the leaf tissue depends on the chelating agent (Zamboni *et al.*, 2016). In that same study the authors have also found that Fe-stress transcriptional response is influenced by the nature of the chelating agent. Hence, based on the molecular targets selected in Chapters 2 and 3, the effect of $Fe(mpp)_3$ on plants' *FRO2*, *IRT1* and *ferritin* gene expression was evaluated. This analysis showed that $Fe(mpp)_3$ treated plants did not elicit Fe-stress related genes, such as *FRO2* and *IRT1* (Chapter 4, Fig. 6), contrarily to Fe-stressed plants that usually elicit these genes, as previously seen in Chapter 3, section 3.1. Additionally, with $Fe(mpp)_3$ treatment, ferritin was highly induced at the leaf level when compared to the commercial chelate FeEDDHA, representing the higher need for Fe storage due to its higher accumulation in the shoots.

Current studies have used chlorophyll production, total Fe content and biomass yield for the evaluation of the effectiveness of certain products as good Fe fertilizers (Bin *et al*., 2016; Carrasco-Gil *et al*., 2016). Other studies have considered different parameters for this evaluation, namely, the stability and reactivity of the compounds, as well as their biodegradability (López-Rayo *et al*., 2015). Here, we concluded that $Fe(mpp)_3$ is a promising alternative to the existing products and the upscaling of its application should be considered.

Highlights

The proposed aim for this research was to find new tools for molecular and physiological determination of IDC response. The mechanisms behind IDC were analysed from different points of view and we have gathered a subset of IDC-specific indicators. These can be useful in the future, not only in the understanding of the effect of Fe deficiency on plant metabolism, but also in the identification of IDC-tolerant cultivars for breeding programs and cultivar selection. We also suggested a new Fe-chelating agent that showed high efficacy in delivering Fe to the plants and that, somehow, promoted Fe translocation to the shoots as a promising alternative to the commercially available synthetic fertilizers. Below we summarize the main highlights of this research:

◊ *G. max*, *P. vulgaris* and *M. truncatula* shared common transcriptomic mechanisms in response to Fe deficiency, namely, the up-regulation of protein kinase, tranferase and metal and zinc ion binding families;

◊ *G. max* and *M. truncatula* up-regulated genes of the isoflavonoid pathway and the three abovementioned species down-regulated oxidoreductase genes;

◊ In *G. max* and *M. truncatula FRO2* and *IRT1* genes were co-regulated; and Fe deficiency caused the up-regulation of *NRAMP3* gene and the down-regulation of *VIT1*, *YSL* and *ferritin*; *GCN2* expression was responsive to Fe deficiency;

◊ In rice, a strategy II grass species, genes utilized by strategy I plants were up-regulated;

◊ Regulation of Fe uptake mechanisms depended on Fe deficiency susceptibility in rice cultivars;

◊ Fe-efficiency trait was related to better Fe translocation capability from root to shoot and to increased ferric reductase activity;

◊ Fe-efficiency was highly associated to lower levels of oxidative stress and to higher activity of ascorbate peroxidase, gluthatione reductase and ferric reductase at the root level under Fe deficiency;

◊ Plants treated with $Fe(mpp)_3$ were bigger (24%) and greener (42%);

◊ $Fe(mpp)_3$ was a promising alternative to the existing fertilizing products.

Suggestions for future work

In the present research study, molecular and physiological mechanisms associated to IDC were investigated. Selected guidelines and specific characteristics were established in order to achieve and select more tolerant crops to IDC and to prevent this problem. However, further studies are needed to fully understand the efficacy of the tools identified in the present work.

From the transcriptomic analysis in Chapter 2.1, a subset of genes was selected to continue the subsequent work. This selection included genes encoding *FRO*, *IRT*, *ferritin*, *NRAMP*, *VIT*, *YSL*, *TOM*, *NAS*, *DMAS*, *IRO* and *IDEF*, given their preponderance in IDC response and vast description in the literature. Besides being interesting to look at homologous of some important genes identified in recent publications, the data obtained through the Illumina analysis could be explored further, paying closer attention to transcripts related to phenolic and flavonoid synthesis, given their recently revealed key intervention in the Fe reduction and uptake processes. Also, when looking at the genome level, genotype-by-sequencing (GBS) is a great high-throughput tool to discover new traits associated to different phenotypes. Besides being utilized for new SNPs discovery, GBS has also been applied in linkage mapping construction and QTL identification for agronomical important traits, being suitable for the identification of important traits in Fe-tolerance.

From a physiological point of view (Chapter 3), two main characteristics were suggested as potential selection parameters for IDC-tolerance. Although these parameters were confirmed to be consistent throughout the experiments, it would be recommended to test their efficacy in different plant growth conditions, such as, IDC induced by bicarbonate-calcareous conditions instead of no addition of Fe, at pH 5.5, as used throughout this thesis. Also at the physiological level, it would be interesting to better establish the correlation between the genetic information and the enzymatic regulation to IDC. Building a network with this data should be of great value to understand the IDC-response mechanisms.

Furthermore, after showing in Chapter 4 that the new chelate, $Fe(mpp)_3$, has potential as a new plant fertilizing agent, it would be interesting to test it in soil, preferably under calcareous conditions. Additionally, the chelate should be tested not only through soil fertilization, but also by spraying at the foliar level, in order to compare its effectiveness in IDC prevention and treatment. Finally, extra tests to infer about the

toxicological profile and potential soil prevalence should be performed, testing its environmentally safe potential.

Although scarce, there is some information on the metabolomic response to IDC and the obtained data showed potential in the identification of metabolites responsive to Fe deficiency. Mass spectrometry, nuclear magnetic resonance, and gas and liquid chromatography are all standard next generation analytical methods that decrease the amount of sample necessary for the analysis and that can provide large amounts of information, being suitable to understand the novel targets for plant breeding and biofortification.

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CHAPTER 5

section 5.2

Critical Reflection

Title: Increasing Atmospheric CO_2 Impact in Plants Nutritional Quality

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Increasing Atmospheric CO_2 Impact in Plants Nutritional Quality

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Abstract

Recent evidences show that legumes, when grown under elevated CO_2 levels, have lower nutrient and protein levels in the seeds. Legumes provide a large share of the global population diet and, considering the increasing atmospheric CO_2 concentration, a reduction in their nutrient levels is a major concern for humanity. Here we discuss the existing evidences of nutrient losses caused by elevated CO_2 and the combination with other stressors, whose effects are yet to be clearly understood. We will pinpoint the constraints associated, particularly, with Fe stress, raising awareness to the fact that new information on these mechanisms can be of utmost importance for the development of breeding programs, which can decrease the vulnerability to the climate changes ahead.

Climate Changes and Agriculture: the increasing CO_2 levels

One of the biggest hazards we're facing nowadays in agriculture and its sustainability is climate change. These unpredictable changes might compromise not only yield, but also the quality of the resulting products, and new measures are being constantly developed in order to find an effective combat strategy. One of the main contributors for climate change is the rise in atmospheric CO_2 levels. The current global CO_2 concentration is about 400 ppm [1], but the monthly mean atmospheric CO_2 at Mauna Loa Observatory, Hawaii has already surpassed this value [2]. Just 150 years ago atmospheric CO_2 levels were at 280 ppm and, given the registered trend for increase in the past years, it is predicted to rise to 550 ppm by 2050 [3].

The development of Free Air Carbon dioxide Enrichment (FACE) technology in the 90's allowed the study of responses to high CO_2 (hCO_2) without the need for chambers

or glasshouses, however, the understanding of how these hCO_2 values will influence crop quality is still sparse. Early studies show that hCO_2 could be related to increased plant growth and biomass, being associated with a putative positive effect in agriculture (referred to as the "CO_2 fertilisation effect"), but recent studies found a significant negative effect of hCO_2 levels in the concentration of zinc, iron and protein in certain grasses and legumes [4].

In this opinion article we review the influence of climate changes in the future of agriculture. To this end, we describe the evidence about the effect of hCO_2 in different crops' growth and mineral content and later discuss its real impact on crops nutritional value. Finally, we hypothesize that iron metabolism could play an important role in plants ability to cope with hCO_2.

Evidences of hCO_2 impact on agriculture

Photosynthesis captures large quantities of atmospheric CO_2 and, when CO_2 concentration increases, photosynthesis is stimulated. Plants are divided in two classes for their CO_2 assimilation mechanism (Box 1) and this differentiation has a strong influence on how CO_2 levels can affect the plant. C3 plants run a low efficiency photosynthetic system, and are more dependent on atmospheric CO_2 levels than C4 plants.

Vegetable cultivation in greenhouses has for long relied on hCO_2 as a "fertilizer", in order to increase yield and improve production [5]. However, the occurrence of photosynthetic acclimation is frequent and causes a down-regulation of photosynthetic capacity, opposing the positive effects of hCO_2 exposure [6,7]. Legume crops and cereals, on the other had, are grown in open-field conditions and are highly dependent on atmospheric conditions therefore, the discussion about the impact of the increasing CO_2 levels targets mainly staple crops.

Another variable to have in consideration when studying this phenomenon is the fact that besides the inter-specific variation in the response to hCO_2, there is also variability within plant species [8] and the selection of cultivars well adapted to hCO_2 could be a smart tactic for agriculture in the future. Whilst the general opinion supports that hCO_2 conditions increase biomass and yield [9, 10, 11], it is important to consider the potential effects and consequences caused by this drastic climate change.

Box 1. The two classes of CO_2 assimilation

Plants can assimilate CO_2 in different ways and have been differentiated between C3 and C4. They both carry out the same photosynthetic functions but differ in where and when C fixation initially occurs [51].

While in both systems CO_2 enters through the stomata, the steps that follow are very distinct (Figure 1). In C3 plants, CO_2 diffuses to the mesophyll cells where the carboxylase ribulose-1,5-biphosphate carboxylase-oxygenase (RuBisCo) fixes both CO_2 and O_2, leading to photosynthesis but also to photorespiration. The first stable product of this process, through the carboxylation of ribulose-1,5-biphosphate, is 3-phosphoglycerate (PGA), which is a three carbon molecule (C3). PGA is then converted into sugars and transported to leaves, roots and reproductive structures. On the other hand, in C4 plants, CO_2 binds to phosphoenolpyruvate (PEP) in the mesophyll cells, where this product is carboxylated to oxaloacetate, which is composed of four carbon atoms (C4). The oxaloacetate is converted to malate and diffused to the bundle sheath cells, where Rubisco exclusively operates in C4 plants. CO_2 is then released and forms sucrose and starch. Due to the fact that C3 plants spend energy and lose CO_2 in the photorespiration process while C4 plants do not, the process of photosynthesis is much more efficient in the latter [51].

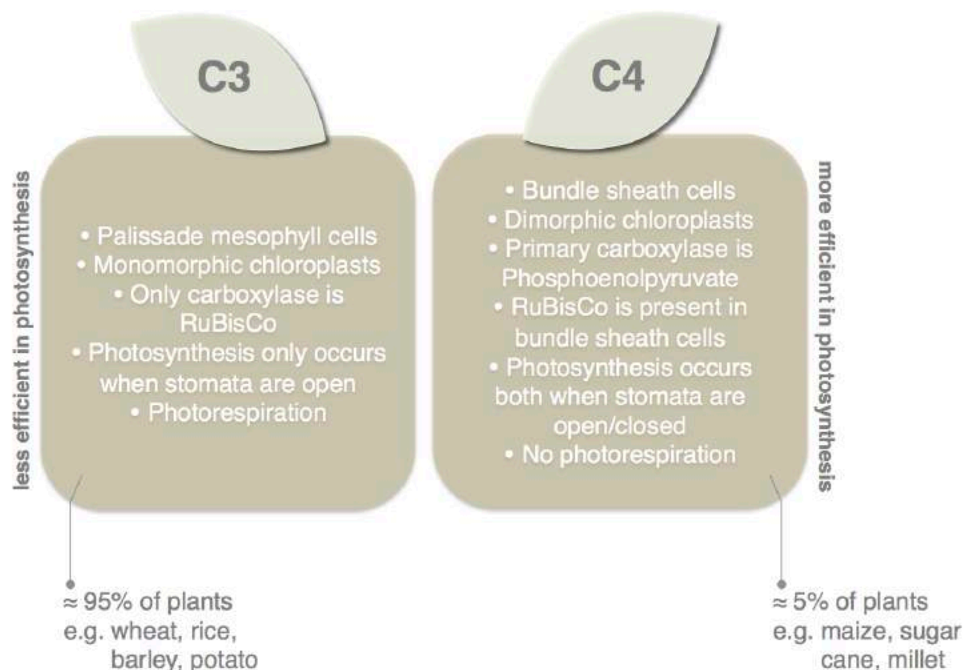

Figure 1. Biochemical and anatomical main differences between C3 and C4 plants.

An early study focused on the nutritional consequences of hCO_2 in the fruit of tomato by testing the influence of a range of CO_2 concentrations between 400 and 10000 ppm in hydroponic conditions [12]. They registered an increase in transpiration and water uptake and, regarding the concentration of different elements (Ca, K, Mg and P), only Ca concentration increased. At the same time, another study showed that hCO_2 delays tomato fruit ripening by impairing ethylene-dependent and independent associated genes [13]. This is to be expected, since CO_2 is a competitive inhibitor of ethylene action. The same study also noticed a significant decrease in extractable protein content.

As the hCO2 conditions lead to higher plant growth, it has been noted that aerial plant parts might decrease its nitrogen (N) content, and C/N ratio tends to increase [14]. Chinese kale plants were maintained in growth chambers under controlled conditions, with ambient (350 ppm) and hCO2 conditions (800 ppm) and, under the latter, plant growth parameters were significantly increased, possibly due to the increase in C content [15].

More recently, a study involving two rice cultivars with contrasting sensitivity to hCO2 revealed that the ability to maintain photosynthetic capacity is a determining factor in plants' adaptation to hCO2 [16]. Whilst the japonica cultivar, which had lower yield (when compared to ambient CO2 concentration), showed reduced N and Rubisco content, the hybrid indica cultivar had increased grain weight and sink:source ratios, with continued stimulation of photosynthesis. Concordantly, another study using a japonica cultivar concluded that hCO2 had no positive effect on grain quality [17].

Besides affecting the photosynthetic activity and the C content, hCO2 also decreases stomatal conductance and transpiration [18]. Hence, although Rubisco does not saturate in C4 plants with hCO2, these are affected as well (Box 1). As stomatal conductance decreases, water use efficiency (WUE) increases, and early evidences show that C4 grass species have reduced water losses when compared to C3 species, having increased above-ground biomass under hCO2 [3,19]. Recent research papers on climate change have focused on plant-water dynamics and drought stress under hCO2 conditions [1,20,21], in order to understand the effects of hCO2 on water-limited lands. The main observations were that as WUE increases, although the biomass is also increased, no other beneficial consequence is obtained, as the amount of water use is also increased and it appears that seed filling and longevity may be impaired. While some studies found no acclimation of stomata in hCO2 [6], a posterior simulation study explains how a potential acclimation results in a significant reduction of the benefits of hCO2 in photosynthesis [22].

Lately, a wave of publications shows how the positive effects of the increasing CO_2 levels could be not so beneficial when considering the consequences from a nutritional point of view.

Dilution effect or the loss of grain quality: why plants lose nutritional quality?

It is important to understand that in cereals and legume crops there are two main growth stages that can be differently impacted by hCO_2. During the vegetative stage the photoassimilates are directed to new shoots and leaves, which are the only sinks; during the reproductive phase the major sinks become the developing grains [8]. So, the biggest impact in growth and yield is during the vegetative stage which might explain why Wheeler et al. (1997) found no impact of hCO_2 on proximate composition, total dietary fiber, nitrate and elemental composition of tomato fruit itself.

But, if on one hand the photoassimilates increase, increasing growth and yield, on the other hand, N content in the leaves decreases under hCO_2, creating an imbalance in C/N ratio [23]. The activity of Rubisco is also decreased as it has a role in N storage and remobilization to the grains. Hence, as the photorespiration is repressed (Box 1) and the nitrate assimilation depends on this process, its translocation to the chloroplast is inhibited [18] and photosynthetic nitrogen use efficiency is increased [6]. As it is largely known, N is a key component of proteins, nucleic acids, chlorophyll, phytohormones and secondary metabolites [24]. Therefore, lower concentrations of N in the leaves could result in lower nutrient and protein levels in the grains.

Besides increasing total dry mass, hCO_2 alters photoassimilates partitioning towards the roots [5,25]. As the plant gets bigger, so does its necessity for more nutrients, and it has been shown that nutrient partitioning is also altered by this climate change. For instance, in rice plants [25], P, N, K, Mg and Ca, generally decreased in all organs under hCO_2 and these nutrients were mostly allocated in the roots, which caused a decrease of their levels in the shoots. This decreased nutrients content in the above-ground organs most likely leads to less available nutrients to be remobilized to the grains and a consequent loss of quality.

For long it was thought that the decreased nutritional value could be a dilution effect of the higher biomass consequent of hCO_2 levels [26]. However, we believe this is not a likely possibility, and recent research proves otherwise. For example, Zhang et al. (2013) found that grain mass and grain nitrogen concentration were negatively correlated but, when looking at the effect of hCO_2 on superior and inferior spikelets independently,

whilst in the first plant structure grain nitrogen concentration decreases and the mass is maintained, on the latter the grain mass increases but the nitrogen concentration does not reduce, hence suggesting that rather than a dilution effect, one should consider differential responses of C and N allocation to the grains [27]. Besides C and N (and protein) concentrations, several other nutrients have been shown to decrease under hCO_2: Zn and Fe were found to decrease significantly in different C3 grasses and legumes [4,28]; and P, K, Cu, Zn, Mg and S decreased in leafy vegetables [29]. Considering the general mass increase caused by hCO_2, it is easy to infer that the plants' mineral requirements are also increased [30].

Therefore, it is our opinion that a dilution effect cannot explain hCO_2 effect on plants, since it would not account for the fact that some minerals, such as Fe and Zn, tend to be lower, whereas other minerals may actually increase.

Iron as a case study

Parallel to hCO2, it is also incontestable that restricted soil Fe supply will impact the nutrition of the foods, which we will consume in the future, as low Fe uptake restricts Fe content and plant fitness [31]. Scientists are starting to assess these issues independently, but studies linking these two important aspects are few, and more targeted analyses are required.

Despite the abundance of Fe in soils (it is the fourth most abundant element in the earth's crust), Fe has low solubility and this is a hurdle that leads to Fe deficiency in plants, especially in aerated calcareous soils, which represent one third of cultivated lands of the whole world [32]. Iron Deficiency Chlorosis (IDC) is one of the main consequences of Fe deprivation in plants and, if left untreated, it leads to stunted growth with reduced total biomass, which together with chlorosis, leads to severe yield losses due to reduced number of seeds per plant and economic problems of great impact amongst farmers [33].

One of the putative explanations behind Fe losses under hCO_2 is its impact on nutrient transportation throughout the plant. It has been shown that Fe partitioning ability can vary within plant cultivars [34] and this may be aggravated by hCO_2 stress. In the case of Fe, it is transported both through the xylem and the phloem [35]. This is an important aspect since, as explained above, one of the consequences of hCO_2 is decreased stomatal conductance, leading to the supposition that the nutrient flux (thrived by transpiration) could decrease too. Hence, although hCO_2 and Fe deficiency have opposite effect on plant

yield, both appear to negatively impact plants' nutritional composition, aggravating nutritional losses and leading to poorer grain quality.

Furthermore, Fe is an interesting case study, as plants, in order to absorb this nutrient through the roots, trigger two distinct uptake strategies – Strategy I, used by dicotyledonous and non-grasses species, and Strategy II, used by graminaceous species (Box 2). A recent report [36] compared and projected the response of two crops, soybean (C3) and maize (C4), to isolated and combined climate change stresses (hCO_2, heat and drought). They observed that the so-called 'fertilization effect' of hCO_2 is dependent, for example, on plants' water condition and that soybean is more likely to be negatively impacted by climate change stresses. But this study did not account for the fact that, besides differing on CO_2 assimilation process, these species are also distinct in terms of Fe uptake strategy utilization: soybean utilizes strategy I, and maize, strategy II. In strategy II plants, phytosiderophores play an important role in the process of Fe uptake (Box 2) and, interestingly, as the majority of C4 plants are grasses, they are mostly strategy II utilizing plants. Thus, we suggest that future studies looking at the response to of different crops to hCO_2, would also look at the influence of these processes on nutritional composition. We predict that strategy II utilizing plants would be less affected than the ones using strategy I, even when belonging to the C3 class. The engineering of C4 photosynthetic machinery into rice [37] could be a resourceful tool to clarify this proposition. Rice is a strategy II utilizing crop and this alteration could decrease its susceptibility to hCO_2, particularly in terms of the impact on mineral composition, besides the added value in terms of photosynthetic machinery [38].

As stated in Box 2, organic acids have the ability to chelate Fe and, as such, they have important role in Fe metabolism. Besides being released into the rhizosphere to aid in the uptake process, they are also present inside the plant, functioning as Fe-carrier molecules for root-to-shoot transport. Recent studies have shown that hCO_2 reduces organic acids production [39,40], leading us to the conclusion that this would be another factor contributing for the decrease in Fe levels both in leaves and in seeds.

Box 2. Iron uptake strategies

Plants developed strategies to acquire Fe from the rhizosphere, which are classically divided in two and that have been thoroughly reviewed [33,52,53,54]. Strategy I (Fig. 2a), which is utilized by all plants except those from Poaceae family, implies an acidification of the rhizosphere by H^+ extrusion to allow the reduction of Fe(III) to Fe(II) by a plasma membrane-bound ferric chelate reductase, FRO2, and consequent absorption into root epidermal cells by transmembrane transporters, IRT-like proteins, that belong to the iron-regulated proteins (ZIP) family (reduction strategy). Alongside with FRO, other compounds have been proposed to have a key role in the reducing step, such as phenolics, organic acids, sugars and flavins [55].

Strategy II (Fig. 2b) is utilized by graminaceous species. In order to increase uptake, these plants synthetize phytosiderophores (PSs), of which nicotianamine (NA) is the biosynthetic precursor, that are released to the rhizosphere and act as chelators with high affinity for Fe(III) (chelation strategy). Phytosiderophores are effluxed to the rhizosphere via TOM1, a transporter whose expression levels augment under Fe-deficient conditions. Once in the rhizosphere, the complex Fe^{3+}-PS is formed and is taken up into the root cells by transmembrane proteins of the yellow-stripe1 (YS1) family. YS1 transporters have been identified in several grass species, and, interestingly, non-graminaceous plants also have YS1-like (YSL) genes that encode proteins essential in metal-NA complexes transporting.

Although this classic division is mostly true, there are few studies showing that some Strategy II plants could use Strategy I mechanisms, as is the example of rice [56,57]. Evidences suggest the use of a ‘combined strategy’, where rice plants besides absorbing Fe(III) via the chelation strategy, also take up Fe(II) directly by the induction of the strategy I transmembrane transporters IRT1/IRT2 [58].

Box 2. Iron uptake strategies (continued)

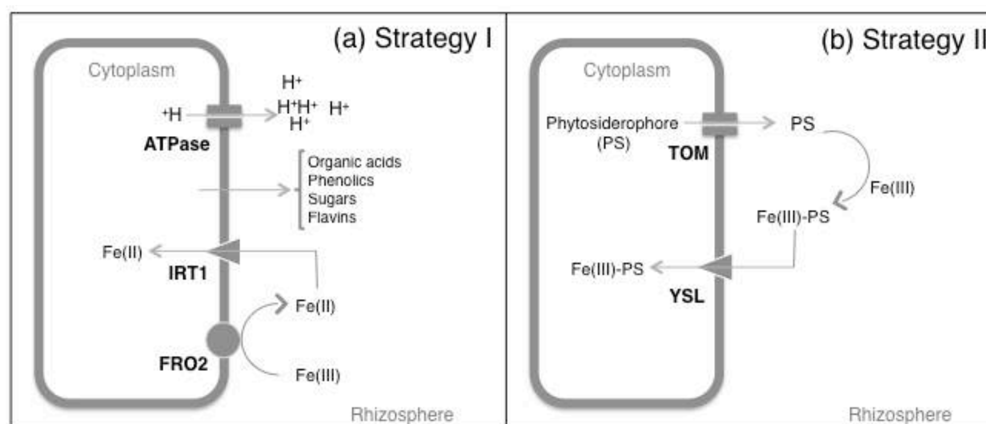

Figure 2. Schematic representation of the strategy I / reduction strategy (a) and strategy II / chelation strategy (b) for Fe uptake in plants.

In the previous section it was already mentioned that N remobilization to the grain is expected to decrease, especially in C3 plants where photorespiration occurs. Strategy II and the nitrate uptake process are closely related and 2'-deoxymugineic acid (DMA) phytosiderophores were shown to link both Fe and nitrate assimilations [41]. Under alkaline conditions the synthesis of phytosiderophores precursor nicotianamine (NA) is expected to increase, thus increasing phytosiderophores release and coping with decreasing Fe levels. This mechanism was hypothesized to be enough to counteract quality grain losses due to hCO_2 [42], however as N becomes less and less available, protein synthesis decreases, and we predict that this will affect photosyderophore synthesis. It is important to stress that soil microbial community composition and structure is also altered under hCO_2 [43,44]. In this context, we predict that future studies might have to look at the effect of hCO_2 on N fixing bacteria or bacteria that assist in mineral absorption.

Mineral losses could also be aggravated by the fact that NA is required to complex with metals like Fe, namely for their transport through the phloem, both in strategy I- and strategy II-utilizing plants [45]. Hence, as phloem transport is impaired by hCO_2, alongside with decreased protein and organic acid synthesis, it seems probable that nutrient losses in the grains would be inevitable. Given the reported changes in soil properties it is also pertinent to reflect on the use of hydroponic growth *versus* soil to study the hCO_2 effect by itself or conjugated with other stresses.

Concluding Remarks

It is important to be mindful that the rising CO_2 has been shown to affect the nutritional value of not only cereals and legume crops, but also fruits and leafy vegetables. Nowadays, we are facing a compromise between the necessity for higher yields and production rates and the loss of the quality of the food. Models for prediction of hCO_2 and the environmental changes due to it have been developed [7,46]. However, other variables like photosynthetic acclimation [47], increasing temperatures [48], fertilization [49], or insect-plant interactions [50] have been shown to influence the extent of hCO_2 consequences in plants. Furthermore, 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 hCO_2 is imperative. The risk of under or overestimating the effect of hCO_2 in real agronomic conditions will decrease as key questions are addressed by modern science and research (see Outstanding Questions).

Outstanding Questions

Could the reduced mineral concentrations be due only to a dilution effect (since under hCO_2 conditions plants have increased biomass)? Or are we facing a possible decrease in nutritional content of our legume plants?

How does the plant balance the increase in photosynthetic reactions caused by increased yield and nutritional value maintenance?

Should we consider hCO_2 “tolerant” cultivars in future agricultural practice? If so, should we select cultivars based on their yield increase under hCO_2 or on their ability to maintain protein and mineral content?

Do plants with different iron uptake strategies (e.g. rice *versus* tomato plants) equally respond to hCO_2? How will nicotianamine synthesis be impacted by N decreasing levels? Shouldn’t this affect phytosiderophore synthesis and metal transport through phloem?

Since evidence show a negative impact of hCO_2 on iron and zinc concentrations in plants, could it also have an effect on the accumulation profile of heavy metals?

Since mineral and N uptake is influenced by soil factors (including microbial diversity), could hCO_2 trigger lower nutrient uptake via a modulation of soil characteristics?

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Outputs of the Thesis

Publications

1. Santos, C. S.; Silva, A. I.; Serrão, I.; Carvalho, A. L.; Vasconcelos, M. W. (2013) Transcriptomic analysis of iron deficiency related genes in the legumes. Food Research International, 54: 1162-1171. **doi: 10.1016/j.foodres.2013.06.024**

2. Pereira, M.P.; Santos, C.S.; Gomes, A.; Vasconcelos, M.W. (2014) Cultivar variability of iron uptake mechanisms in rice (*Oryza sativa* L.). Plant Physiology and Biochemistry, 85: 21-30. **doi: 10.1016/j.plaphy.2014.10.007**

3. Santos, C.; Roriz, M.; Carvalho, M.P.; Vasconcelos, M.W. (2015) Iron partitioning at an early growth stage impacts iron deficiency responses in soybean plants (*Glycine max* L.). Frontiers in Plant Science, 6: 325. **doi: 10.3389/fpls.2015.00325**

4. Santos, C.S.; Serrão, I.; Vasconcelos, M.W. (2016) Comparative analysis of Iron Deficiency Chlorosis responses in soybean (*Glycine max*) and barrel medic (*Medicago truncatula*). Revista de Ciências Agrárias, 39: 70-81.
doi: http://dx.doi.org/10.19084/RCA16090

5. Santos, C.S.; Carvalho, S.M.P.; Leite, A.; Moniz, T.; Roriz, M.; Rangel, A.O.S.S.; Rangel, M.; Vasconcelos, M.W. (2016) Effect of tris(3-hydroxy-4-pyridinonate) iron(III) complexes on iron uptake and storage in soybean (*Glycine max* L.). Plant Physiology and Biochemistry, 106: 91-100. **doi: 10.1016/j.plaphy.2016.04.050**

6. Santos, C.S.; Benkeblia, N.; Vasconcelos, M.W. (2017) Strategies for enhancing phytonutrient content in plant based foods. In N. Benkeblia (Ed.) Phytonutritional improvement of crops. John Wiley & Sons, Ltd., West Sussex – UK. (in press)

7. Lima, M.R.M.; Santos, C.S.; Vasconcelos, M.W. (2017) The use of genetic engineering to improve the nutritional profile of traditional plant foods. In N. Benkeblia (Ed.) Phytonutritional improvement of crops. John Wiley & Sons, Ltd., West Sussex – UK. (in press)

8. Santos, C.S.; Roriz, M.; Rangel, A.O.S.S.; Carvalho, SM.P.; Vasconcelos, M.W. (2017) Oxidative stress impairs iron efficiency in soybean plants. (submitted)

9. Santos, C.S.; Carvalho, S.M.P.; Vasconcelos, M.W. (2017) Increasing atmospheric CO_2 impact in plants nutritional quality. (submitted)

Conference papers

1. Santos, C. S.; Roriz, M.; Carvalho, S. M. P.; Rangel, A. O. S. S.; Vasconcelos, M. W. (2015). The impact of iron deficiency chlorosis on the tetrapyrrole and antioxidative systems in soybean plants (Glycine max L.). In Eucarpia International Symposium on Protein Crops, Actas AEL 6, pp. 55-56.

Oral communications

1. Santos, C. S.; Roriz, M.; Carvalho, S. M. P.; Rangel, A. O. S. S.; Vasconcelos, M. W. (2015). The impact of iron deficiency chlorosis on the tetrapyrrole and antioxidative systems in soybean plants (*Glycine max* L.). In Eucarpia International Symposium on Protein Crops, May 6.

Scientific Posters

1. Santos, C. S.; Silva, A. I.; Serrão, I.; Carvalho, A. L.; Vasconcelos, M. W. (2012) Identification of mineral related genes in the legumes using high throughput sequencing. VI International Conference on Legume Genetics and Genomics, Hyderabad, India.

2. Santos, C.S.; Carvalho, S.M.P.; Vasconcelos, M.W. (2013). Morphological and molecular responses of efficient and inefficient soybean accessions to iron deficiency chlorosis. XIII Congresso Luso-Espanhol de Fisiologia Vegetal. Lisboa, Portugal. 24-27 July.

3. Silva, A.I.; Santos, C.S.; Vasconcelos, M.W. (2013). Transcriptomic analysis of iron nutrition genes in soybean [(*Glycine max* (L.) Merr.] XVII International Plant Nutrition Colloquium. Istanbul, Turkey. 19-22 August.

4. Serrão, I.; Santos, C.S.; Vasconcelos, M.W. (2013). Evaluation of Iron Deficiency Chlorosis (IDC) mechanisms in soybean (*Glycine max*) and barrel medic (Medicago truncatula). XVII International Plant Nutrition Colloquium. Istanbul, Turkey. 19-22 August.

5. Santos, C.S.; Roriz, M.; Carvalho, S.M.P., Vasconcelos, M.W. (2014) Iron partitioning at an early growth stage impacts iron deficiency responses in soybean plants. IFLRC VI & ICLGG VII. Saskatoon, Saskatchewan, Canada. Pp. 160

6. Santos, C.S.; Carvalho, S.M.P.; Rangel, A.O.S.S.; Rangel, M.; Vasconcelos, M.W. (2014) Evaluation of the effect of new iron (III)-chelates in mineral nutrition of soybean

plants (*Glycine max* L.). IFLRC VI & ICLGG VII. Saskatoon, Saskatchewan, Canada. Pp. 160

7. Vasconcelos, M.W.; Santos, C.S.; Machado, A.; Pinheiro, C.; Ricardo, C.P. (2016) Combined 'omics' approaches to dissect the root's iron uptake system in soybean plants. COST action FA 1306. Diving into integrative cell phenotyping through "omics". Versailles, France.

8. Santos, C.S.; Araújo, D.; Carvalho, S.M.P.; Mohammad-Reza, H.; Giehl, R.; von Wirén, N.; Vasconcelos, M.W. (2016) Phenotyping for iron deficiency chlorosis at a morphological, biochemical and multispectral level. Second International Legume Society Conference: Legumes for a sustainable world. Tróia, Portugal. Pp. 329.