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## EVALUATION OF THE PHYSIOLOGICAL AND MOLECULAR IMPACT OF IRON DEFICIENCY IN TWO LEGUME SPECIES

Inês Valadares Serrão Branco Mendes

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EVALUATION OF THE PHYSIOLOGICAL AND MOLECULAR IMPACT  
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AVALIAÇÃO DO IMPACTO FISIOLÓGICO E MOLECULAR DA  
DEFICIÊNCIA DE FERRO EM DUAS ESPÉCIES DE LEGUMINOSAS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Food Engineering

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## Resumo

As leguminosas apresentam um papel socio-económico importante e fazem parte da nutrição humana e animal. As suas sementes comestíveis são uma fonte de micronutrientes, com alto conteúdo proteico.

Apesar do ferro (Fe) ser o quarto elemento mais abundante na superfície terrestre, a sua baixa solubilidade torna-o pouco biodisponível para as plantas, baixando o conteúdo de Fe nas suas sementes. Em países pobres, onde as sementes são componentes predominantes das dietas, o acesso a fontes de nutrientes variadas e fortificadas é limitado, aumentando a prevalência de doenças associadas à subnutrição, tal como a Anemia por Deficiência de Ferro. Várias estratégias têm sido utilizadas para atenuar esta doença, sendo uma delas a biofortificação. Contudo, para aumentar o conteúdo nutricional das sementes é necessário perceber os mecanismos relacionados com as vias dos minerais até às mesmas. Assim, genes relacionados com a absorção (*FRO2*, *IRT1*), transporte (*NRAMP3*, *VIT1*, *YSL1*) e armazenamento (*Ferritin*) do Fe, bem como novos genes candidatos (*GCN2* and *WEE1*), foram estudados, em duas espécies de leguminosas utilizadas na nutrição humana e animal: *Glycine max* e *Medicago sp.* Este trabalho foca também os mecanismos fisiológicos comuns relativos à deficiência de Fe destas duas espécies, crescidas na presença e na ausência deste nutriente. Neste trabalho, ambas as espécies revelaram-se suscetíveis à clorose por deficiência de ferro (IDC), desenvolvendo vários sintomas característicos, tais como redução do crescimento, amarelecimento das folhas e desenvolvimento de raízes secundárias. Os genes *FRO2* e *IRT1* apresentaram níveis de expressão similares, o que sugere que o *IRT1* é co-regulado pelo *FRO2*. Quanto ao transporte, *NRAMP3* e *VIT1* apresentaram padrões de expressão opostos, sendo estes mais elevados nas folhas. *YSL1* e *Ferritin* apresentaram maior expressão na suficiência de Fe, em ambos os tecidos. Os resultados obtidos para o *GCN2* indicam que este gene participa no metabolismo do Fe, visto que na falta de Fe é sobre-expresso, principalmente nas raízes. Quanto ao *WEE1*, os resultados sugerem que este gene é sub-expresso em ausência de Fe onde o crescimento é reduzido. Quando submetidas a condições de stress (ausência de Fe), ambas as espécies apresentaram um impacto nutricional semelhante, com diminuição do conteúdo nutricional, e com maior concentração de zinco na parte aérea e de Fe nas raízes.

Este trabalho contribui para uma melhor compreensão, tanto dos mecanismos de resposta à deficiência de Fe, como de novos genes relacionados com o metabolismo do Fe, crucial não só para combater o IDC e consequente impacto económico, mas também para o aumento do conteúdo nutricional de leguminosas importantes na nutrição humana e animal.



## Abstract

Legume grains have an important socio-economical role, being highly utilized in human and animal nutrition. They are mainly cultivated for their edible seeds, sources of micronutrients, and their high protein content.

Although iron (Fe) is the fourth most abundant element in the earth's crust, its limited solubility makes it poorly bioavailable for plants, lowering the Fe content of their seeds. In poorer countries, where seeds are the main components of their diets, the access to diverse food sources and fortified food sources is often limited, increasing the prevalence of several diseases associated with undernourishment, namely Iron Deficiency Anemia (IDA). Various strategies are being used to withstand this disease and one of them is biofortification. However, to increase the seeds mineral content, one must understand the mechanisms underlying the minerals pathway to the plants edible parts. Thus, Fe related genes involved in nutrient uptake (*FRO2*, *IRT1*), transport (*NRAMP3*, *VIT1*, *YSL1*), and storage (*Ferritin*), as well as novel candidate genes (*GCN2* and *WEE1*), were studied, in two significant crops utilized in human and animal nutrition: *Glycine max* and *Medicago* sp. This work also focused on the common physiological mechanisms underlying the response to Fe deficiency, of these two species, grown under Fe-deficient and Fe-sufficient conditions. In the current work, both species were very susceptible to Iron Deficiency Chlorosis (IDC), developing several characteristic symptoms, such as yield reduction, yellowing of leaves and development of secondary roots. Fe acquisition genes, *FRO2* and *IRT1*, presented similar expression patterns, suggesting that *IRT1* is co-regulated with *FRO2*. In what concerns Fe transport, *NRAMP3* and *VIT1*, showed opposite functions and appeared to have a stronger expression in the leaves. *YSL1* and *Ferritin* presented higher expression levels under Fe-sufficient conditions in shoots and roots. The results obtained for *GCN2* strongly indicate that this gene plays a role in Fe metabolism, since in the lack of Fe it is over-expressed, mainly in roots. In what concerns to *WEE1*, the results show that this gene seems to have a more important role in Fe-sufficient roots, being under-expressed in stress situations, where growth is diminished. When submitted to stress conditions, both species presented a similar nutritional impact, with a decreased nutrient content, where Zn predominates in the aerial part, whereas Fe predominates in the roots.

This work contributes to an enhanced understanding, on Fe deficiency response mechanisms, as well as new Fe-metabolism related genes, crucial 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.



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## 1 Introduction

### 1.1 Legumes grains: the importance to human and animal nutrition

The *Fabaceae*, or legumes, broadly defined by their unusual flower structure, podded fruit and the ability to form nodules with rhizobia, economically represent the second most important family of crop plants after *Poaceae* (grass family). The unique capacity of legumes to obtain fixed nitrogen from the symbiotic nitrogen-fixing bacteria offers a growth advantage, as soil nitrogen often limits plant growth (Graham and Vance, 2003). Not only is the symbiosis beneficial to the legume, but also symbiotic nitrogen fixation introduces approximately 40 million tons of nitrogen into agricultural soils each year (Herridge *et al.*, 2008).

The legumes are highly diverse and can be divided into three subfamilies: *Mimosoideae*, *Caesalpinioideae*, and *Papilionoideae* (Doyle and Luckow, 2003). Among these, the *Papilionoideae* subfamily contains nearly all economically important crop legumes, including soybean (*Glycine max*), peanut (*Arachis hypogaea*), mungbean (*Vigna radiata*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), and alfalfa (*Medicago sativa*) (Zhu *et al.*, 2005).

Legume grains have been consumed by humans since the earliest practice of agriculture and have been used both for their medicinal, cultural as well as nutritional properties, providing an important source of protein and oil, which can also be converted into biodiesel (Libault *et al.* 2010). Legumes also have an important socio-economical role, being highly present in the Mediterranean diet (Santos *et al.*, 2013). They are cultivated for their edible seeds, sources of micronutrients, such as iron (Fe) and zinc (Zn), and their high protein content, being one of the most important foods, for humans and animals (Vasconcelos and Grusak, 2006). On a worldwide basis, legumes contribute with about one third of humankind's protein intake, while also serving as an important source of forage for animals and of edible and industrial oils (Graham and Vance, 2003). There are some population groups where 100% of their diet is plant based, being in most cases not a matter of personal choice, but of economic constraints, due to the high cost of proteins of animal origin and their inaccessibility by the poorer segment of the population (Tharanathan and Mahadevamma, 2003).

In intensive animal and milk production, where grain crops are major feed sources, forage legumes are required to maintain animal health (Jezierny *et al.*, 2010). This is why legume production is so important for helping to improve human and animal health and nutrition, attenuating the incidence of several diseases, especially the ones related to micronutrient deficiency.

### 1.1.1 Soybean and alfalfa

The Food and Agriculture Organization (FAO) statistics for 2012 show that about 253 million metric tonnes of soybean were produced across the world, ranking it one of the world's top commodity production. Much of the world's protein and oil comes from soybean and, in fact, soybean contains more protein (40%) than any other ordinary food source, including meat, cheese and fish, and high oil content (20%) (Krishnan, 2005; Bolon *et al.*, 2010). Soybean is considered an important food source worldwide, especially in the developing countries, for its nutritional quality, easy cultivation, low cost and high production, and is also an excellent raw material for the production of derived food (e.g. milk, sour cream) thanks to the versatility of application of its products in food and feed (Silva *et al.*, 2006). The appropriate addition of soy products results in lower calorie food products, with reduced lipid and high protein content, proper nutritional needs of adults, besides preserving the physical and sensory characteristics of traditional product (Dhingra and Jood, 2001). This feature allows soybean to be an important alternative food for the population in general, mostly for people who cannot or do not want to consume animal products and specific substances (e.g. lactose intolerant and vegetarians), as well as malnourished populations.

Moreover, *G. max* is a good crop to study genetic and molecular mechanisms since, in 2010, its genome was sequenced, assembled and published (Schmutz *et al.*, 2010). This discovery enabled the advance of studies of genetic and molecular analysis, contributing to a better understanding of nutritional problems related to this legume.

In ruminant nutrition, legume forages such as alfalfa have a higher feeding value than other typical grasses and cereals commonly used in animal feeds, because of their rapid particle degradation, faster rumen fermentation and reduced rumen retention time, besides having greater voluntary intake, resulting in increased dry matter intake, growth rate and milk yield (Dewhurst *et al.*, 2009). Alfalfa is grown all over the world on an estimated 32 million hectares of land (Yuegao and Cash, 2009). Crude protein content in alfalfa ranges between 18-25 %, with an oil and mineral proportion content of 1.78% and 9.87%, respectively (Katić *et al.*, 2009).

A closely related species to *M. sativa* is *Medicago truncatula*. Although *M. truncatula* is not as used in animal feeding as *M. sativa*, it has been chosen as a model species for genomic and molecular studies in view of its rapid life cycle, small diploid genome, self-fertilization, high seed production and high transformation efficiency, reduced size and easiness of growing (Trieu *et al.*, 2000).

To be convenient as a model for legume genomics, it is essential that *M. truncatula* exhibits genome conservation with other crop legumes. Choi *et al.* (2004) made a detailed comparison between *M. truncatula* and *M. sativa*, and observed that marker relationships were uniformly syntenic. In addition, Bell *et al.* (2001) reported that genes from *M. truncatula* share very high sequence identity to their counterparts from alfalfa, so it serves as an excellent, genetically tractable model for alfalfa, serving also as a model organism for soybean and other economically important legumes.

## 1.2 Iron

### 1.2.1 Anemia: a major disorder around the world

Although the effort on poverty reduction spearheaded by the United Nations and numerous civil society organizations is high, malnutrition, hunger and food insecurity are still affecting large portions of the global population, which consequently affects human and economic health and development (Charles, 2012). In most poor countries, the access to diverse food sources (quality and quantity) and fortified food sources, as well as the access to health services and interventions (e.g. supplementation) is often limited, increasing the prevalence of several diseases associated with undernourishment (e.g. anemia) (Balarajan *et al.*, 2011).

The prevalence of anemia is estimated at 9% in countries with high development, whereas in countries with low development the prevalence is 43% (McLean *et al.*, 2009), with Africa and Asia accounting for more than 85% of the absolute anemia burden in high-risk groups (Balarajan *et al.*, 2011). Children and women of reproductive age are most at risk, with global anemia prevalence estimated in 2011 at 43% in children younger than 5 years, 38% in pregnant women and 29% in non-pregnant women aged 15–49 years (Stevens *et al.*, 2013).

Anemia is a condition characterized by reduction in hemoglobin concentration, diminished red blood cells, or packed-cell volume, leading to the subsequent diminishing in meeting the oxygen demands of tissues. Nutritional anemia results from insufficient bioavailability of hematopoietic nutrients (e.g. including folate, vitamin B12 and vitamin A) needed to meet the demands of hemoglobin and erythrocyte synthesis (Semba and Bloem, 2008). Anemia can also be caused by acute and chronic inflammation, parasitic infections, and inherited or acquired disorders that affect haemoglobin synthesis, red blood cell production or red blood cell survival. Iron deficiency is among the most common nutritional problems of human populations throughout the world, affecting more than two billion people (Bouis and Welch, 2010), which is caused by dietary iron deficiency, iron mal-absorption, and blood losses.

### 1.2.2 Biofortification: how legume plants can improve our health

Various strategies are being used to withstand Iron Deficiency Anemia (IDA) (Bouis and Welch, 2010). One of them is supplementation with Fe, which can be practiced at health centers through a liquid or injectable medicine. However, this strategy has some drawbacks such as the taste of the medicine, teeth staining and need for an outreach as well as a delivery mechanism (Frossard *et al.*, 2000). Another approach to attenuate IDA in susceptible populations is fortification, usually done with flours produced from cereals, but tends to be expensive and is not an option for whole grains, like legumes (Blair *et al.*, 2010). Although these interventions have been considerably successful in the past, they have not been able to eradicate the problem, mainly owing to the significant and expensive distribution logistics required which are insufficiently developed in poorer countries (Beyer, 2010). Hereupon, innovative, cost-effective, accessible and long-lasting treatment and prevention options are needed (Charles, 2012).

An advantageous alternative to the techniques referred above is to increase the concentration of Fe in diets through biofortification. Biofortification is characterized by the ‘fortification’ of agronomical important crop plant tissues by means of their own biochemical capacity (Beyer, 2010). In other words, biofortification is the process by which the nutritional quality of staple crops is enhanced, using the best traditional breeding practices and modern biotechnology methods (Nestel *et al.*, 2006). This strategy implicitly targets poorer households, where staple foods predominate in their diets.

One of the major advantages of biofortified crops is that they capitalize on the regular daily intake of a consistent and large amount of food staples, by all family members (Nestel *et al.*, 2006). Another major advantage is that, after the one-time investment in order to develop seeds that fortify themselves, recurrent costs are low, and germplasm can be shared internationally, making biofortification a cost-effective approach (Beyer, 2010). Besides that, once the biofortified crop system is placed and stabilized, it is highly sustainable, and nutritionally improved varieties can still be grown and consumed, even if government attention and international funding for micronutrient issues fade (Nestel *et al.*, 2006). Probably the major advantage of biofortification, when compared to other approaches, is that it provides feasible means of reaching undernourished populations in relatively remote rural areas and to health care centers where supplementation programs are made. In an environmentally beneficial way, biofortification may have important spinoff effects for increasing farm productivity in developing countries.

The seeds mineral content depends on the uptake from the rhizosphere into the roots, translocation to the transpiring shoots through xylem, transference into leaves or other tissues, and finally, translocation into the seeds through phloem. An incomplete understanding of the pathways and the rate limiting steps involved in translocating minerals to the seeds represents a major challenge of biofortification (Waters and Sankaran, 2011). Thus, in order to develop a successful biofortification strategy, one must identify the key molecular players involved in nutrient uptake, transport and storage, preferably in crops heavily used in human and animal diets.

### 1.2.3 From root to seed: the mechanisms are still unclear

Although Fe is the fourth most abundant element in the earth's crust, its limited solubility makes it poorly bioavailable for plants (Morrissey and Guerinot, 2009), which consequently results in seeds with insufficient Fe content to meet the body's requirements. Fe availability is dictated by the soil redox potential and pH. In aerobic or alkaline soils, Fe is readily oxidized, and is predominately in the form of insoluble ferric oxides. At lower pH, the ferric Fe is free from the oxide, and becomes more available for uptake by roots (Morrissey and Guerinot, 2009). Because 30% of the world's cropland is too alkaline for optimal plant growth (Marschner, 1995) and some staple crops, like rice and soybean, are very susceptible to Fe deficiency (Takahashi *et al.*, 2001), much research has focused on how plants cope with Fe limitation. Crops growing in alkaline soils fail to accumulate Fe in edible organs causing nutrient deficiency in humans (Gómez-Galera *et al.*, 2010).

Plants require Fe for innumerable life-sustaining processes from respiration to photosynthesis, where it participates in electron transfer through reversible redox reactions, cycling between Fe<sup>2+</sup> (ferrous) and Fe<sup>3+</sup> (ferric) form. Fe homeostasis in plants is achieved through very dynamic processes requiring proteins and small organic molecules in order to take up the metal from the soil, to transport it throughout the plant, to compartmentalize it in the cells, and ultimately to buffer and to store it in case of excess (Briat *et al.*, 2009).

Insufficient Fe uptake leads to Fe deficiency chlorosis (IDC) symptoms, such as yellowing of the upper leaves, interveinal chlorosis and stunted growth, as well as reduction of crop yields (Prasad *et al.*, 2003; Kim and Guerinot, 2007). Besides influencing chlorophyll synthesis, IDC also 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 this context, the acquisition, usage and detoxification of

Fe pose a considerable challenge to cells and organisms, which have evolved sophisticated mechanisms to satisfy their metabolic needs and minimize the risk of toxicity (Domenico *et al.*, 2008; Andrews, 2008; Hentze *et al.*, 2010).

In order to uptake Fe from the soil, dicotyledonous plants, such as soybean and alfalfa, utilize Strategy I, which is characterized by the requirement of reducing Fe<sup>3+</sup> to Fe<sup>2+</sup> before its uptake. The reduction is carried out by a plasma membrane-bound ferric reductase, the Ferric Reduction Oxidase (FRO). This reduction step appears to be the rate-limiting stage in Fe uptake in *Arabidopsis thaliana* (Connolly *et al.*, 2003), a model species commonly used in plant biology. In fact, the transgenic overexpression of ferric chelate reductases in the roots of rice, tobacco and soybeans has been successful in increasing tolerance to Fe-limiting conditions (Vasconcelos *et al.*, 2006; Ishimaru *et al.*, 2007). The FRO family of metal reductases contains seven additional members in *Arabidopsis* spp. (Wu *et al.*, 2005; Mukherjee *et al.*, 2006) however *FRO2* is thought to be the main Fe<sup>3+</sup> chelate reductase in roots and over-expressed in the epidermal cells of Fe-deficient roots (Kim and Guerinot, 2007). Plants overexpressing *FRO2* are resistant to low Fe growth conditions (Connolly *et al.*, 2003).

The expression of FRO genes in various locations suggests that different sets of FRO proteins are participating in Fe uptake in different plant tissues. For example, *FRO2*, *FRO3* and *FRO5* are expressed in roots, and *FRO3* is predominantly expressed in the vascular cylinder of roots, suggesting a role in Fe re-absorption from the root apoplast (Kim and Guerinot 2007); and *FRO6*, *FRO7* and *FRO8* are described as shoot-specific (Kim *et al.*, 2006).

After its uptake, Fe<sup>2+</sup> is transported into the plant to leaves and seeds by specific membrane transporters (Grotz and Guerinot, 2006), such as the Iron-Regulated Transporter 1 (*IRT1*), a member of the Iron-Regulated Transporter-Like Protein (ZIP) gene family (Waters *et al.*, 2002; Walker and Connolly, 2008; Ivanov *et al.*, 2011). *IRT1* is expressed in the epidermal cells of roots and is localized in the plasma membrane (Kim and Guerinot, 2007). Both *FRO2* and *IRT1* genes are up-regulated under Fe deficiency and are activated by specific bHLH transcription factors, which are also up-regulated by Fe deficiency (García *et al.*, 2012).

Once Fe enters the root symplast, it must be bound by chelating compounds, such as nicotianamine (NA) and citrate. Fe-chelator complexes then move through intercellular connections into the stele along the diffusion gradient. A broad spectrum of transporters has been characterized in model plants including the Natural Resistance Associated Macrophage (NRAMP) family protein, the Vacuolar Iron Transporter (VIT), and the Yellow Stripe 1-Like (YSL). Plant NRAMP proteins are generally involved in Fe import into the cytoplasm (Brear *et al.*, 2013) and they can transport Fe (Lanquar *et al.*, 2005), manganese (Mn) (Cailliatte *et al.*,

2010; Lanquar *et al.*, 2010; Sasaki *et al.*, 2012), cobalt (Cailliatte *et al.*, 2010), cadmium (Sasaki *et al.*, 2012), and aluminum (Xia *et al.*, 2010), having a broad specificity. *AtNRAMP3* and *AtNRAMP4* are H<sup>+</sup> metal symporters responsible for Fe and Mn mobilization from the vacuole (Lanquar *et al.*, 2010). *VIT1* is involved in the uptake of Fe<sup>2+</sup> into the vacuole for storage (Brear *et al.*, 2013). YSL transporters, on the other hand, are likely involved in the transport of Fe<sup>2+</sup>-NA complexes and the genes they encode are expressed in various tissues, suggesting roles in Fe uptake at diverse locations (Kim *et al.*, 2006).

Fe must also be transported through the phloem bounded to chelators, because the transpiration flow in the xylem vessels is inefficient in developing organs such as the apex, seeds and root tips (Kim and Guerinot, 2007). Fe remobilization from older leaves to younger leaves also takes place via phloem transport (Kim *et al.*, 2006). In 1993, Stephan and Scholz suggested that Fe traveled in the phloem as a Fe-NA complex, however, later on, this idea became controversial (Schmidke *et al.* 1999), but NA is still thought to play a role in Fe loading and, probably, unloading of the phloem.

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 Fe deficiency and toxicity (Liao *et al.*, 2012). Therefore, storage proteins play an important role in Fe homeostasis, since they assure that ferric Fe is in a way that is still bio-available in case of cellular needs but yet nonreactive with oxygen (Briat *et al.*, 2010). Ferritins are a broad superfamily of ubiquitous Fe storage and detoxification proteins, found in the entire living kingdom, except in yeast (Andrews *et al.*, 2003; Briat *et al.*, 2006; Arosio *et al.*, 2008). Ferritin naturally occurs in plant tissues such as cotyledons, root and shoot apices, cells of vessels, reproductive cells and senescing cells, and are abundant when plants are grown under high Fe conditions (Briat *et al.*, 2010). Besides their role in Fe storage, ferritins are also involved in protection against oxidative stress through their potential detoxification properties of excess iron, dioxygen and, to some extent, hydrogen peroxide (Zhao *et al.*, 2002; Theil *et al.*, 2006; Arosio *et al.*, 2008).

The pathways and mechanisms that regulate the expression of these Fe-related genes are still unclear. Several published reports (Waters *et al.*, 2007; Bacaicoa *et al.*, 2011; Lingam *et al.*, 2011; Meiser *et al.*, 2011; Ramírez *et al.* 2011; Romera *et al.*, 2011; Wu *et al.* 2012) suggest that some hormones, such as auxin, ethylene and nitric oxide (NO), can up-regulate the expression of these genes, in plants grown in Fe-deficient conditions. However, in plants grown with high levels of Fe, these hormones showed almost no effect in the expression of Fe-related genes, suggesting that their up-regulation does not exclusively depend on activator hormones,

but also on Fe availability (Lucena *et al.* 2006; Graziano and Lamattina, 2007; Chen *et al.*, 2010; García *et al.* 2011).

Another question that is still unsolved, is how Fe reacts to repress gene expression and which pool of Fe (e.g. from root, phloem, intracellular, apoplastic, etc.) is monitored by the plant to mediate this control. Maas *et al.* (1998) proposed phloem Fe as an inhibitor of Fe deficiency responses in Strategy I plants, and they found that the application of Fe-EDTA to leaves decreased some of the Fe deficiency responses, such as proton extrusion and ferric reductase activity. Based on these results they proposed that leaves could modulate Fe deficiency responses through phloem Fe, leading to suppression of gene expression under Fe-sufficient conditions (Maas *et al.*, 1998). Beyond this, there are studies also suggesting that application of Fe, chelated to other compounds (e.g. citrate), to leaves of Fe-deficient plants suppressed root Fe deficiency responses (Venkatraju and Marschner, 1981; Romera *et al.*, 1992; Enomoto *et al.*, 2007).

Even though much has been learned about the physiology of Fe uptake in model species, which are relevant to human and animal nutrition, there is no clear understanding of the physiology of tolerance to Fe deficiency in crop species such as soybean and alfalfa, and this has hampered breeding programs (Vasconcelos and Grusak, 2013). 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 seed Fe levels (Santos *et al.*, 2013). 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.

#### 1.2.3.1 *GCN2* and *WEE1*: novel candidate genes

To clearly understand the mechanisms of Fe uptake and translocation to the edible parts of the plant, the search for new candidate genes and new molecules that could be involved in the process should be intensive. The quick understanding of these mechanisms is essential to build a successful biofortification program, in order to combat malnutrition around the world.

It is known that the phosphorylation of the  $\alpha$  subunit of the eukaryotic translation initiation factor 2 (eIF2 $\alpha$ ) provides a key mechanism for down-regulating protein synthesis in response to nutrient starvation or stresses (Wek *et al.*, 2006; Hinnebusch, 2005). In mammals, GCN2 is the primary eIF2 $\alpha$ -kinase in response to nutrient limitation (Zhang *et al.*, 2002).

Studies in mouse (Dang Do *et al.*, 2009) showed that, in stress situations, the expression of proteins encoded by mRNAs related to eIF2 $\alpha$  phosphorylation, namely GCN2, is increased, consequently enhancing Fe uptake.

A GCN2-like ortholog (AtGCN2) has been identified in *A. thaliana* (Zhang *et al.*, 2003) and was shown to be activated 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). The activation of GCN2 protein following wounding or exposure to methyl jasmonate, 1-Aminocyclopropane-1-carboxylic acid (ACC) and salicylic acid, further suggests that this enzyme could play a role in plant defense responses to insect herbivores and may represent a key but yet uncharacterized player linking biotic and abiotic stresses (Lageix *et al.*, 2008). Lageix *et al.* (2008) confirmed that AtGCN2 is essential for plant growth in stress situations, by introducing an intact copy of AtGCN2 in the *gcn2* mutant line and observing that plants grow identically to wild-type seedlings during amino acid deprivation. To this date, there are no published studies on the role of GCN2 on Fe uptake, which makes the study of this gene an important innovation in Fe nutrition in plants.

Ataxia Telangiectasia Mutated (ATM) and RAD3-Related (ATR) proteins are the main regulators of the DNA damage pathway and perform functions in plants very similar to those of their orthologs in mammals (Garcia *et al.*, 2003; Culligan *et al.*, 2004). These proteins sense DNA damage and induce the coordinated expression of DNA repair and cell cycle–arresting genes. It has been known that WEE1 kinase is a key protein for cell cycle arrest in plants at times of DNA damage and interferes directly with cell cycle progression phosphorylating Cyclin-dependent kinases (CDKs) and preventing the cell from entering mitosis (Jones R. *et al.*, 2013). Plants lacking a functional *WEE1* do not accumulate phosphorylated CDKs, thereby entering mitosis with incompletely replicated or damaged DNA, with growth arrest as a consequence (De Schutter *et al.*, 2007; Jones R. *et al.*, 2013). Recent works (Santos *et al.*, 2012) suggest that *WEE1* may have a role in Fe homeostasis, in *M. truncatula* plants, as this gene was found to be up-regulated in Fe deficient plants, using a transcriptomic approach. However, confirmation of this observation is lacking, which makes it relevant to study this gene, in order to understand how its expression is affected by Fe deficiency and which mechanisms is *WEE1* associated with.



## 2 Objectives

This project aims at identifying genes that are important in the uptake of Fe in two significant crops utilized in human and animal nutrition, *G. max* and *M. truncatula*, as well as evaluating the impact of low Fe availability on mineral accumulators in the plant food. It also aims at unravelling the common mechanisms underlying the response to Fe deficiency at a physiological and molecular level, of these two species, grown under Fe-deficient (0  $\mu\text{M}$   $\text{Fe}^{3+}$ -EDDHA [ethylenediamine-N,N'bis(o-hydroxyphenyl)acetic acid]) and Fe-sufficient (20  $\mu\text{M}$   $\text{Fe}^{3+}$ -EDDHA) hydroponic conditions. To this end,

The specific goals of this work are:

- To analyze the common morphological responses of the two species in study, such as plant weight, root length and secondary structure development;
- To understand how Fe deficiency affects photosynthetic pigment accumulators.
- To quantify and analyze, at a molecular level, selected genes related to Fe metabolism, in order to understand which genes are over expressed or repressed by the plant in Fe stress conditions.
- To evaluate the impact of Fe deficiency on Fe and Zn accumulation, in plants submitted to both treatments, in order to understand how Fe deficiency affects nutrient content.
- To identify novel genes that may be involved in Fe uptake or translocation and that may be candidates for improved biofortification strategies.

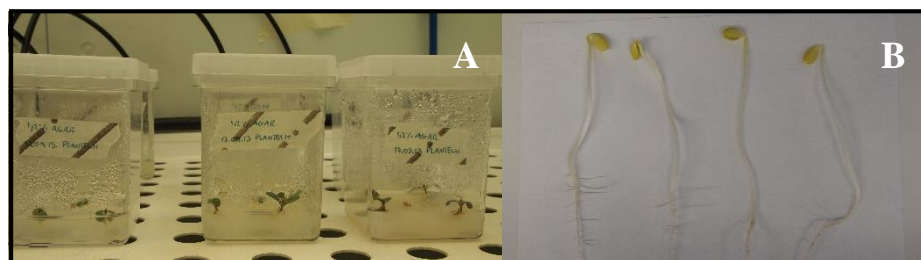


### 3 Materials and Methods

#### 3.1 Plant growth conditions

Plants of *Medicago truncatula* cultivar Luzerna revilheira and *Glycine max* cultivar Williams 82, were grown in an Aralab Fitoclima 10000EHF with 16h day / 8h night photoperiod. The temperature was kept at 20°C during the light period, with 350  $\mu\text{mol s}^{-1} \text{m}^{-2}$  of photon flux density, and at 18°C during the dark period, with 75% of relative humidity.

Scarified seeds of *M. truncatula* were germinated in 1.2% agar (Fig. 3.1-A) and of *G. max* were rolled in filter paper and placed vertically in a solution of 250 mM  $\text{CaCl}_2$  (Fig.3.1-B), at 25°C, in the dark.



**Figure 3.1** - Germination conditions of *M. truncatula* (A) and *G. max* (B) seeds.

Germinated seeds of *M. truncatula* and *G. max* with 5 cm and 15 cm length, respectively, were then transferred to hydroponic solutions with different Fe treatments: Fe-sufficiency (20  $\mu\text{M Fe}^{3+}$ -EDDHA) (+Fe) and Fe deficiency (0  $\mu\text{M Fe}^{3+}$ -EDDHA) (-Fe). The standard solution for hydroponic growth of *M. truncatula* contained: 3 mM  $\text{KNO}_3$ ; 1 mM  $\text{Ca}(\text{NO}_3)_2$ ; 0.5 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.5 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ ; 0.75 mM  $\text{K}_2\text{SO}_4$ ; 25  $\mu\text{M CaCl}_2$ ; 25  $\mu\text{M H}_3\text{BO}_3$ ; 2  $\mu\text{M MnSO}_4$ ; 2  $\mu\text{M ZnSO}_4 \cdot \text{H}_2\text{O}$ ; 0.5  $\mu\text{M CuSO}_4 \cdot \text{H}_2\text{O}$ ; 0.5  $\mu\text{M MoO}_3$ ; 0.5  $\mu\text{M NiSO}_4$ . The conditions used for *G. max* included: 1.2 mM  $\text{KNO}_3$ ; 0.8 mM  $\text{Ca}(\text{NO}_3)_2$ ; 0.3 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.2 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ ; 25  $\mu\text{M CaCl}_2$ ; 25  $\mu\text{M H}_3\text{BO}_3$ ; 0.5  $\mu\text{M MnSO}_4$ ; 2  $\mu\text{M ZnSO}_4 \cdot \text{H}_2\text{O}$ ; 0.5  $\mu\text{M CuSO}_4 \cdot \text{H}_2\text{O}$ ; 0.5  $\mu\text{M MoO}_3$ ; 0.1  $\mu\text{M NiSO}_4$ . Both hydroponic solutions were buffered by the addition of 1mM MES, pH 5.5.

After 14 days (14d) of hydroponic growth, the length and weight of shoots and roots was measured and the number of secondary roots was counted.

#### 3.2 Total chlorophyll extraction and quantification

The total chlorophyll extraction and quantification was performed according to an optimized method from Abadía *et al.* (1984).

In short, 12.5 ml of 4 g/L solution of CaCO<sub>3</sub> in methanol were added to 0.5 g of fresh leaves. After an incubation period of 48 hours in the dark, at room temperature, the material was white due to the loss of the pigments to the methanolic solution. One ml of the supernatant was diluted in 25 ml of deionized water and absorbance measures were taken at 663 nm and 645 nm. Spectrophotometric chlorophyll quantification of total chlorophylls was calculated following the equation 3.2.1.

$$\text{Total Chlorophylls} = (8.02 \cdot A_{663} + 20.21 \cdot A_{645}) \frac{0.0125 \times \text{Dilution Factor}}{\text{Fresh weight (g)}} \quad (3.2.1)$$

### 3.3 Mineral Analysis

Three replicates of 200 mg of dried shoot and root of *G. max* and *M. truncatula* were mixed with five milliliters of 65% HNO<sub>3</sub> in a Teflon reaction vessel and heated in a Speedwave<sup>TM</sup> MWS-3+ (Berghof, Germany) microwave system. Digestion procedure was conducted in five steps: 130°C for five minutes; 160°C for 10 min; 170°C for 10 min; 100°C for two minutes and 100°C for two minutes. Leaf tomato standard (Sigma) was digested to ensure digestion efficiency. The resulting clear solutions of the digestion procedure were then brought to 20 mL with ultrapure water for further analysis.

The determination of mineral concentration was performed using the Inductively Coupled Plasma – Optical Emission (ICP-OE) spectrometer Optima 7000 DV (PerkinElmer, Massachusetts, USA) with radial configuration.

### 3.4 RNA extraction

To extract RNA from leaves and roots, the Qiagen RNeasy Plant Mini Kit (USA, #74904) was used. Briefly, roots and leaves were grounded with liquid nitrogen, until a fine powder was obtained. To 75 mg of leaf powder and 100 mg of root powder, RLC buffer and RLT buffer were added, respectively, with 1% of β-mercaptoethanol. Roots and leaves were vortexed at 56°C for 3 minutes and at room temperature (RT) for 6 minutes, respectively. The lysates were transferred to a QIAshredder spin column sitting in a 2 ml collection tube. Roots and shoots were centrifuged at maximum speed for 5 and 10 minutes, respectively. After the flow through was transferred to another 1.5 ml tube, 225 µl and 375 µl of 100% ethanol was added to roots and leaves respectively. Samples were transferred to an RNeasy mini spin

column sitting in a 2 ml collection tube, and they were centrifuged for 15 seconds at 10000 rpm. The column was then washed twice with 500 µl of RW1 buffer, centrifuged for 15 seconds, and then washed again with 500 µl of RPE buffer and centrifuged for another 15 seconds. RNeasy column was finally transferred to a 1.5 ml tube and RNA was eluted with 30-50 µl of sterilized water.

Possible DNA contamination was cleaned with Turbo DNA-free kit (Ambion, Austin, TX, USA), accordingly to manufacturer's instructions. RNA quality and quantity were checked with UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal).

### 3.5 Gene expression analysis

About two µg of total RNA were used to synthesize cDNA, using the First Strand cDNA Synthesis Kit (Fermentas), following manufacturer's instructions. In short, one µl of Random Hexamer primer and eight µl of nuclease-free water were added to two µg of RNA. To this mixture it was added four µl of 5X Reaction Buffer, one µl of RiboLock RNase Inhibitor (20 u/µl), two µl of dNTP Mix (10 mM) and two µl of M-MuLV Reverse Transcriptase (20 u/µl), making a total volume of 20 µl. The mixture was finally incubated at 37°C for 60 min, and then heated at 70°C for 5 min.

Candidate genes were selected according to their established (*FRO2*, *IRT1*, *NRAMP3*, *VIT1*, *YSL1*, *Ferritin*) or possible (*WEE1* and *GCN2*) role on Fe metabolism. Primers targeting Fe stress metabolism 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 3.1. Quantitative Real-Time PCR (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 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. The amplification of all genes was performed accordingly to Han *et al.* (2013). Briefly, samples were heated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15s and 68 °C for 15s. Melt curves profiles were analysed for each gene tested. The comparative CT method ( $\Delta\Delta CT$ ) (Livak, & 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).

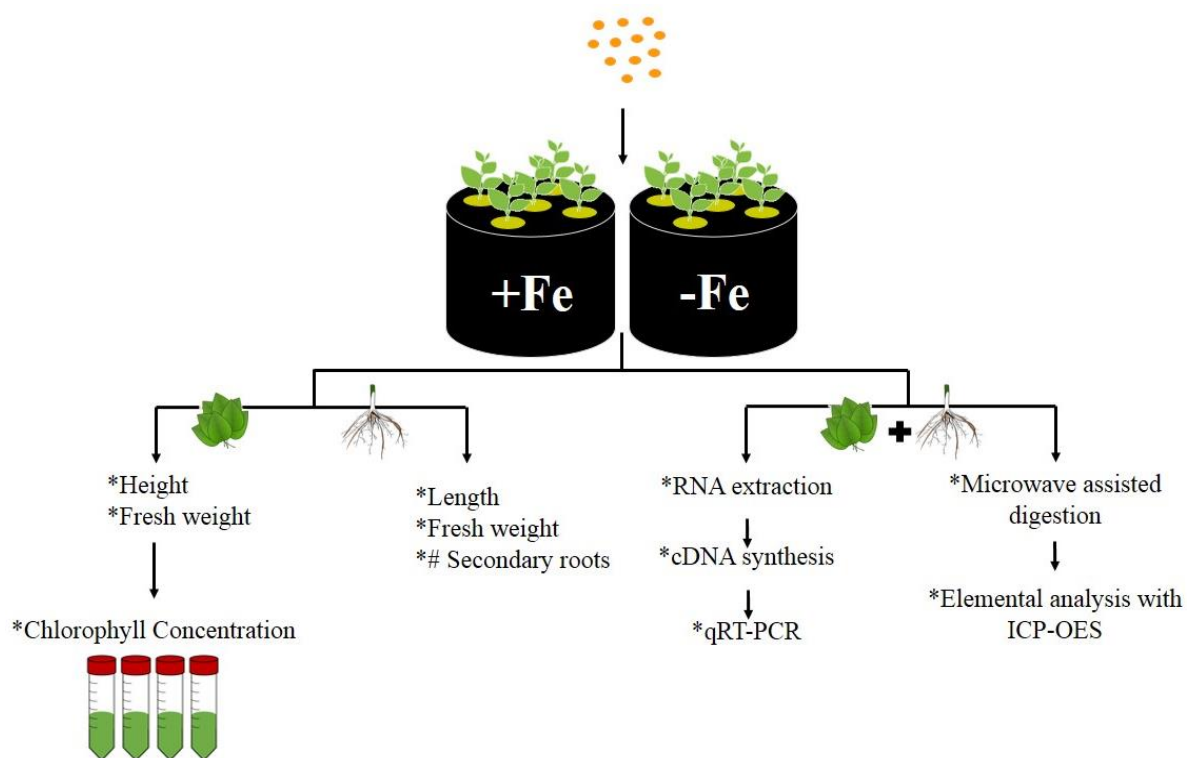
**Table 3.1** – Forward and reverse primer sequences used in quantitative Real-Time PCR reactions.

Gene	Specie	Sequence
<i>18S</i>	-	F 5'- TTAGGCCATGGAGGTTTGAG -3' R 5'- GAGTTGATGACACGCGCTTA -3'
<i>FRO2</i>	<i>G. max</i>	F 5'- TGCTTGGACTCACACCAGAG -3' R 5'- AGAGGTAGAAACCGGGGAGA -3'
	<i>M. truncatula</i>	F 5'- CACTTGTGATGGTGAGTGGA -3' R 5'- GATGGTGTGCCAGAAATAGG -3'
<i>IRT1</i>	<i>G. max</i>	F 5'- GATTGCACCTGTGACACAAA -3' R 5'- CAGCAAAGGCCTTAACCATA -3'
	<i>M. truncatula</i>	F 5'- GACAAAGGAACCGGAACAAA -3' R 5'- TTGATGGAAGCAAAGTGACAG -3'
<i>YSL1</i>	<i>G. max</i>	F 5'- GCTTTTGGAGCAGGTCTCAC -3' R 5'- AGACCACAACCCACAAGTCC -3'
	<i>M. truncatula</i>	F 5'- GATCTTGGCCCACAACAAGT -3' R 5'- ACTGCAGGAACCATCAAACC -3'
<i>VIT1</i>	<i>G. max</i>	F 5'- TTGTTAGCTTGGCGTGACAG -3' R 5'- TGCAACCAAGGTAACCACAA -3'
	<i>M. truncatula</i>	F 5'- GGGTGGAAATTGTTCTCTCA -3' R 5'- AGCACTCCTGATTGGCTTGT -3'
<i>FER</i>	<i>G. max</i>	F 5'- CCCCTTATGCCTCTTTCTC -3' R 5'- GCTTTTCAGCGTGCTCTCTT -3'
	<i>M. truncatula</i>	F 5'- GTAAGAAATGGGGTGGTGGA -3' R 5'- CGAGCCAAAGAACTTGAGG -3'
<i>NRAMP3</i>	<i>G. max</i>	F 5'- TG TTCAGTCAAGGCAGGTTG -3' R 5'- CCAGCATTTACAAGGCCAAT -3'
	<i>M. truncatula</i>	F 5'- TTTGGATCCTGGAACTTGG -3' R 5'- GCTGAATCAAAAAGCCCCATA -3'
<i>GCN2</i>	<i>G. max</i>	F 5'- ATCCTTGCCTCATCACCAAC -3' R 5'- ATGGGGAACTGTGTTTGAGC -3'
	<i>M. truncatula</i>	F 5'- GTAACCGAGGTCCGAGATGA -3' R 5'- CTCCACCATGGGTCAGAAGT -3'
<i>WEE1</i>	<i>G. max</i>	F 5'- TTGCGATAGGGAAAATACGC -3' R 5'- AGGGGTTCTTAAAGCAAGGA -3'
	<i>M. truncatula</i>	F 5'- TGCATATCGGGTACCACTCA -3' R 5'- TATGCACAATTGGGAGGTCA -3'

### 3.6 Statistical Analysis

The obtained data concerning shoot and root length and weight, number of secondary roots, chlorophyll concentration, and Fe and Zn content, were analyzed using GraphPad InStat for Windows (Version 3.05, 16 bit, GraphPad Software, Inc.). Treatment differences were tested by t-test ( $p < 0.05$ ).

Figure 3.2 summarizes all the methodologies utilized in this work to evaluate the mechanisms underlying the response to Fe deficiency at a molecular and physiological level, of *G. max* and *M. truncatula* plants grown in Fe-sufficiency and Fe-deficiency.



**Figure 3.2** - Schematic representation of the methodology applied in this work.



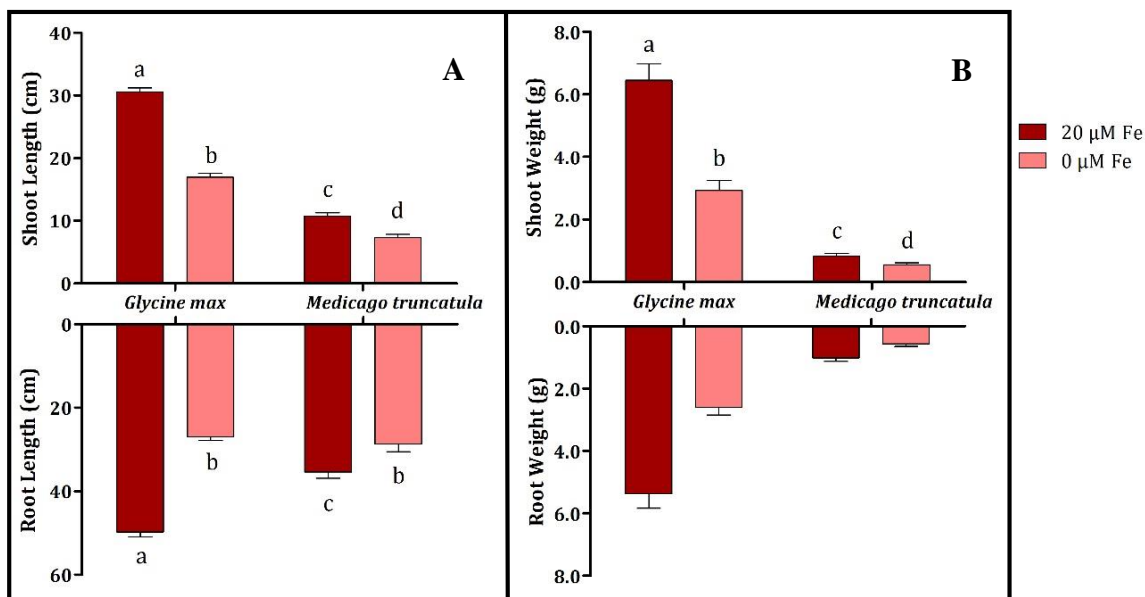
## 4 Results and Discussion

From all the strategies being used to withstand Iron Deficiency Anemia, biofortification seems to be the one with greater advantages and it may have important spinoff effects for increasing farm productivity in developing countries. Thus, Fe is not synthesized in the plant, it must be absorbed from the rhizosphere. The amount of Fe that a plant food can accumulate in its edible tissues will be directly dependent on the uptake of Fe from the soil. It is crucial to analyze the morphological mechanisms developed by the plant in response to iron limitations in the soil, in order to understand how the plant is nutritionally affected by Fe deficiency. The growth, the development of secondary structures and the chlorophyll levels of soybean and *M. truncatula* plants grown in Fe-sufficiency and Fe deficiency was evaluated.

The seed mineral content depends on the uptake of minerals into the roots, translocation to shoots, and efficient transference to the seeds. Increasing nutrient concentration in the seeds, via genetic manipulation or conventional breeding, also requires a better understanding of these pathways, especially the rate limiting steps involved in loading minerals to the seeds. Therefore, in order to develop a successful biofortification strategy, one must identify the key 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 novel candidate genes, that could have important roles in Fe metabolism (e.g. *GCN2* and *WEE1*).

### 4.1 IDC symptoms: visible response mechanisms

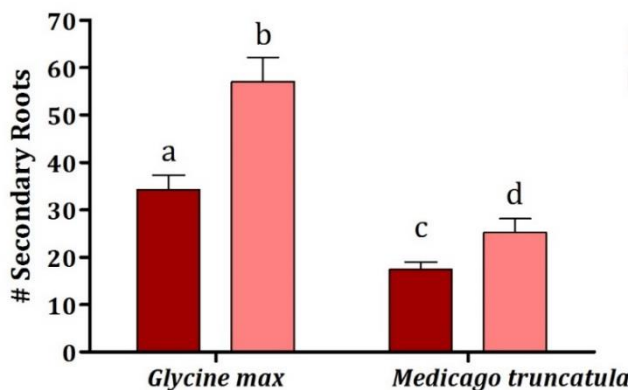
When Fe is scarce, plants develop several morphological and biochemical changes that lead to an increase in their Fe uptake capacity. One of the main consequences of IDC is the yield reduction that leads to plants with diminished length and growth. In order to study how Fe affects plant development, these parameters were measured, in both *G. max* and *M. truncatula*, after the 14d trial (Figure 4.1).



**Figure 4.1** - Shoot and root length (A) and weight (B) of *G. max* and *M. truncatula* grown hydroponically in Fe-sufficient (●) and Fe-deficient (●) conditions. Means +SE separation were performed independently for each structure, using 12 independent replications. Different letters represent significant differences between samples ( $p < 0.05$ ).

In general, both species behaved similarly when faced with the absence of Fe. Both plants developed visible IDC symptoms, resulting in a drastic yield reduction. Root and shoot length, decreased 45.6% and 44.5%, respectively, for *G. max*, and 18.8% and 31.8% for *M. truncatula* (Figure 4.1-A). In what concerns root and shoot weight, *G. max* had 51.7% and 54.6% lower fresh weight in roots and shoots, respectively, under Fe deficiency, which was more pronounced than *M. truncatula* that had a reduction of 44.7% and 31.7% (Figure 4.1-B).

Another important characteristic associated with the absence of Fe is the development of secondary structures. Thus, secondary roots were counted and compared between the two treatments in both species (Figure 4.2).



**Figure 4.2** – Number of secondary roots of *G. max* and *M. truncatula* grown hydroponically in Fe-sufficient (●) and Fe-deficient (●) conditions. Data are means +SE of 12 independent replications. Different letters represent significant differences between samples ( $p < 0.05$ ).

As previously seen by others (Schmidt, 1999; Andaluz *et al.*, 2009), plants submitted to Fe deficiency showed swelling of root tips and increased the number of secondary structures, namely an average of more 40% for *G. max* and 30% more for *M. truncatula*. 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, 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.

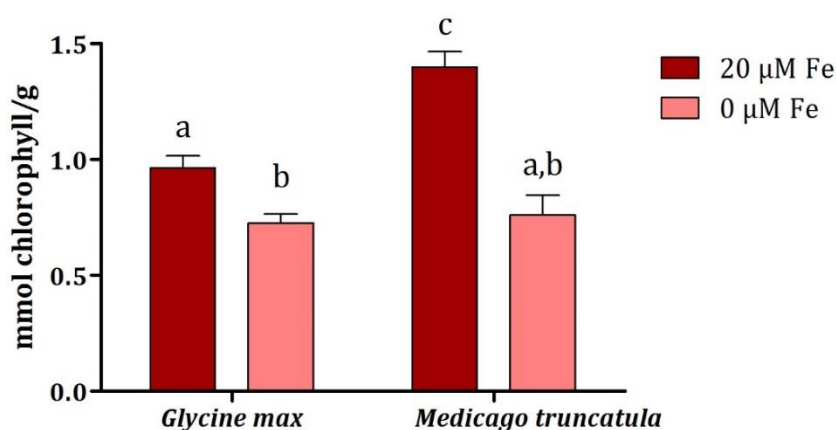
At shoot level, when plants were faced with Fe-deficiency, they developed visible symptoms of chlorosis - e.g. yellowing of leaves - whereas plants grown in Fe-sufficient conditions remained green throughout the experiment, as it can be seen in figure 4.3.



**Figure 4.3** - *G. max* plants grown hydroponically in Fe-deficient (left) and Fe-sufficient (right) conditions.

The yield reduction observed, as previously reported by others (Jolley *et al.*, 1996), is according with the expected, since Fe is essential for several mechanisms that regulate plants growth. The absent of Fe inhibits chloroplast biogenesis and chlorophyll biosynthesis, leading to the development of chlorosis, especially in younger leaves (Henriques *et al.*, 2002).

To quantify the visible symptoms observed (as in Figure 4.3) chlorophyll levels of both species were measured (Figure 4.4).



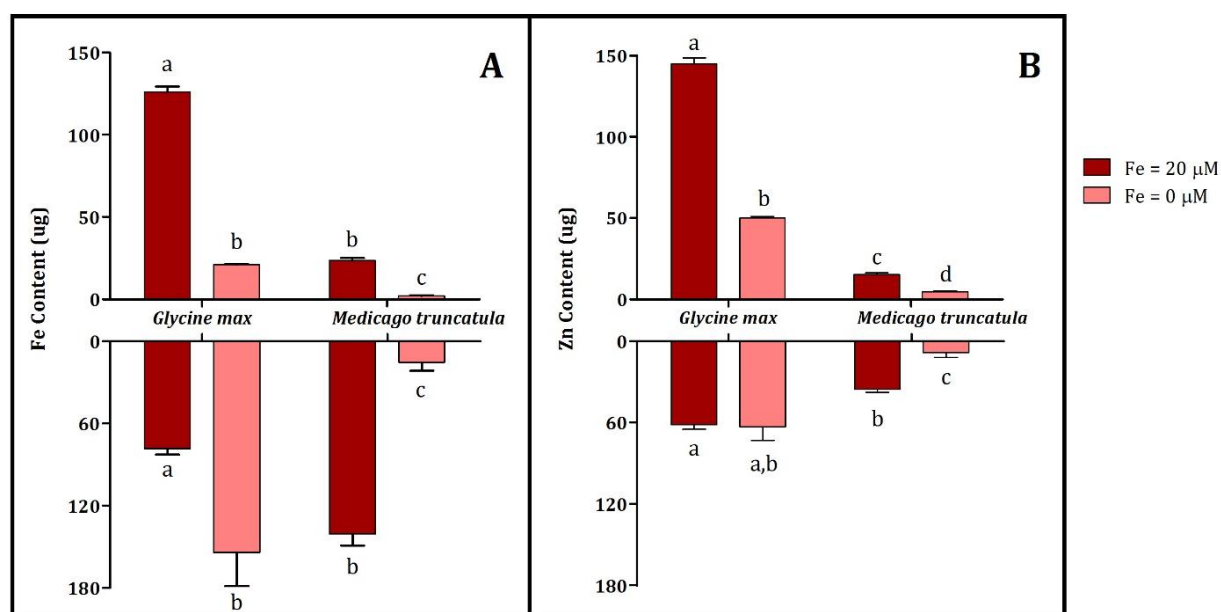
**Figure 4.4** - Chlorophyll concentration of *G. max* and *M. truncatula* grown hydroponically in Fe-sufficient (●) and Fe-deficient (●) conditions. Data are means +SE of 12 independent replications. Different letters represent significant differences between samples ( $p < 0.05$ ).

When challenged with the lack of Fe, both plants presented lower chlorophyll levels, namely 24.6% less in *G. max* and 45.6% less in *M. truncatula* (Figure 4.4), corroborating the visible symptoms observed in figure 4.1.3. Fe starved plants may be expected to be more prone to oxidative damage since Fe is a constituent of enzymes associated with the cellular antioxidant system, e.g. ascorbate peroxidase, catalase, peroxidase, and Fe-superoxide dismutase (Kumar *et al.*, 2010). When in lack of this nutrient, these proteins would not function properly, leading to the accumulation of ROS, to oxidative stress, and to lower chlorophyll levels, as observed in figure 4.4.

As leaves are affected by root Fe concentration, it is possible that the seeds have a similar behavior. However, root Fe uptake capacity is linked with the solubilization 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  $Fe^{2+}$ . Blair *et al.* (2010) have concluded that, at least in common bean, high seed Fe accumulation is generally correlated with higher Fe reductase activity. This might suggest a relation between root uptake and seed loading of Fe in common bean. Therefore, the supplementation of Fe at root level may help biofortification programs, since some of that additional Fe could also be remobilized to the grains.

## 4.2 Nutritional impact of low Fe availability

In order to understand how Fe deficiency affects the nutritional composition of Fe and Zn in soybean and *M. truncatula*, Fe and Zn were quantified by ICP-OES, after the 14d trial (Figure 4.5).



**Figure 4.5** – Iron (A) and Zinc (B) content of *G. max* and *M. truncatula* grown hydroponically in Fe-sufficient (●) and Fe-deficient (●) conditions. Data are means +SE of 3 independent replications. Different letters represent significant differences between samples ( $p < 0.05$ ).

Under Fe deficiency, both *G. max* and *M. truncatula*, showed lower Fe content, in particular at the shoot level. The implications of this observation are that when grown under Fe deficiency, crop plants will be nutritionally poorer, especially when considering the aerial parts (which include leaf, stem and ultimately, seeds). As Zn shares common transporters to the ones utilized for Fe uptake, and since Zn is also an essential nutrient for human and animal health, Zn content was also analyzed in both plant species. As expected, Zn content was negatively impacted in plants grown under Fe deficiency (Figure 4.5-B). This shows that the impact of Fe deficiency on the plants nutritional status will go beyond the levels of Fe in the edible plant parts to other transportable cations such as Zn and possibly Calcium (Ca), Magnesium (Mg) and Manganese (Mn). This has already been observed in previous studies conducted in rice (Sperotto *et al.*, 2012) and soybean (Vasconcelos *et al.*, 2010).

When a great amount of Fe is available around the root surface, plants tend to uptake this important nutrient and it will be stored when is no longer needed. In optimal growth conditions, since *G. max* is much more susceptible to IDC than *M. truncatula*, the Fe was mobilized to the storage spots in the aerial part of the plant, thus presenting higher Fe content in the leaves (Figure 4.5-A). Regarding the root levels of Fe and Zn, it can be seen that *M. truncatula* presented higher Fe content in sufficient conditions, as expected. However, when Fe is absent, the uptake of Zn also decreases, suggesting that Fe may act as a co-factor in Zn uptake at root level. On the other hand, *G. max* presented similar values of Zn in the roots in both

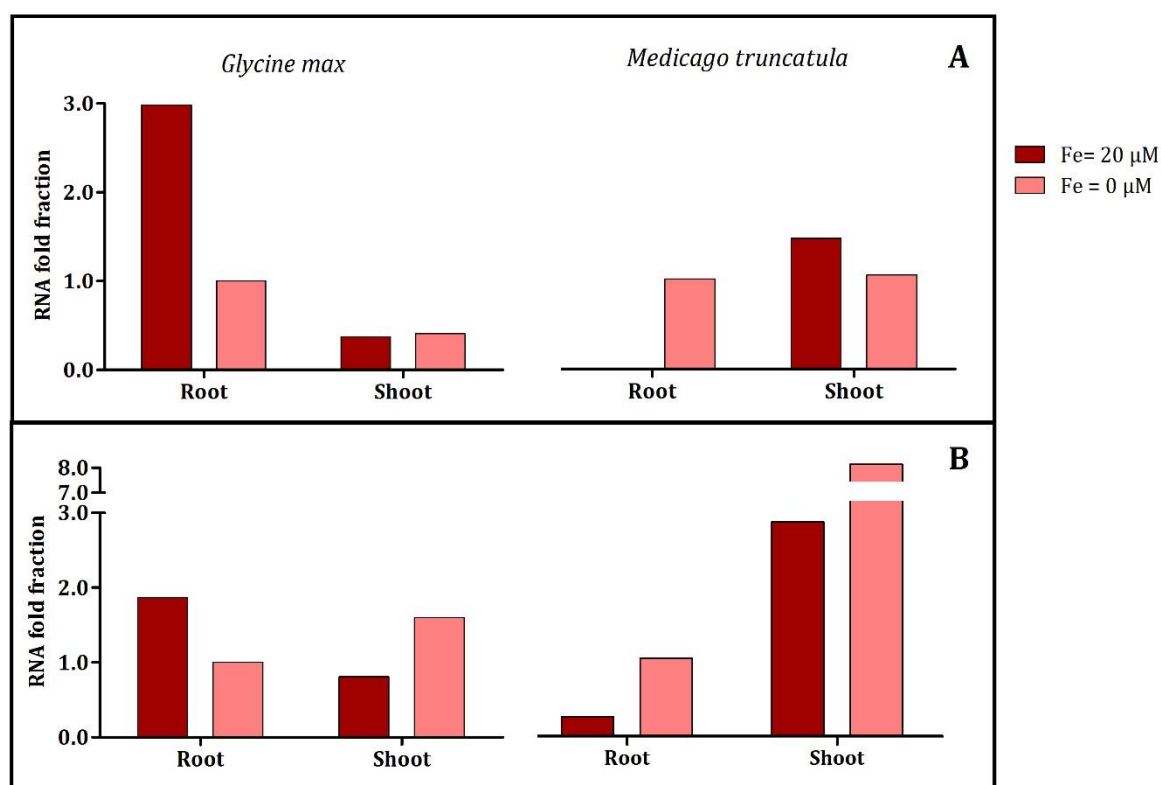
conditions. When the plant is faced with lack of Fe, it mobilizes the internal Fe to the roots (Figure 4.5-A), as an internal signal to induce Fe uptake, which leads to the development of IDC symptoms in the leaves, as described before. Faced with that, plants uptake Zn and mobilize it to the shoots, possibly to counterwork IDC, which can be observed in the obtained results (Fig.4.5-B), where *G. max* presents higher Zn content than Fe in shoots under Fe deficiency.

### 4.3 Gene Expression Analysis

Since the crops do not all respond to Fe stress equally, it is essential to understand the mechanisms underlying the translocation of Fe from roots and leaves to seeds, in order to regulate Fe accumulation in seeds and design an efficient biofortification program. To this end, it is essential to look at the molecular regulators of these processes, and to identify the rate limiting steps, at a molecular level. Thus, the expression of several selected genes related to Fe metabolism was evaluated.

#### 4.3.1 Fe acquisition: *FRO2* and *IRT1*

The root Fe uptake related genes *FRO2* and *IRT1* are extremely important since they participate in one of the most critical steps concerning plant defense mechanisms to Fe deficiency, and control the efficiency of Fe uptake. The present work suggests that *G. max* and *M. truncatula* regulate these genes differently, as seen in figure 4.6.



**Figure 4.6-** Root Fe uptake related genes *FRO2* (A) and *IRT1* (B) fold of expression, in roots and shoot tissues, of *G. max* and *M. truncatula*, grown in Fe-sufficient (●) and Fe-deficient (●) conditions. Roots in Fe deficient conditions were used as comparison term and *18S* was used as internal control.

*FRO2* is responsible for reducing  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , being mainly expressed by the roots, the organ responsible for mineral uptake. Studies show that roots are responsible for Fe deficiency sensing (Vigani *et al.*, 2013). Thus, when *M. truncatula* was submitted to stress conditions, the expression of *FRO2* in roots increased, as expected (Figure 4.6-A). Once the plant is faced with this stress, it needs to increase the rate of nutrient uptake, to compensate the lack of Fe and, for that, root membrane reductases must increase their activity and their uptake rate. When Fe was present in sufficient amounts, *M. truncatula* had almost no *FRO2* expression: since from the beginning of the trial, plants have been in optimal conditions, maybe they captured sufficient Fe to meet their daily requirements, inhibiting the *FRO2* gene and decreasing Fe uptake in order to avoid Fe toxicity.

On the other hand, *G. max* presented an opposite expression pattern, showing higher expression in roots, especially in Fe-sufficiency, decreasing 2.98 fold when submitted to the stress condition (Figure 4.6-A). Blair *et al.* (2010), observed that in some of the genotypes studied, Fe reductase activity, which is directly linked to *FRO2* expression, in Fe deficiency conditions, showed lower values than plants grown in Fe-sufficiency. This suggests that the expression may be reduced due to a need for Fe in the *FRO2* enzyme itself. As previously seen

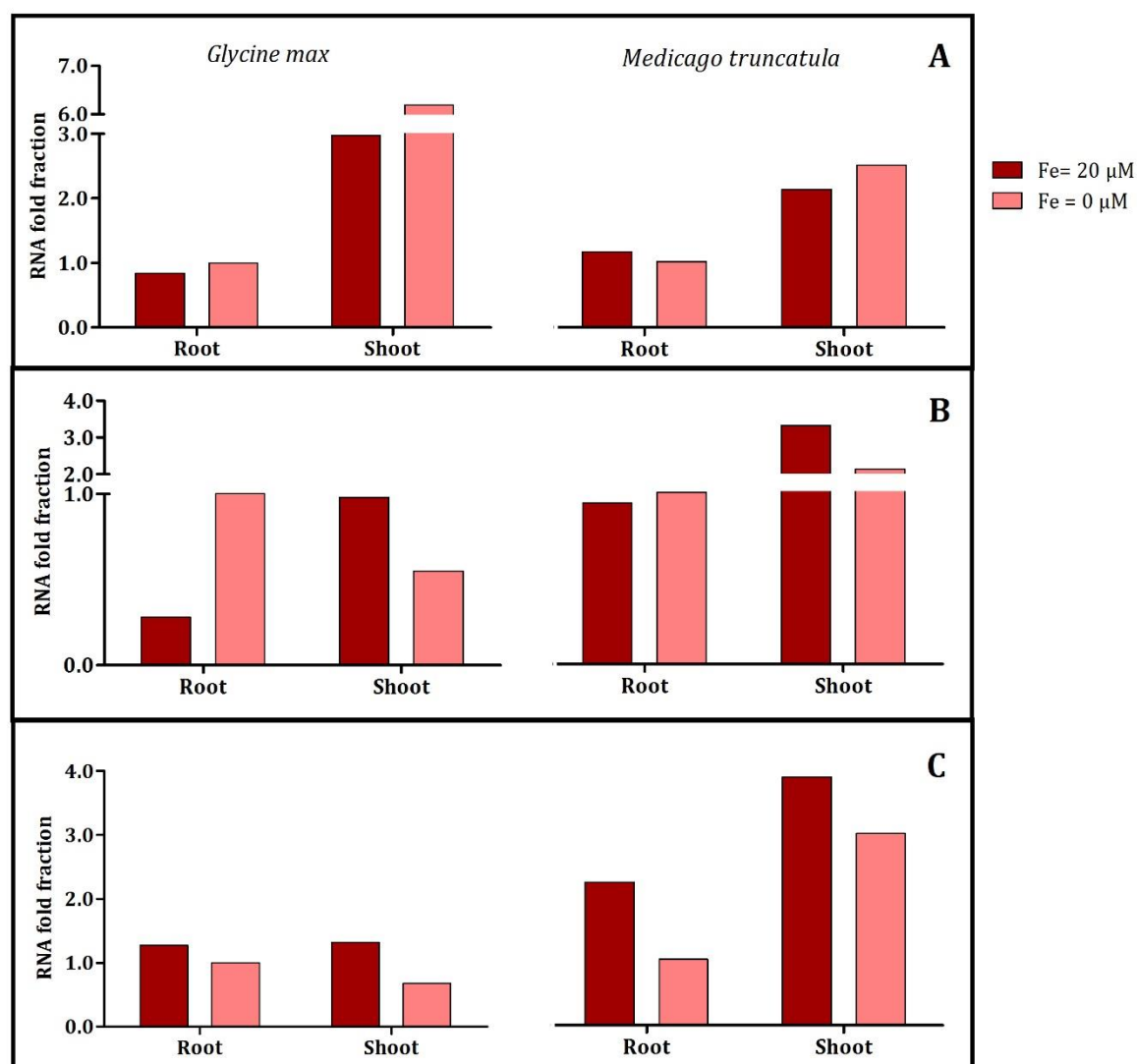
in figure 4.5, *G. max* roots, presented higher Fe content in Fe-deficient conditions, which would enhance the reductase system, that seem to need some Fe to be activated. Fe reductase is known to be the rate-limiting enzyme for Fe uptake since Fe transporters, such as *IRT1*, do not reach saturation at normally achieved concentrations of  $Fe^{2+}$  (Grusak *et al.*, 1990). If there is no Fe being reduced, *IRT1* should consequently present lower activity in *G. max* Fe-deficient roots, which is clearly observed in figure 4.6-B.

In addition to transporting  $Fe^{2+}$ , *IRT1* non-selectively mediates the uptake of several divalent transition metal cations into the root symplasm, thus acting as a major pathway for the influx of  $Zn^{2+}$  (Vert, G. 2002). In general, the levels of expression of *IRT1* in both species and in both tissues are very similar to those obtained for *FRO2*, suggesting that *IRT1* is co-regulated with this gene, as previously seen by others for *A. thaliana* (Kim and Guerinot, 2007). If *FRO2* gene is more activated, it will reduce more Fe, and consequently *IRT1* will transport more Fe into root tissues, showing a higher level of expression. If, on the other hand, Fe is not being reduced by *FRO2*, *IRT1* has no need to be activated, showing low expression levels.

Studies on Fe homeostasis (Vert, G. 2002) showed evidence for long-distance systemic signalling of metal status in different crop species. Experiments with *Arabidopsis*, in which half the root system of was placed in Fe-deficient hydroponic medium and the other half was placed in Fe-replete medium, showed that *IRT1* and *FRO2* transcript levels are regulated by both local Fe levels in the apoplast and by a shoot-derived systemic Fe deficiency signal (Vert, G. 2002).

#### 4.3.2 Fe transport: *NRAMP3*, *VIT1* and *YSL1*

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 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, other plant edible parts and storage organs.



**Figure 4.7** – Fe transporter genes *NRAMP3* (A), *VIT1* (B) and *YSL1* (C) fold of expression, in roots and shoot tissues, of *G. max* and *M. truncatula*, grown in Fe-sufficient (●) and Fe-deficient (●) conditions. Roots in Fe deficient conditions were used as comparison term and *18S* was used as internal control.

*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).

In figure 4.7 it can be seen that these genes are mainly expressed in shoots, whereas in roots they present lower expression. In shoots, an increase of *NRAMP3* expression of 2.08 and 1.17 fold was observed for *G. max* and *M. truncatula*, respectively, when submitted to Fe deficiency (Figure 4.7-A). Studies in *A. thaliana* demonstrate that *NRAMP3* is an H<sup>+</sup> metal symporter responsible for Fe and Mn remobilization from the vacuole, a crucial step during early seedling development (Lanquar *et al.*, 2010). Thus, the results obtained here are coherent because, when under Fe deficiency, plants need more remobilization of Fe to respond to their needs, which means that *NRAMP3* will be more expressed. In roots, the expression levels were

lower and similar in both conditions, for both species (Figure 4.7-A), again supporting the hypothesis that this gene's role is predominant in the leaves, the principal storage organ of Fe.

Knowing the role of *VIT1*, the expression of this gene is expected to be higher in Fe-sufficient leaves, where there is a need for Fe storage, which was clearly observed in both species (Figure 4.7-B). In shoots, the expression levels were 1.78 and 1.56 fold higher in Fe sufficient leaves, for *G. max* and *M. truncatula*, respectively (Figure 4.7-B). These results are in accordance with previous reports, because when Fe is in sufficient conditions, plants activate *VIT1* to increase Fe<sup>2+</sup> uptake into the vacuole for storage purposes (Brear *et al.*, 2013), in order to minimize waste, and reduce the risk of oxidative damage. In roots, *M. truncatula* had similar expression for both conditions, whereas in *G. max* the expression increased 3.57 fold in Fe deficiency (Figure 4.7-B).

Studies in *A. thaliana* (Kim and Guerinot, 2007) demonstrate that *AtNRAMP3* and *AtVIT1* mutants present arrested seedling growth on plants grown on Fe-limiting soil. 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. Taken together, these results suggest that vacuoles are an important site of Fe storage and Fe remobilization during germination, and without leaf accumulation, there is enhanced Fe and Zn translocation to the seed, providing one successful strategy to biofortify Fe and Zn.

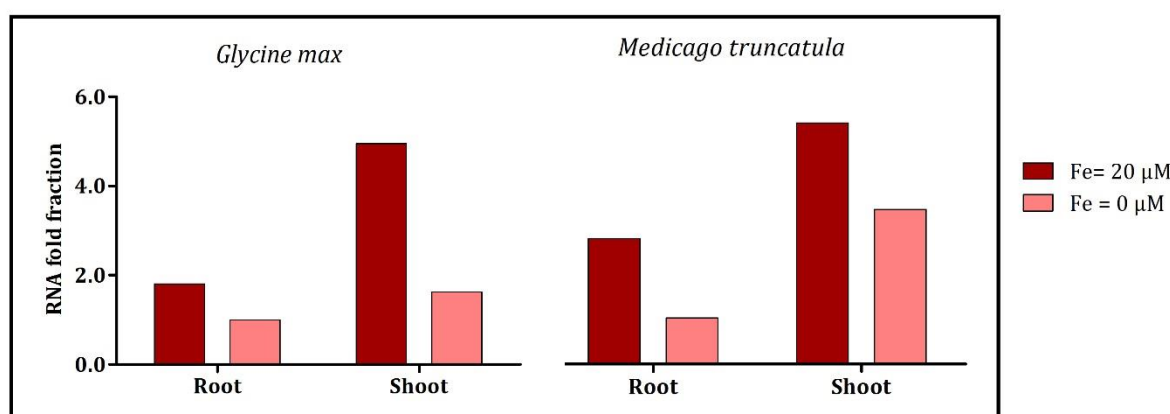
Besides *NRAMP3* and *VIT1*, *YSL1* has an important role in Fe metabolism, being involved in the transport of the Fe<sup>2+</sup>-NA complexes (Kim *et al.*, 2006). This complex, as mentioned in the introduction section is hypothesized as the main transportable Fe form in the phloem.

Hence, it was expected that the expression levels of *YSL1* would be higher in Fe-sufficient conditions, because when there is more amount of Fe, more transporters are needed to satisfy the plant's demand. Both species presented the expected results in both studied tissues (Figure 4.7-C), since both presented higher *YSL1* relative expression values under Fe-sufficient conditions. Similar results in both tissues suggest roles in Fe translocation at diverse plant organs (Kim *et al.*, 2006). Jean *et al.* (2005) showed that by knocking out *AtYSL1*, the levels of NA and Fe in leaves and seeds decreased, as well as the germination rate, even when plants were grown in the presence of an excess of Fe, suggesting that Fe and NA levels in seeds rely in part on *YSL1* function. Thus, as well as *NRAMP3* and *VIT1*, *YSL1* transporter may also play a crucial role in the control of the amount of Fe translocated to the seeds. Overexpressing this

gene could lead to an increased translocation of Fe to the edible plant parts, resulting in seeds with higher Fe concentration.

#### 4.3.3 Fe storage: *Ferritin*

Storage proteins like 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).



**Figure 4.8** – Fe storage related gene *Ferritin* fold of expression, in roots and shoot tissues, of *G. max* and *M. truncatula*, grown in Fe-sufficient (●) and Fe-deficient (●) conditions. Roots in Fe deficient conditions were used as comparison term and *18S* was used as internal control.

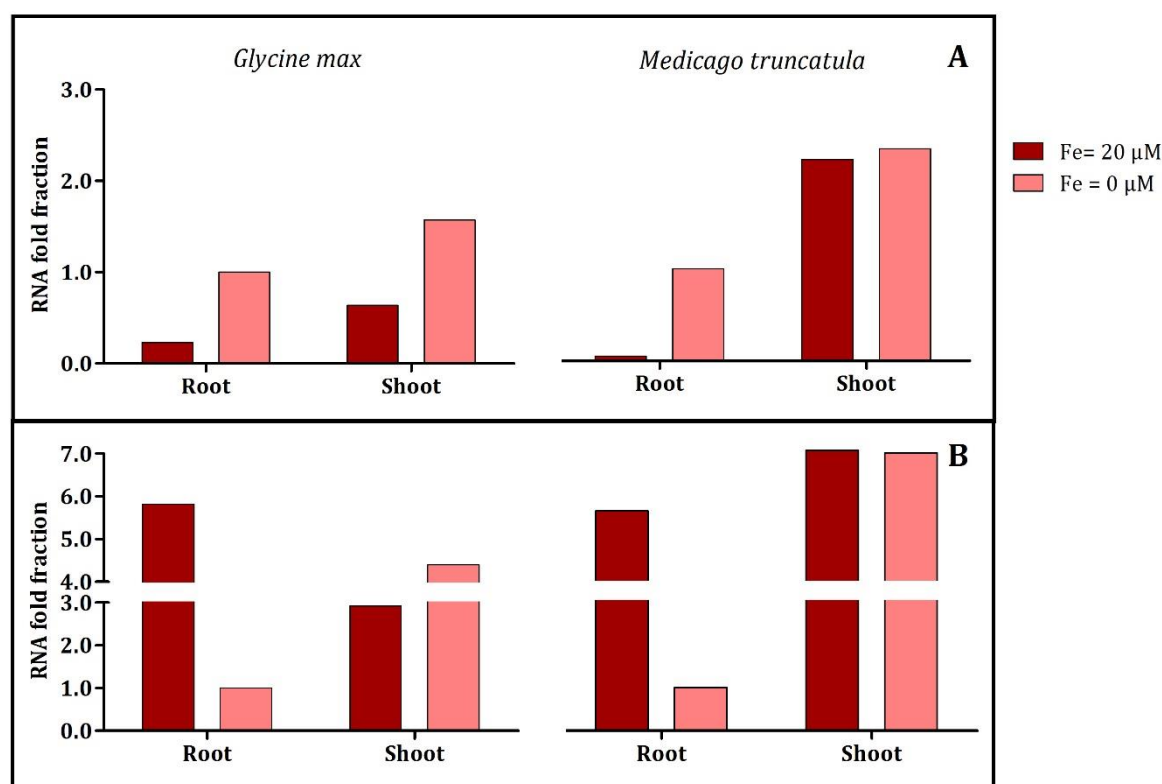
Both *G. max* and *M. truncatula* down-regulated the *Ferritin* gene under stress conditions. In shoots, when submitted to Fe deficiency, the expression decreased 3.06 and 1.56 fold, for *G. max* and *M. truncatula*, respectively (Figure 4.8). At root level, the expression decreased 1.81 and 2.73 fold, for *G. max* and *M. truncatula*, respectively (Figure 4.8). These results are in accordance with the expected since *Ferritin* plays an important role in Fe storage, so when there is sufficient amount of Fe within the plant, it needs to be stored, and *Ferritin* expression increases. Thus, 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, 1995). By manipulating the expression of the vacuolar Fe transport systems *AtVIT1*, *AtNRAMP3*, and *AtNRAMP4*, Ravet *et al.* (2009) have shown that the control of Fe fluxes between vacuoles and plastids is an important factor of Fe homeostasis in seeds.

One of the strategies studied for Fe biofortification has been the overexpression of the *Ferritin* gene (Vasconcelos *et al.*, 2003). However, it has been shown that, independently of Fe content increase in shoots, the content in the grain does not increase proportionally (Drakakaki *et al.*, 2000), suggesting that leaves act as a stronger sink for Fe than the seeds (Rawat *et al.*,

2012). Besides, Ravet *et al* (2009) have shown that when Fe is loaded into seeds, at its maximum rate, *Ferritin* gene expression is almost lacking. Therefore, when designing new biofortification approaches, it is crucial to study all stages of development and the genes involved in each phase. Down-regulating *Ferritin* gene expression in leaves could allow more Fe storage in the seeds. However, one must be careful with the possible negative consequences in terms of oxidative stress.

#### 4.3.4 Novel genes: *GCN2* and *WEE1*

Even though several gene families are known to be involved in the Fe uptake, transport and storage, there are still many undiscovered genes that may have important roles in these processes. Therefore, to efficiently construct a biofortification strategy it is worthwhile to find candidate genes that could have an important role in Fe metabolism. To this end, two novel genes were studied in the current work: *GCN2* and *WEE1*. To ascertain their putative role in mineral nutrition, the expression of these selected genes was evaluated (Figure 4.9).



**Figure 4.9** – Novel candidate genes *GCN2* (A), *WEE1* (B) fold of expression, in roots and shoot tissues, of *G. max* and *M. truncatula*, grown in Fe-sufficient (●) and Fe-deficient (●) conditions. Roots in Fe deficient conditions were used as comparison term and *18S* was used as internal control.

Previous work show that *GCN2* is essential for plant growth under different stress situations, such as amino acid deprivation (Lageix *et al.*, 2008), but no studies have looked at the possible role of *GCN2* and Fe nutrition. The over-expression of *GCN2* under Fe deficiency conditions, as observed in figure 4.9-A, particularly at the root level, seems to indicate role for *GCN2* alleviating Fe stress, for both species. In roots, *GCN2* expression increased 4.35 and 20 fold for *G. max* and *M. truncatula*, respectively. In shoots, the difference for both treatments in *M. truncatula* was minimal, but *G. max* had an increase of 24.5 fold when in stress condition (Figure 4.9-A). Although the relation between *GCN2* and Fe is not well known, the results obtained strongly indicate that *GCN2* plays a role in Fe metabolism, since after the signal for the lack of Fe it is over-expressed, mainly in roots, in order to maintain plant growth and act as a defense mechanism.

In what concerns to *WEE1*, the results show that this gene was down-regulated in the roots of deficient *G. max* and *M. truncatula* plants, whereas it showed a constitutive expression in the leaves of both plant species in Fe deficiency (Figure 4.9-B).

*WEE1* has been identified as a protein involved in cell cycle regulation (De Schutter *et al.*, 2007). According to Jones R. *et al.* (2013), the lacking of a functional *WEE1* in plants is related to growth arrest. Basically, in stress situations, the expression of *WEE1* is diminished, which means that plants will not have that cell cycle control system, entering mitosis with incompletely replicated or damaged DNA, which consequently leads to an arrested growth. The results of the current work seem to indicate that the down-regulation pattern was expected, since the growth was diminished in Fe deficiency (Figure 4.1).



## 5 Conclusion

Biofortification of crops is a feasible and economical approach for overcoming nutrient deficiency disorders around the world. To increase mineral concentration in edible parts, it is essential to understand how one can improve uptake from soil, translocation from roots and leaves to grains and finally enhance the sequestration in the endosperm. The struggle to quickly develop successful biofortification programs relies on the fact that plants behave differently, e.g. 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.

In the current work, both *G. max* and *M. truncatula* were very susceptible to IDC, developing several characteristic symptoms, such as yield reduction, yellowing of leaves and development of secondary roots. All these physiological aspects were corroborated by molecular studies, to identify the molecular players involved in the response to Fe deficiency stress and understand if Fe metabolism mechanisms are transverse amongst two important legume species for human and animal nutrition.

Fe acquisition genes, *FRO2* and *IRT1*, presented similar expression patterns, suggesting that *IRT1* is co-regulated with *FRO2*. Both revealed to be very important players in Fe homeostasis, especially *FRO2*, which is known to be the rate-limiting enzyme for Fe uptake. In what concerns Fe transport, *NRAMP3* and *VIT1*, showed opposite functions and appeared to have a stronger expression in the leaves. Several studies related to these genes concluded that vacuoles are an important Fe storage and remobilization system and, without leaf accumulation, there is enhanced Fe and Zn translocation to the seed, providing one successful strategy to biofortify Fe and Zn plants. Fe and NA levels in seeds also rely in part on *YSL1*, which is responsible for the transport of the Fe-NA complex. This gene presented higher expression levels under Fe-sufficient conditions in both species and for both root and shoot, as expected. The results obtained suggest that this gene has functions at diverse locations and, as well as *NRAMP3* and *VIT1*, also plays a crucial role in the control of the amount of Fe translocated to the seeds.

Ferritin assures that Fe in excess is in a bio-available way for cellular needs but yet nonreactive with oxygen, being involved in oxidative protection. Both species down-regulated *Ferritin* gene under stress conditions, as expected.

To better understand the transportation and loading of Fe into the edible parts, it is essential to continually explore new candidate genes with possible important roles related to

these mechanisms. The results obtained for *GCN2* strongly indicate that this gene plays a role in Fe metabolism, since after the signal for the lack of Fe it is over-expressed, mainly in roots, in order to maintain plant growth and counteract the stress. In what concerns to *WEE1*, the results show that this gene seems to have a more important role in the roots, being under-expressed in stress situations, where growth is deficient, which corroborates the theory that the lacking of a functional *WEE1* in plants is related to growth arrest (Jones R. *et al.* 2013). Fe and Zn homeostasis relies on the fact that they present a chemical similarity between their divalent cations, which allows some proteins, such as *IRT1*, to work with either one or another, depending on their availability. When submitted to stress conditions, both species presented a similar nutritional impact, decreasing Fe content, especially in the aerial parts. In stress condition, both plants captured more Zn, in order to compensate the lack of Fe.

Since Fe deficiency is the leading human nutritional disorder in the world today, it is important to enhance the knowledge on Fe metabolism, not only to combat IDC crop devastation with 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 *et al.*, 2012).

## 6 Future Work

This experiment was conducted in optimal environmental growth conditions, with sufficient and controlled amounts of specific nutrients, to understand the physiologic and molecular mechanisms important in crops biofortification. The next step, to closely mimic real growth conditions, would be the utilization of soil instead of a hydroponic system, and compare both sets of data.

After analyzing roots and leaves, the next step would be to focus the attention in other edible plant parts, such as the seeds. This would imply growing plants to full maturity under hydroponic and/or soil conditions. Since some studies suggest that leaves act as a stronger sink for Fe than the seeds, another study that could be done would be the effect of Fe restriction at the shoot level, and its consequences on seeds concentration. This could be done by removing some leaves or by blocking some genes responsible for Fe accumulation in leaves. After understand the mechanisms responsible for the translocation of Fe from roots and leaves to seeds, one can easily regulate Fe accumulation in seeds, and design an efficient biofortification program.

Another topic that should be consider is that throughout plant growth, genes present different levels of expression, concerning their specific needs. Therefore, it would be interesting to develop an experiment where the genes and the nutrient concentration within the plant are analyzed at different time-points. Besides, it is crucial that new candidate genes are studied, in order to better understand the pathways related to the translocation of nutrients to the edible parts of the plant. In this context, more candidate genes should be considered in further studies.



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