CATÓLICA

UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

STUDY OF THE FE UPTAKE SYSTEMS IN TWO DIFFERENT RICE CULTIVARS

by

Ana Margarida Patrício Pereira

December 2013

CATÓLICA
UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO
Escola Superior de Biotecnologia

STUDY OF THE FE UPTAKE SYSTEMS IN TWO DIFFERENT RICE CULTIVARS

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

by

Ana Margarida Patrício Pereira

Place: Escola Superior de Biotecnologia da Universidade Católica Portuguesa

Supervision: Doctor Marta Wilton de Vasconcelos

Co-supervision: Prof. Doctor Ana Gomes

December 2013

RESUMO

O arroz (*Oryza sativa* L.) alimenta mais de metade da população do mundo e é rico em diversidade genética, com milhares de variedades cultivadas em todo o mundo. A deficiência de ferro (Fe) constitui um dos principais stresses abióticos na produção agrícola dado que, em solos calcários, que abrangem cerca de 30 % dos solos cultivados do mundo, o Fe forma complexos solúveis e não está prontamente disponível para absorção pelas plantas. Durante muito tempo, as plantas foram divididas em dois grupos distintos, pela sua capacidade de absorção de Fe: dicotiledóneas, que utilizam a Estratégia I e utilizam um sistema de redução e transporte de Fe^{2+}; e as monocotiledóneas gramíneas, plantas da Estratégia II, que usam um sistema baseado nos fitosideróforos (PS). Recentemente, evidências sugerem que o arroz, além de usar a Estratégia II, pode também usar um transportador de Fe^{2+}, típico das plantas de Estratégia I, conferindo vantagem em solos alagados onde o Fe^{2+} está mais disponível.

O objetivo deste estudo foi compreender se as plantas de arroz têm ou não a capacidade de sobre expressar mecanismos relacionados com a Estratégia I para a absorção de Fe. De forma a cumprir estes objetivos, duas cultivares diferentes de arroz com suscetibilidades diferentes para a deficiência de Fe foram cultivadas em sistema hidropónico, nomeadamente, Nipponbare (cujo genoma foi já sequenciado) e Bico Branco (nunca antes estudada), para analisar vários parâmetros ao nível fisiológico e molecular.

Os resultados obtidos mostraram que a cultivar Bico Branco acumulou mais minerais nas raízes e a Nipponbare na parte aérea e que, quando a absorção de Fe é reduzida, há um aumento na absorção de outros minerais, principalmente de zinco (Zn), manganês (Mn) e cobre (Cu). No que diz respeito aos pigmentos fotossintéticos, a cultivar Bico Branco mostrou-se mais suscetível à deficiência de Fe do que Nipponbare, por ter desenvolvido maior clorose. Além disso, teve maior atividade da enzima reductase de Fe sob deficiência de Fe e apresentou níveis elevados de expressão do gene *OsFRO2* nas raízes, gene responsável pela redução de Fe. Esta nova descoberta mostra que certas cultivares de arroz podem beneficiar do sistema de redução de Fe, principalmente porque o arroz é conhecido por produzir PS em baixas quantidades e crescer em solos aeróbicos/terras altas, onde o Fe^{3+} é abundante.

Foram também identificados novos genes candidatos no arroz, nomeadamente, *OsFPN1*, *OsFPN2*, *OsMYB2* e *OsMYBS3*, que revelaram ser importantes na homeostase do Fe em arroz.

ABSTRACT

Rice (*Oryza sativa* L.) feeds more than half of the world's population and is rich in genetic diversity, with thousands of varieties grown throughout the world. Iron (Fe) deficiency is a major abiotic stress in crop production, since in calcareous soils, which account for about 30% of the world's cultivated soils, Fe form soluble complexes and is not readily available for uptake. For a long time plants have been divided into two distinct groups, by their capacity for Fe uptake: dicotyledoneas, that belong to Strategy I and utilize an Fe reduction and Fe^{2+} transporter system; and graminaceous monocotyledoneas, strategy II plants, that use an phytosiderophore (PS)-based system. Recently, evidences suggest that rice, in addition to use Strategy II, can also use an Fe^{2+} transporter, typical in Strategy I plants, that could confer advantage in flooded soils where Fe^{2+} is more available.

The aim of this study was to understand if rice plants have or not the capacity to up-regulate Strategy I mechanisms for Fe uptake. To meet these purposes, two different rice cultivars with different susceptibilities to Fe deficiency, were grown hydroponically, namely, Nipponbare (whose genome has already been sequenced) and Bico Branco (never studied before) to analyze various parameters at a physiological and molecular level.

The results obtained showed that Bico Branco cultivar accumulated more minerals in roots and Nipponbare in shoots and that when Fe uptake is decreased there is an increase on the uptake of some other minerals, mainly zinc (Zn), manganese (Mn) and copper (Cu). In what concerns the photosynthetic pigments, the Bico Branco cultivar showed to be more susceptible to Fe deficiency than the Nipponbare cultivar, as the first developed more chlorosis than the latter. Furthermore, the Nipponbare cultivar revealed the highest Fe-reductase activity under Fe deficient conditions and revealed higher levels of expression of *OsFRO2* gene in the roots, a gene that is responsible for Fe reduction. These new findings show that some rice cultivars may utilize the Fe-reduction system, mainly because rice is known for producing PS in low amounts and grow in aerobic/upland where Fe^{3+} is more available.

Also, new candidate genes in rice were identified, namely *OsFPN1*, *OsFPN2*, *OsMYB2* and *OsMYBS3*, and they revealed to be important in Fe homeostasis in rice.

ACKNOWLEDGMENTS

First of all, I want to thank my parents for the opportunity they gave me to complete my studies at a graduate level, for always supporting my decisions and for always being by my side.

I would also like to express my sincere gratitude to my thesis supervisors, Marta Wilton Vasconcelos, for the guidance, support and constructive advice throughout my entire research work; and Ana Gomes, for the encouragement in this stage of my academic life. I thank both for the review of the scientific writing and for giving me the opportunity to complete my master's degree under your guidance.

To "my" research group, Plantech, I would like to thank for all the support, friendship, knowledge and brainstorming that we have shared, and for the help they gave me whenever I needed, especially to Carla Santos, my sincere appreciation.

I am also thankful to my boyfriend, António, and to my friends Patrícia, Filipa, Venessa, Ana Vaz, Marta, Joana and Vicente, for their unconditional support, patience, help, for their love and friendship, and for always believing in me!

Finally, my thanks to all the people who have crossed my path and those who, in some way or another, allowed this work to take place.

My deepest thanks to all!

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Al	Aluminum
At	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
B	Boron
B.C.	Before Christ
bHLH	Basic helix-loop-helix
BPDS	Bathophenanthroline disulfonic acid
$CaCl_2$	Calcium chloride
$Ca(NO_3)_2$	Calcium nitrate
cDNA	Complementary deoxyribonucleic acid
Cd	Cadmium
Cl	Chlorine
cm	Centimeters
Co	Cobalt
Cu	Copper
$CuSO_4$	Copper sulphate
DMA	Deoxymugineic acid
DMAS	Deoxymugineic acid synthase
dNTP	Deoxyribonucleotide triphosphate
DW	Dry weight
Dw	*Dendrobium* Woo Leng
EDDHA	Ethylenediamine-N,N'-bis(2-hydroxyphenylacetic acid
EDTA	Ethylenediamine tetraacetic acid
Fe	Iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FIT1	Fe-deficiency induced transcription factor 1
FPN	Ferroportin
FRO	Ferric reductase-oxidase
FW	Fresh Weight
g	Gram
GUS	β-Glucuronidase
h	Hours
H^+	Hydrogen ion
H_2MoO_4	Molybdic acid
H_3BO_3	Boric acid
Ha	Hectare
HNO_3	Nitric acid
I	Iodine
ICP-OES	Inductively Coupled Plasma- Optical Emission Spectroscopy
IDC	Iron deficiency chlorosis

IRO2	Iron-related transcription factor 2
IRT	Iron-regulated transporter
K	Potassium
K_2SO_4	Potassium sulphate
KH_2PO_4	Potassium dihydrogen orthophosphate
KNO_3	Potassium nitrate
MA	Mugineic acid
Mb	Mega base pairs
MES	2,4-morpholino-ethane sulfonic acid
mg	Milligrams
min	Minutes
mL	Milliliters
mM	Millimolar
Mn	Manganese
$MnSO_4$	Manganese sulphate
Mo	Molybdenum
Mx	*Malus xiaojinensis*
N	Nitrogen
NA	Nicotianamine
Na	Sodium
NAAT1	Nicotianamine amino transferase 1
NAS	Nicotianamine synthase
Ni	Nickel
$NiSO_4$	Nickel sulfate
nm	Nanometer
Os	*Oryza sativa* L.
P	Phosphorus
Pb	Lead
PS	Phytosiderophores
qRT-PCR	Real Time quantitative Reverse Transcription polymerase chain reaction
RNA	Ribonucleic acid
UV	Ultra-violet
WHO	World Health Organization
YSL	Yellow stripe-like
ZIP	Zinc Regulated Transport/Iron Regulated Transport-like Protein
Zn	Zinc
$ZnSO_4$	Zinc sulphate
µ	Micro (10^{-6})

1. INTRODUCTION

1.1 History and production

Rice is the oldest food of the world and at the same time the most current (Maclean *et al*., 2002). Its origins have been debated for some time, but it is thought that rice plant remains from 10,000 B.C. were discovered in a Spirit Cave on the Thailand-Myanmar border. Migrant people from southern China or perhaps northern Vietnam carried the traditions of wetland rice cultivation to the Philippines during the second millennium B.C. (Maclean *et al*., 2002). The crop may well have been introduced in Europe, more accurately in Greece and the neighboring areas of the Mediterranean by Alexander Magno in expedition to India around 344-324 B.C. From central positioning in Greece and Sicily, rice gradually spread throughout southern Europe and to a few locations in northern Africa (Maclean *et al*., 2002).

The Arabs introduced rice in the Iberian Peninsula in the 8th century (771), but it was during the reign of D. Dinis, O Lavrador (1279-1325), that the first references to rice cultivation appear in Baixo Mondego, in the area of Montemor-o-Velho. After his reign, rice growing was abandoned and then restored in the 18th century (MADRP, 2002). During World War I, rice consumption was strongly increased and became embedded in Portuguese agriculture (Lains, 2003). In 1929, under the dictatorial regime of Salazar, began a strong regulatory protectionist stance for cereals, known as the "Wheat Campaign" where wetland areas were reclaimed and these arable lands that were unproductive, were chosen to plant rice (MADRP, 2002). In 1972 the total amount of land harvested for rice reached its maximum at 43.487 ha, showing the great success of Salazar's protectionist policies (Vianna Silva, 1975). The first reform in 1995 (COUNCIL REGULATION (EC) 3072/1995) provided further stimulus to grow rice, the production-dependent subsidies were seen as an incentive for greater improvement in yields that led to an unexpected increase in rice production (Commission of the European Communities, 2002). Currently, rice is grown in the basins of Mondego (Figueira da Foz, Coimbra), Sado (Alcácer do Sal), Tejo, Beira Baixa and other regions in smaller scale (Panzone *et al.,* 2009).

Rice is grown in more than a hundred countries, with a total harvested area in 2012 of approximately 163 million ha (FAO, 2012), producing more than 700 million tons annually. The main producer in the world is Asia, which accounts for over 90 % of the world production of rice (Figure 1.1.1A), with China and India producing the most, according to the latest data provided by FAO (2012). Thailand is the world largest exporter of milled rice, with about 10 million tons exported in 2011, followed by Vietnam, India and Pakistan.

Rice production in Europe is indicated in Figure 1.1.1B. Italy and the Russian Federation, in that order, are the largest producers with about 1,500,000.00 and 1,051,891.00 tons in 2012, respectively, followed closely by Spain with a production of 881,000.00 tons. Portugal is currently the sixth producer in Europe with 184,100.00 tons produced in 2012, supplying more than 236 million tons of rice in 2009, more exactly 151 Kcal/capita/day. In 2011, Portugal exported about 25,609.00 tons of rice (FAO, 2012).

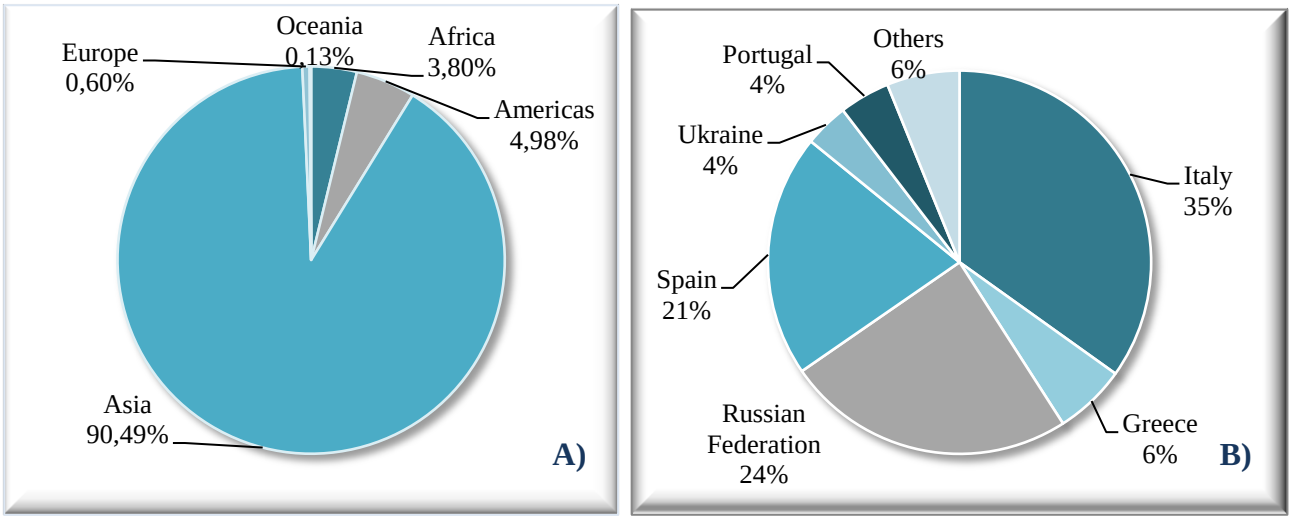

Figure 1.1.1 A) Percent of global rice production by region in 2012; B) Rice production in Europe in 2012 (data from FAO, 2012)

In addition to being the most important staple food in the world, rice is also part of many cultures and traditions, being used in several religious ceremonies and festivals (Maclean *et al*., 2002). In 2004 the United Nations declared it the International Year of Rice, as a "symbol of cultural identity and unity among peoples".

1.2 Rice growing habitats and grain types

Rice, as a very diverse plant, can grow in a wide range of environments where other crops would fail. Currently, rice-growing environments throughout the world (Figure 1.2.1) include irrigated rice, rain-fed lowland and rain-fed upland (IRRIa, 2013), as follows:

- Irrigated rice is grown in fields with 5–10 centimeters (cm) of water ("floodwater") in the field, receiving about 40 % of the water from irrigation. This anaerobic environment is the most common method used worldwide, having about 80 million ha of this cultivation providing 75 % of the world's rice production;

- Rainfed lowland rice is grown in bunded fields that are flooded with rainwater for at least part of the cropping season. This can originate multiple abiotic stresses and high uncertainty in timing duration, and intensity of rainfall. About 60 million ha of rainfed lowlands supply about 20 % of the world's rice production. This technique predominates in areas of greatest poverty: parts of Southeast Asia, South Asia, and essentially all of Africa. Thus, yields are very low (1–2.5 t/ha) which leads to poverty of these families;

- Upland rice is grown under dry land conditions in mixed farming systems without irrigation and without puddling. These aerobic environments are highly variable in terms of moisture and soil fertility and sometimes there are problems of nutrient deficiencies, in particular of Fe-deficiency. Although this method constitutes around 13 % of the total rice area worldwide, it is the predominant rice-growing method Latin America and West Africa, where poverty is widespread.

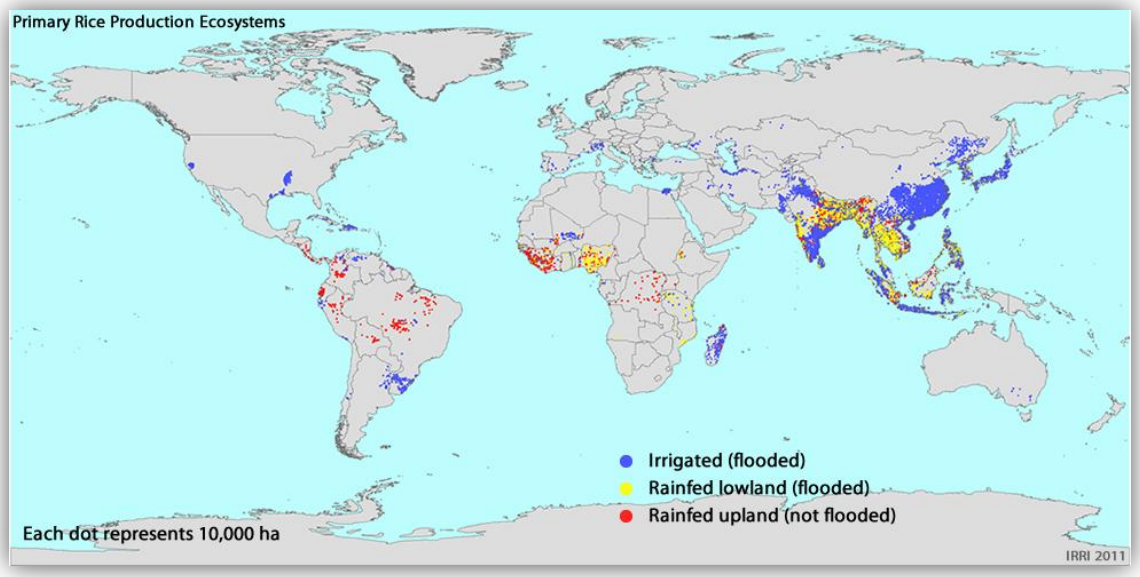

Figure 1.2.1 Distribution map of irrigated rice, rainfed lowland rice and rainfed upland rice in different world regions (from IRRIa, 2009).

Rice belongs to the family *Gramineae*, that includes other cereals such as wheat, corn or barley (IRRIb, 2009), and to the genus *Oryza*, which includes 21 wild species and 2 cultivated species, *Oryza glaberrina* Steur and *O. sativa* L. (Ge *et al*., 1999). While *O. glaberrina* is cultivated in restricted areas of western Africa, *O. sativa* is cultivated all over the world (Londo *et al*., 2006).

Oryza sativa L. was the first fully sequenced crop genome, and besides having the smallest genome (430 Mb across 12 chromosomes), it is easy to genetically modify and it is a model organism used for research in cereals and other monocotyledonous plants (Ohnishi *et al*., 2011). In 1928, the pioneering work of Kato *et al*. showed the existence of two main variety types, designated as *indica* and *japonica*. In ecogeographical terms, the first one is produced in Southern Asia, while the second is typically found in upland areas of Southeast Asia, temperate East Asia, and high elevations in South Asia (IRRIb, 2013). However, more recent studies to interpret the evolutionary relationships between groups revealed a total of five distinct groups known, corresponding to *aus, aromatic, indica, tropical japonica*, and *temperate japonica*, where group differences were explained through contrasting demographic histories (Garris *et al*., 2005). It is estimated that about 120,000 rice cultivars

exist in the world (Khush, 1997). This ecological diversity is result of natural and human selection, diverse seasons, climates and soils, and varied cultural practices (Maclean *et al*., 2002).

Rice has physical characteristics that distinguish one variety from others. It can be of short, medium or long grain size. Its pericarp can also vary in terms of color, including brown, red, purple and black (FAO, 2004). Three different types of rice are produced, namely white rice, which has long grain without the hull and bran; brown rice, which retains most of the cuticle that covers the grain, having a certain brown tone, and a fiber content higher than that of other varieties; and the steamed rice, which has the same nutritional value and a golden color similar to the "integral" rice (Decree-Law nr. 62/2000).

In Europe, the most cultivated variety is *japonica*, with a medium round grain. In Portugal the main types produced are *japonica* (or Carolino) and *indica* (Agulha) (Pacheco Dias and Nunes da Rocha, 2012; ANIA, 2006), and the Decree-Law number 62/2000 establishes the classification of varieties, the methods of analysis, types of commercial class, and rules of their marketing, packaging and labeling. The *indica* variety, with long grain, despite being preferred in the Nordic countries constitutes 80 % of world rice.

1.3 Nutritional characteristics of rice

Rice feeds more than half of the world's population, most of whom in developing countries, predominating in 17 countries in Asia and the Pacific, nine countries in South and North America and eight countries in Africa (FAO, 2004) where this food is, at least during certain seasons, their sole source of nutrients (Sautter *et al*., 2007). Unpolished rice is rich in nutrients, fibers, vitamins and minerals (Maclean *et al*., 2002) that supply the majority of daily dietary nutrients for billions of people (FAO, 2004). Rice also contains all of the amino acids essential for humans except lysine (Maclean *et al*., 2002). It is a source of complex carbohydrates, and provides 21 % of global human per capita energy and 15 % per capita protein (Maclean *et al*., 2002), while wheat supplies 19 % and maize 5 % of energy (FAO, 2004).

Table 1.3.1 summarizes the nutritional composition of brown (unpolished) rice when compared to white (polished) rice.

Table 1.3.1 Nutrient composition of rice grain types (adapted from FAO, 2004; Agricultural Engineering Unit and IRRI, 2013).

Type of Rice	Protein (%)	Fat (%)	Carbohydrates (%)	Iron (mg/100g)	Zinc (mg/100g)	Fiber (g/100g)
Unpolished	7.1 – 8.3	1.6 – 2.8	73.0 – 87.0	2.2	0.5	2.8
Polished	6.3 – 7.1	0.3 – 0.5	77.0 – 89.0	1.2	0.5	0.6

Most rice is consumed in its polished form and in this process some important nutrients such as iron (Fe), zinc (Zn) and Vitamin A are lost. White rice alone is not enough to provide the nutrient needed in each meal, being very important to compensate these shortages of nutrients by consuming other vegetables, fish and meat, to prevent nutritional deficiencies and other problems (Maclean *et al*., 2002).

The major portion of minerals in the rice seeds are likely supplied through continuous uptake and translocation during reproductive growth to developing seeds (Hocking and Pate, 1977; Sperotto *et al*., 2012a). Environmental factors (soil fertility, wet or dry season, solar radiation, temperature during grain development) and crop management (added N fertilizer, plant spacing) can affect rice nutrient content. When rice grows in calcareous soils with low amount of available Fe, nutrient content is also lower in rice seeds (Grusak and Dellapenna, 1999). Previous studies show that when rice is grown in solutions with different Fe concentrations, this is reflected in the content of minerals in the rice seeds (Sperotto *et al*., 2012a).

1.4 Iron deficiency, a global problem

Human metabolism requires various nutrients to function properly, and all of them can be supplied by an appropriate diet (Welch and Graham, 2004; White and Broadley, 2009). This diet should not only supply the energy nutrients but also the essential amino acids, particularly the uncommon S-rich amino acids, lysine, methionine, vitamins A, C, D and E, B vitamins, folic acid and ionic elements such as iodine (I), Fe, Zn and sodium (Na) (Sautter et al., 2007).

When physiological requirements cannot be met through the absorption of nutrients in the diet, mineral malnutrition can occur (Zimmermann and Hurrell, 2007). Mineral malnutrition is considered to be the most serious global challenge to humankind (White and Broadley, 2009). Nearly two-thirds of all deaths of children are associated with nutritional deficiencies, many from micronutrients deficiencies (Caballero, 2002; Walker and Waters, 2011), which increases the risk of death from common diseases such as acute gastroenteritis, pneumonia and measles (Caballero, 2002).

Fe deficiency is one of the most widespread dietary challenges in human health (Lee et al. 2009b), affecting about 30% of the world population. It is the only nutrient deficiency which is also significantly prevalent in industrialized countries (WHO, 2013).

Dietary Fe is usually divided into two types (Theil, 2004): heme Fe, found almost exclusively in meat, and non-heme Fe, found in animal and plant tissues as Fe^{2+} bound to insoluble proteins, phytates, oxalates, phosphates and carbonates, which are inhibitors of Fe absorption (Reddy et al., 1992). The first one is 2-6 times more available for absorption from the diet than non-heme Fe (SACN, 2010). In human metabolism, Fe plays important roles, namely in the synthesis of heme found in hemoglobin, which distributes oxygen around the body and in myoglobin, which stores oxygen in muscles and tissue. It also serves as a transport medium for electrons within cells (FAO and WHO, 2001).

According to the World Health Organization (WHO), anemia is considered the main consequence of Fe deficiency (WHO, 2013). It can affect anyone at any part of the world, however, it is more prevalent in developing countries as South Asia and Africa, where young children and pregnant women are the most affected (WHO, 2005). Anemia is associated with clinical symptoms such as weakness, decreased respiratory capacity and dizziness. In areas with limited resources, this is frequently exacerbated by infectious diseases, because of a

depressed immune function. Even in the absence of anemia, Fe deficiency can cause neuro-cognitive disorders.

Mineral malnutrition can be addressed through dietary diversification, increasing mineral concentrations in edible crops, Fe supplements, food fortification and/or biofortification (Zhao and Shewry, 2011). However, dietary diversification and Fe supplements by tablets are not easily available in developing countries (Gillespie and Haddad, 2001) and in spite of food fortification being considered the best long-term strategy for prevention, Fe compounds of relatively high Fe availability, such as ferrous sulfate, often originate unacceptable color and flavor changes, whereas those compounds which are organoleptically inert, such as elemental Fe, are usually poorly absorbed (Hurrell, 1992).

For various reasons, none of these intervention strategies has been very successful in reducing the prevalence of Fe deficiency anemia in developing countries. Nutritional health and well-being of humans are entirely dependent on plant foods. Plant foods contain almost all of the mineral and organic nutrients established as essential for human nutrition, as well as organic phytochemicals that have been linked to the promotion of good health (Grusak and Dellapenna, 1999). Because the concentrations of many of these constituents are often low in edible plant sources, studies have been done to understand the physiological, biochemical and molecular mechanisms that contribute to their synthesis, transport and accumulation in plants (Grusak and Dellapenna, 1999). Thus, to improve the nutrition and health of rice consumers, development of high-quality rice varieties seems to be an alternative approach which is more sustainable (Duan and Sun, 2005).

The use of plant breeding and/or transgenic approaches to develop new cultivars with the potential to increase the nutrient concentration of edible portions of crop plants is named biofortification (White and Broadley, 2005). Currently, two of the various techniques that are being used in rice to improve the nutritional status of populations are the following:

- use of traditional plant breeding techniques to select rice varieties with superior nutrients content and breed these with the most commonly grown varieties to enhance the nutrient content of the grains (FAO, 2004);
- develop more nutritional rice using genetic modification techniques. The best-known example of this technology is “Golden Rice”. This variety was incorporated with beta-carotenoids, precursors of vitamin A, whose deficiency causes irreversible blindness.

This rice is being tested in nutrition trials before it can be approved by national authorities (Barry, 2013).

However, before increasing the mineral content of plants, it is necessary to understand not only how minerals are obtained from the rhizosphere, but also how the minerals are then distributed throughout the plant (Krämer *et al*., 2007). There is a lack of knowledge about how minerals are moved into or out of vascular tissues, translocated to vegetative tissues and accumulated in seeds, the edible portion of the rice plant (Colangelo and Guerinot, 2006). Fe translocation and Fe homeostasis in rice has already begun to be understood at the molecular level (Masuda *et al*., 2012), but the mechanisms behind these processes still need deep research in order to be clearly comprehended.

1.5 Iron deficiency in plants

Similar to humans, Fe is essential for plant growth and plays important roles in general plant metabolism (Clark, 1983). Since Fe accepts and donates electrons it serves as a cofactor of several proteins that are involved in a number of physiological processes in plants, such as respiration, chlorophyll biosynthesis and photosynthetic electron transport, hormone biosynthesis, production and scavenging of reactive oxygen species and pathogen defense (Jeong and Guerinot, 2009).

Plants must maintain Fe homeostasis, and to achieve this they developed complex mechanisms to regulate the acquisition, storage and distribution of Fe to the specific compartments (Puig *et al*., 2007; Walker and Connolly, 2008), providing the necessary amounts of this micronutrient and preventing internal cation excess (Zimmermann and Hurrell, 2007). Fe deficiency is a major problem for plants that grow in aerobic soils at neutral or alkaline pH (calcareous soils), which cover approximately 30% of world land. Under these conditions, Fe forms insoluble complexes and despite its abundance in the soil, it is not readily bioavailable for uptake (Jeong and Guerinot, 2009). Fe deficiency is a widespread agricultural problem and one of the main symptoms is chlorosis, usually called "Fe deficiency chlorosis" (IDC) (Curie and Briat, 2003) IDC is associated with decreased photosynthetic rate and inhibition of chlorophyll biosynthesis (Belkhodia *et al*., 1998), and if

severe, it can lead to reduction of plant growth and crop yield or even complete crop failure (Guerinot and Yi, 1994).

Fe stress alters chloroplast ultrastructure (Spiller and Terry, 1980), protein and lipid composition of thylakoid membranes (Nishio *et al*., 1985), reduces electron transport capacity (Spiller and Terry, 1980), diminishes noncyclic ATP formation (Terry, 1980) and leaf ATP levels (Arulanantham, 1990). Morphological and physiological characteristics of roots are also modified under Fe deficiency in dicotyledonous and monocotyledonous (non-graminaceous) plants. Fe deficiency is associated with inhibition of root elongation, increased diameter of the root apical zone and abundant root hair formation (Romheld and Marschner, 1981; Chaney *et al*., 1992).

Another major class of plant pigments are the anthocyanins, a group in the diverse flavonoid family, responsible for the red-blue coloration of berries, red grapes, purple maize and vegetables, and are found in the cell vacuole of flowers, fruits, leaves, stems, and roots (Harbone, 1993; Escribano-Bailón *et al*., 2004). Anthocyanins serve multiple eco-physiological functions, and it has been shown that their accumulation in leaves can be induced by diverse environmental and anthropogenic stressors, such as high light, UV-exposure, chilling, pathogen infection, wounding, osmotic stress, pollution, and nutrient deficiencies, such as N and P, however, correlation with Fe deficiency has not yet been reported (Neill, 1994; Hodges and Nozzolillo, 1995).

To cope with Fe deficiency, plants developed sophisticated and tightly regulated mechanisms to mobilize Fe in the rhizosphere and take it up across the plasma membrane of root cells (Puig *et a*l., 2007). These acquisition strategies are based on two distinct mechanisms, namely, Strategy I and II which are depicted in Figure 1.4.1 (Grotz and Guerinot, 2006; for reviews please see Palmer and Guerinot (2009) and Gross *et al*., (2003)).

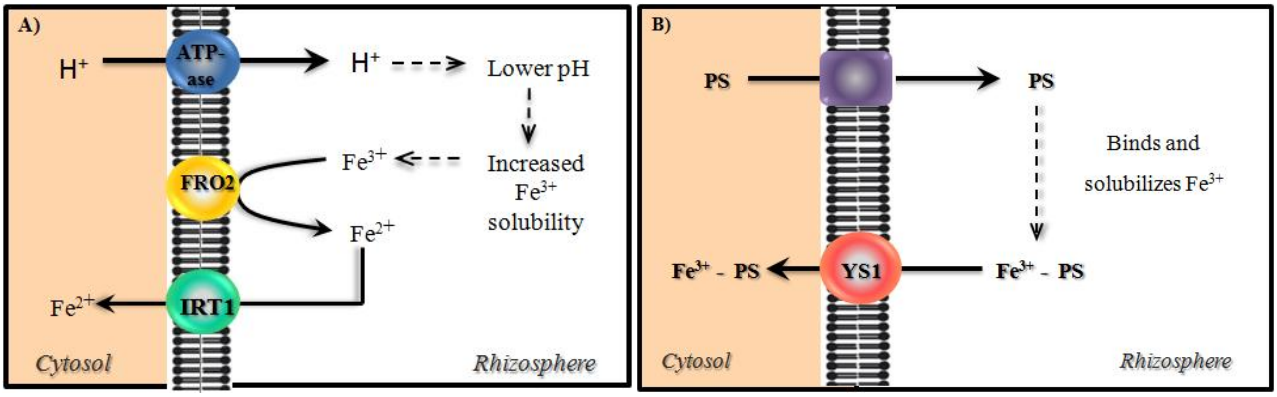

Figure 1.4.1 Strategies for Fe uptake from the soil. (A) Strategy I; (B) Strategy II (adapted from Sperotto *et al*., 2012b; Walker and Connolly, 2008).

The Strategy I response (Figure 1.4.1A) is used by all dicotyledonous species such as *Arabidopsis*, and by non-graminaceous monocotyledonous species (Mukherjee *et al*., 2006; Jeong and Connolly, 2009), and involves the release of protons into the rhizosphere to acidify the soil and increase Fe^{3+} solubility (Fox and Guerinot, 1998). Ferric iron (Fe^{3+}) is subsequently reduced to ferrous iron (Fe^{2+}) in the plasma membrane of root epidermal cells, by a ferric reductase-oxidase (FRO) (Robinson, 1999) and the Fe^{2+} 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*., *2001*), have been isolated and characterized in *Arabidopsis*. The *FRO2* gene encodes an enzyme thought to pass electrons across the plasma membrane to reduce ferric Fe chelates using two intramembrane heme groups (Yi and Guerinot, 1996) and 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).

The Strategy II response (Figure 1.4.1B) is used by the monocotyledonous species (grasses, graminaceous), such as rice, wheat, corn and barley, in which phytosiderophores (PS), that have a high affinity for Fe^{3+}, are released into the rhizosphere by OsTOM1/OsZIFL4 (Nozoye *et al*., 2011). The Fe^{3+}-PS complex 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), compounds obtained by the subsequent action of nicotianamine synthase (NAS) and deoxymugineic acid synthase (DMAS), are biosynthesis precursors of PS which chelate with metals, such as Fe, to transport them through the plant (Mori *et al.,* 1991; Inoue *et al*., 2003).

Different gramineae species produce different types and amounts of PS (Bashir *et al*., 2006). The quantity of PS released into the soil is correlated with the ability of the plant to tolerate Fe deficiency (Bashir and Nishizawa, 2006). Unlike barley and rye, rice secretes PS in relatively smaller amounts in response to Fe deficiency and is, thus, susceptible to low Fe availability, especially in the earlier stages of rice development (Marschner and Hohenheim, 1990; Bashir *et al*., 2006).

Although responses to Fe deficiency in graminaceous plants, such as increased secretion and production of MAs have been described, the mechanisms of gene regulation related to these responses are largely unknown (Ogo *et al*., 2007). Recently, Ogo *et al*. (2006) isolated and identified in rice a basic helix-loop-helix (bHLH) transcription factor, *OsIRO2*, involved in the response to Fe deficiency in graminaceous plants. *OsIRO2* was demonstrated to be strongly expressed in roots and shoots under Fe-deficiency and to be 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*. *IDEF1* is 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). Kobayashi *et al*. (2012) suggested that *OsIDEF1* is essential to sense the cellular Fe status in rice, especially at early stages, but not necessarily at subsequent stages.

Until recently, Strategy II plants were thought to only use the above-described response to obtain Fe from the soil (Ishimaru *et al*., 2006). However, an ortholog of the major root Fe transporter in *Arabidopsis*, IRT1, has already been 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). In accordance to that, a loss-of-function mutation in *OsNAAT1* results in less DMA secretion and decreased growth in media with Fe^{3+}. However, this mutant is still able to grow

under waterlogged conditions or when Fe^{2+} is provided (Cheng *et al*., 2007), which supports the hypothesis that rice has combined features of both strategies.

On the other hand, previous studies suggested that rice does not have the ability to reduce Fe^{3+} (Ishimaru *et al*., 2006), a limiting-step of Strategy I plants (Grusak *et al*., 1990). Moreover, attempts to produce rice with the ability to create more Fe available for absorption in conditions of Fe deficiency by introducing the gene *AtFRO2* in rice failed (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).

1.6 New candidate genes

After uptake from the soil, Fe is transported into the roots and is loaded in the xylem. Citrate seems to be the major Fe chelator in the xylem (Abadía *et al.*, 2002). AtFRD3 is a plasma membrane transporter that mediates citrate efflux into the root xylem, a process important for Fe translocation to shoots (Durrett e*t al.*, 2007). However, relatively little is known about how metals such as Fe are effluxed from cells, an indispensable step for transport from the root to the shoot (Durrett e*t al.*, 2007). Ferroportin (FPN) is the sole Fe efflux protein identified to date in mammals, functioning in both Fe absorption in the intestine and Fe recycling in macrophages (Muckenthaler *et al*., 2008). Recently, two closely related orthologs were identified in *Arabidopsis*: IRON REGULATED1 (IREG1/FPN1) and IREG2/FPN2 (Morrissey *et al*., 2009).

Morrissey *et al*. (2009) showed that FPN2 is localized in the vacuole and is expressed in the two outermost layers of the root in response to Fe deficiency, transporting Fe and Co into the vacuole. No orthologs for FPN have been described in rice. Evidence of direct Fe transport is still lacking, and while FPN1 could be a good candidate to mediate Fe efflux to the xylem, FPN2 seems to have a role in buffering metal influx (Morrissey *et al*., 2009).

Other candidate genes for Fe homeostasis in rice belong to the MYB transcription factor family. Previously, it was shown that the expression of two MYB genes was upregulated by

Fe deficiency (Colangelo and Guerinot, 2004), suggesting that MYB genes perhaps also play a role in Fe metabolism. In plants, MYB transcription factors play a key role in plant development, hormone signal transduction, secondary metabolism, abiotic stress tolerance and disease resistance (Stracke *et al*., 2001).

This family of genes was identified in a number of monocotyledonous and dicotyledonous plants (Yanhui *et al*., 2006). In *Dendrobium* hybrid Woo Leng, one of the most popular cut orchids in Southeast Asia, was described a MYB gene, *DwMYB2*, related with Fe deficiency (Chen *et al*., 2006). Moreover, the expression of *DwMYB2* in *Arabidopsis* promoted Fe uptake and impaired the Fe transportation from roots to shoots. This gene was never identified in rice.

OsMYBS3 is another transcription factor belonging to the MYB transcription factors family described to be regulated by sugars, where its expression is increased in the absence of sugars (Lu *et al*., 2002). *OsMYBS3* is the homologous of *MxMYB1*, isolated in one apple species in genus *Malus*, which is induced under Fe deficient conditions in *Arabidopsis* (Shen *et al*., 2008). There are no studies to show whether *OsMYBS3* plays a role in Fe homeostasis in rice plant or not.

2. OBJECTIVES

2.1 General objectives

There has been some controversy about the mechanisms used by rice plant for Fe uptake from the rhizosphere. The aim of this study was to understand if in fact rice plants have or not the capacity to up-regulate Strategy I mechanisms for Fe uptake, and if its capacity is dependent on the rice cultivar. Thus, two different rice cultivars, namely Nipponbare (cv. *japonica*) and Bico Branco (cv. *tropical japonica*) were studied at the physiological and molecular level when grown hydroponically under Fe deficiency (0 μM Fe(III)EDDHA) and Fe sufficiency (20 μM Fe(III)EDDHA).

2.2 Specific objectives

Nipponbare and Bico Branco cultivars were selected and grown for three weeks under Fe sufficiency and Fe deficiency conditions in order to,

- analyze the effect of Fe concentration in the growth medium on the accumulation of Fe and other micro micronutrients in different tissues (roots and shoots);
- understand the impact of Fe deficiency on photosynthetic pigment accumulation in rice shoots;
- understand if rice plants induce the Fe reductase enzyme in roots (a typical mechanism of Strategy I plants);
- analyze the expression of previously described mineral-related genes involved in Strategy I and II of Fe uptake, in different tissues (roots and shoots) and understand if their expression is cultivar dependent;
- identify new candidate genes that may be important in regulating Fe uptake in plants.

3. MATERIALS AND METHODS

3.1 Cultivars selection

Two major parameters were considered for cultivar selection: germination rate and Fe concentration. Table 3.1.1 shows the seven ecotypes of *Oryza sativa* L. and respective cultivars used in the present work.

Table 3.1.1 *Oryza sativa* L. ecotypes, cultivar names and country of origin

Ecotype	Cultivar Names	Country of Origin
Tropical Japonica	Carolina Gold	United States
	Peh-Pi-Nuo	China
	Bico Branco	Brazil
Temperate Japonica	Shinchiku-iku 103	Taiwan
	Aichi Asahi	Aichi Asahi
	Preto Regado 142	Morocco
Aromatic	Dom-Zard	Iran
	Lambayque 1	Peru
	Mana Muri	Nepal
Japonica	Eh-ia-Chiu	Taiwan
	Nipponbare	Japan
	Kalo Moni	Bangladesh
Aus	Sada Solay	Pakistan
	Hasawi	Saudi Arabia
	Dhali Khama	Bangladesh
Indica	Wie	Malasya
	Pin Kaeo	Thailand
	Padi Oro	Indonesia
Admix-Aus-Indica	Sareina	India
	Tak Siah	Pakistan
	Bhadoia 685	Bangladesh

3.1.1 Germination rate

For determination of the germination rate, between nine and ten seeds of each variety were germinated in germination bags. The seeds were maintained at room temperature under natural light (~20 ºC, 8 h light), and watered every three days. At seven and 14 days, the germination rate was calculated using the following equation:

$$Germination\ Rate = \frac{Number\ of\ geminated\ seeds}{Inicial\ number\ of\ seeds} * 100 \qquad \textbf{(3.1.1.1)}$$

3.1.2 Seed mineral analysis

For seed Fe concentration analysis, 200 mg of each variety, *ca.* ten seeds, were manually peeled and 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^{TM} MWS-3+ (Berghof, Germany) microwave system.

After digestion, the resulting clear solutions were diluted to 20 mL with ultrapure water. Mineral concentrations were measured using inductively coupled plasma atomic emission spectrometry (Optima 7000 DV ICP-OES, PerkinElmer) (Massachusetts, USA). The element was quantified using the axial alternate method.

3.2 Plant growth

Rice (*Oryza sativa* L.) seeds of two cultivars, cv. Bico Branco (*tropical japonica*) and cv. Nipponbare (*japonica*), were germinated on filter paper moistened with deionized water, wrapped in silver paper and incubated in a greenhouse at 25 ºC in the dark. They were watered with $CaCl_2$ 250 mM every three days. After three weeks of germination, 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.

Five rice plants were transferred to an Fe-free nutrient solution (Fe deficiency) and another five plants were transferred to a nutrient solution containing 20 μM Fe(III)-EDDHA (Fe sufficiency) as control, for more three 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 8h night (with temperatures of 19 – 20 °C). Growth solutions were changed weekly.

3.3. Photosynthetic pigment extraction

Anthocyanin, chlorophyll and carotenoid concentrations were measured in plants grown in Fe deficient and Fe sufficient conditions, as described previously. The referred compounds were extracted and quantified according to a modified protocol of Sims *et al*. (2002). Briefly, 0.1 g of shoot samples were grinded with a mortar and pestle using liquid nitrogen. Photosynthetic pigments were extracted with 0.4 mL of a cold acetone/Tris buffer solution (80:20 vol:vol, pH = 7.8). After homogenization, samples were incubated at 4 ºC for 1h. The supernatants were transferred to a new tube and diluted fivefold with additional acetone/Tris buffer to measure absorbance at 470, 537, 647 and 663 nm with a spectrophotometer (Implen, Isaza, Portugal).

The amount of anthocyanins, chlorophyll a and b and carotenoids were determined through the following equations (3.3.1):

$$\begin{aligned}
Anthocyanins &= (0.08173 \times \mathrm{A537} - 0.0697 \times \mathrm{A647} - 0.002228 \times \mathrm{A663}) \times \frac{Dilution\ Factor}{Fresh\ weight\ (g)} \\
Chlorophyll\ a &= (0.01373 \times A663 - 0.000897 \times A537 - 0.003046 \times A647) \times \frac{Dilution\ Factor}{Fresh\ weight\ (g)} \\
Chlorophyll\ b &= (0.02405 \times A647 - 0.004305 \times A537 - 0.005507 \times A663) \times \frac{Dilution\ Factor}{Fresh\ weight\ (g)} \\
Carotenoids &= \frac{(A470 - (17.1 \times (Chla + Chlb) - 9.479 \times Anthoccyanin))}{119.26} \times \frac{Dilution\ Factor}{Fresh\ weight\ (g)}
\end{aligned} \quad \textbf{(3.3.1)}$$

3.4. Root Fe-reductase activity assay

Root capacity to reduce Fe(III)-EDTA in Bico Branco and Nipponbare cultivars, was analyzed according to Vasconcelos *et al.* (2006), via the spectrophotometric measurement of Fe^{2+} chelated to BPDS (bathophenanthroline disulfonic acid). Roots of each single intact (Fe sufficient or Fe deficient) rice plant were submerged in an 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. The solution was buffered with 1 mM MES, pH 5.5. The assays were conducted in the dark at room temperature and were terminated after 45 minutes by removal of the roots. The absorbances were obtained spectrophotometrically (Implen, Isaza, Portugal) at 535 nm, and an aliquot of the solution that had no roots during the assay was used as blank. The amount of Fe^{3+} reduced was calculated using the molar extinction coefficient of 22.14 mM^{-1} cm^{-1}.

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.

3.5 Total RNA extraction

Rice 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 stored at – 80 ºC for RNA extraction. Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit (USA, Nr. #74904), according to the manufacturer's instructions with some modifications, and treated with RNase-free DNase I to remove contaminating genomic DNA.

Briefly, about 50 mg of shoots and 100 mg of roots were weighed, and placed in an eppendorf tube containing 750 µL and 450 µL of RLC and RLT extraction buffer, respectively, with 1 % of β-mercaptoethanol. Samples were vigorously vortexed and, while shoots samples were incubated at room temperature for six min, root samples were incubated for three min at 56 ºC. The lysates were transferred to a QIAshredder spin column placed in a two mL collection tube, and centrifuged for five min at full speed. Then, the supernatants were carefully transferred to a new microcentrifuge tube to which was added 0.5 volume of 100 % ethanol to the cleared lysate, mixed immediately by pipetting and transferred to an RNeasy spin column placed in a two mL collection tube and centrifuged for 15 seconds at 10,000 rpm. After discarding the flow through, 700 µL of RW1 buffer was added to the RNeasy spin column of each sample and centrifuged for 15 seconds at 10,000 rpm. The column was washed with 500 µl Buffer RPE twice in shoots samples and three times in roots samples, centrifuging for 15 seconds at 10,000 rpm between each addition and discarding the flow-through. Lastly, each spin column was transferred to another sterile tube and 30 µl of RNase-free water was added and centrifuged for one min at 10,000 rpm to elute the RNA. The previous step was repeated, passing the eluate through the membrane again and submitting the tubes to a new centrifugation to ensure maximal yield. RNA quality and quantity were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal). Samples were stored at – 80 ºC for further analyses.

3.6. cDNA Synthesis

Single-stranded cDNA was synthesized from extracted RNA using the First Strand cDNA Synthesis Kit (Fermentas UAB, Cat. Nr. #K1612), according to manufacturer's instructions. Briefly, about 1000 ng of RNA was added one µL of Random Hexamer Primer and nuclease-free water to a final volume of 11 µL. To this mixture four µL of 5x Reaction Buffer, one µL of RiboLock RNase Inhibitor (20 u/ µL), two µL of ten mM dNTP Mix and M-MuLV Reverse Transcriptase (20 u/µL) were added. This mixture was incubated in a Thermal cycler (VWR, Doppio, Belgium) for five min at 25 °C followed by 60 min at 37 °C. The reaction was terminated by heating at 70 °C for five min.

cDNA quantity and quality were checked by UV-spectrophotometry, using a nanophotometer (Implen, Isaza, Portugal). cDNA samples were stored for further analyses.

3.7 Gene expression analysis

Accession numbers of genes identified in Fe nutrition in rice plants were chosen using NCBI databases. Accession orthologs to AtTOM1, AtFPN1/IREG1, AtFPN2/IREG2 and DwMYB2 were identified using the TBLASTN tool against the GenBank databases with search specifications for *Oryza sativa* [Organism]. The new sequences in rice were named *Oryza sativa* TOM1 (*OsTOM1*), *OsFPN1* and *OsMYB2*. Only sequences that showed an $e^{-value} \leq 6e^{-14}$ were considered significant (Annexes, Table 3.7.1).

Primer sequences were designed for 11 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 3.7.2 (Annexes). The primers used were hydrated according manufacturer's instructions.

cDNA, extracted from roots and shoots of Bico Branco and Nipponbare cultivar growing under Fe sufficient and Fe deficient conditions, was amplified by qRT-PCR in a Chromo4 Thermocycler (Bio-Rad, CA, USA).

Amplifications were carried out 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 ºC and then 10 min at 95 ºC, followed by 39 cycles of 15 s at 95 ºC and 1 min at 57 ºC, and a final dissociation step of 1 min at 72 ºC.

Melting curve from 50.0 ºC to 65 ºC was read every 1 ºC holding 1 s. Then, melt curves profiles were analyzed for each gene tested. The comparative CT method (ΔΔCT) (Livak and Schmittgen *et al*., 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.

3.8 Elemental analysis

To determine mineral concentrations, Bico Branco and Nipponbare cultivars were grown under Fe deficient and Fe sufficient conditions 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 ºC. Samples were reduced to a fine powder digested and analyzed with ICP-OES, as described in section 3.1.2

3.9 Statistical analysis

Data processing and statistical analysis of ICP-OES data, root Fe reductase activity assay, anthocyanins, chlorophyll a and b, total chlorophylls and carotenoids analyses were performed using Microsoft Excel and GraphPad Software. Differences between treatments were tested with an unpaired t-test.

4. RESULTS AND DISCUSSION

One important gap in the understanding of Fe allocation to the rice grain is the knowledge about how Fe is acquired from the environment (Sperotto *et al*, 2012). When plants are grown in aerobic conditions, where Fe is not available, they induce a set of mechanisms that function at the root–soil interface to solubilize Fe and subsequently transfer it across the plasma membrane of root cells (Palmer and Guerinot, 2009).

Dicotyledonous plants use Strategy I for Fe uptake from the soil under Fe deficiency, and Strategy II was described to be used by all grasses (Romheld, 1987). However, even though rice (*Oryza sativa* L.) is a Strategy II plant, reports suggest that it could have the ability to use both strategies for Fe uptake. Ishimaru *et al.* (2006) showed that rice has an efficient Fe^{2+} uptake system, since its genome encodes two proteins - OsIRT1 and OsIRT2 - which are higly similar to the Strategy I transporter IRT1. This could be an adaptation of rice plants to flooded/anaerobic soils, where low redox conditions occur and Fe^{3+} is reduced to Fe^{2+}, being the latter directly transported through OsIRT1 (Ishimaru *et al.*, 2006). On the other hand, in upland rice, where aerobic conditions prevail, Fe^{3+} is present in greater quantity than in anaerobic soils. Since rice is, of all grasses, the one which produces lower amounts of PS (Mori, 1991), one could hypothesize that the utilization of Strategy I mechanisms would represent an environmental advantage to compensate for the lack of available Fe in aerobic conditions.

In strategy I plants, it is known that Fe deficiency induces an increase in root Fe^{3+}-reductase activity (Kochian and Lucas, 1991), but Strategy II plants were described to not possess this reduction capacity. Ishimaru *et al.* (2006) measured the Fe^{3+}-chelate reductase activity in the surface of rice roots under Fe deficient conditions and showed that rice has very low Fe^{3+} reductase activity. They also found increased chlorosis of the fully expanded youngest rice leaves showing, like others, that Fe deficiency inhibits the biosynthesis of chlorophyll (Belkhodia *et al*. 1998).

Besides altering chlorophyll synthesis, mineral deficiencies were described to increase the production of anthocyanins pigments, that are thought to protect plants from this type of stress conditions (Neill, 1994; Hodges and Nozzolillo, 1995; Gould, 2004). However, there are no studies available to demonstrate the relationship between this pigment in rice and Fe deficiency stress.

To sum up, due to the major enigma surrounding the Fe uptake mechanisms utilized by rice, in this report, a focus on physiological and molecular parameters was taken to elucidate the question behind rice strategies alternation. *Oryza sativa* L. cv. Nipponbare and cv. Bico Branco were grown under Fe deficient and Fe sufficient conditions to evaluate mineral concentration, photosynthetic pigment accumulation, Fe-reductase activity and expression of Fe metabolism related genes.

4.1 Rice cultivar selection

Given the high degree of variability in molecular and physiological responses between cultivars, an initial screening stage was conducted in order to select two cultivars with contrasting responses to Fe deficiency. The criteria for selection were a high germination capacity, as well as a good nutritional Fe status of the seeds, and an optimum growth in hydroponic conditions. Moreover, we were interested in selecting two cultivars with different ecotypes, as growth habitats may influence the plants response to Fe deficiency.

The germination rates of 21 rice cultivars were evaluated at seven and 14 days, as shows Figure 4.1.1

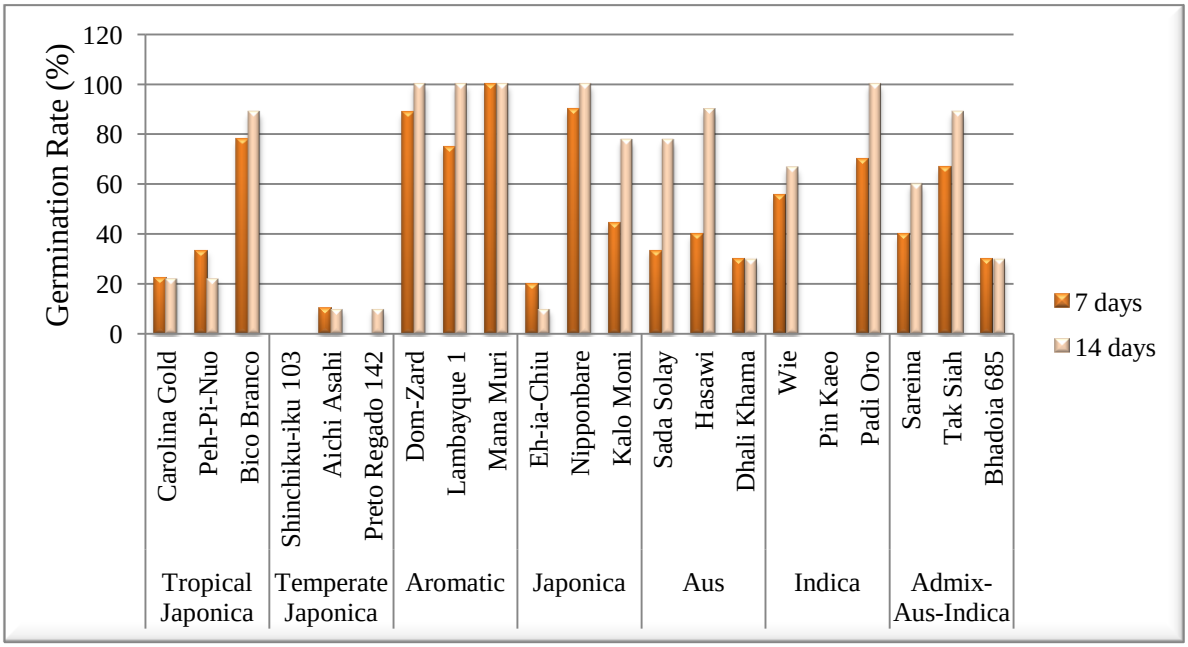

Figure 4.1.1 Germination rate of 21 rice (*Oryza sativa* L.) cultivars at seven and 14 days after germination.

In general, Bico Branco, Dom-Zard, Lambayque 1, Mana Muri, Nipponbare, Padi Oro, Hasawi and Tak Siah had the highest germination rate, ranging from 89 to 100 %. Cultivars of *temperate japonica* ecotype revealed the lowest germination rates, with values reaching only 10 % for Aichi Asahi and for Preto Regado 142, and no germination of Shinchiku-iku 103 (Figure 4.1.1).

Another criterium utilized was the seed Fe concentration. This enabled us to understand the nutritional variability of our cultivars, and to select the varieties to use in the subsequent analyses.

Figure 4.1.2 shows that, in general, Mana Muri seeds had the highest concentration of Fe, followed by Nipponbare and Lambayque 1. The cultivars with lowest Fe concentrations were Pin Kaeo, an *indica*-type, and Bhadoia 685, an *admix-aus-indica*-type.

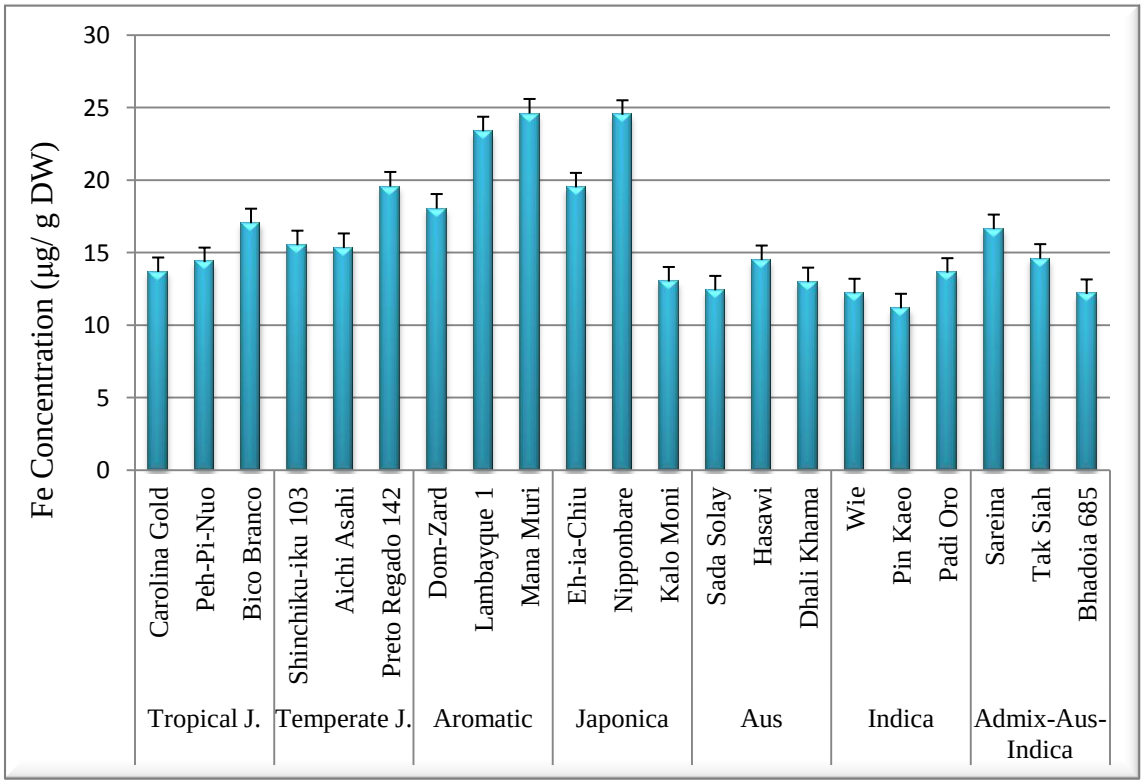

Figure 4.1.2 Concentration (µg/g dry weight (DW)) of Fe in seeds of 21 rice (*Oryza sativa* L.) cultivars. Data are mean±standard error. An average of three technical replicates were analyzed.

The values obtained ranged from 11 μg/g DW to 24 μg/g DW in seeds, from Pin Kaeo to Mana Muri, respectively. In other studies, Fe concentration in rice grains had the lowest levels when compared to other cereals, ranging from 6 to 22 μg/g compared to 10 to 160 μg/g in maize and 15 to 360 μg/g in wheat (Gómez-Galera *et al*., 2010). Gregorio *et al*. (2000) evaluated the genetic variability of Fe concentration in other rice varieties and, among the 1,138 samples analyzed, the Fe concentration in brown rice ranged from 6.3 to 24.4 ppm. The highest grain-Fe concentrations (ranging from about 18 to 22 μg/g) were found in several aromatic rice varieties, such as Zuchem, Jalmagna and Xua Bue Nuo.

Excluding Padi Oro and Tak Siah, the cultivars with highest germination rates also had higher seed Fe concentrations. Therefore, experimentally, the seven cultivars with highest germination rates were grown under Fe deficient and Fe sufficient hydroponic conditions to evaluate their growth capacity in Fe limiting conditions. In this work we aimed at understanding the physiological molecular response to Fe deficiency in two rice cultivars with different growth habitats and ecotypes. As Bico Branco and Nipponbare were the cultivars that grew better and also had good nutritional value and high germination rates these were the ones selected for further studies.

4.2 Physiological responses to Fe deficiency

When plants are under mineral stress conditions, they develop a range of mechanisms to cope with these fluctuations, such as storage and remobilization of mineral nutrients and changes in morphology and physiology (Marschner, 1995). In aerobic conditions, Fe is highly unavailable for plant uptake, and its deficiency can be severe in plants grown in calcareous soils (Jeong and Guerinot, 2009).

Here, the photosynthetic pigment accumulation in shoots, as well as micronutrients concentration in rice tissues and root Fe-reductase activity of two different rice plants grown hydroponically under Fe deficient and Fe sufficient conditions will be analyzed.

4.2.1 Photosynthetic pigment accumulation

As referred in the Introduction section, IDC is one of the earliest symptoms observed in the leaves of plants growing in soils with low Fe availability (Abadía *et al.*, 1999*).* In rice, Sperotto *et al*. (2007) visualized the first symptoms of chlorosis after 11–13 days of Fe deficient treatment, which was well established after 18 days, with significant decreases in chlorophyll concentration.

Figure 4.2.1.1 shows the growth differences between shoots of plants after three weeks under Fe deficient and Fe sufficient conditions, where shoots of Fe deficient plants showed more yellowing than under Fe sufficient ones. Abbott (1967) previously described an evident difference in the size of shoots between treatments that was also observed in this study.

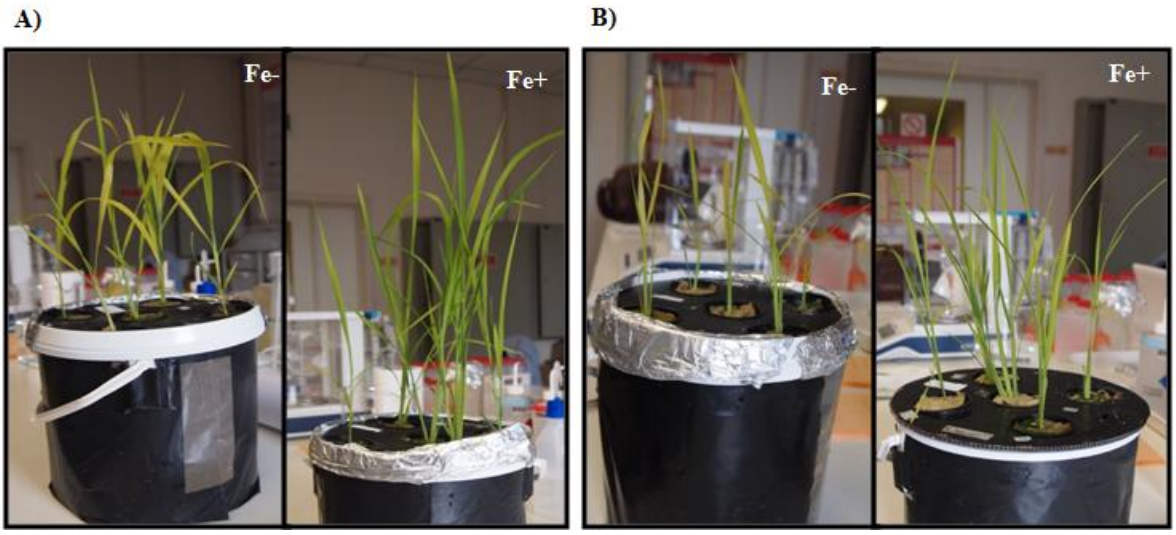

Figure 4.2.1.1 Fe deficiency chlorosis in shoots of (A) Bico Branco and (B) Nipponbare cultivars after three weeks under Fe deficiency (Fe-) (0 μM Fe(III)-EDDHA) (left panels) and Fe sufficiency (Fe+) (20 μM Fe(III)-EDDHA) (right panels) hydroponic conditions.

IDC has been usually attributed to inhibition of chlorophyll synthesis, since Fe plays a role in the biosynthesis of this photosynthetic pigment and its precursors in leaves (Pushnika *et al*., 1984). Thus, under Fe deficiency, the loss of chlorophylls as well as carotenoids, are the primary responses associated with the unavailability of this element (Belkhodia *et al*., 1998; Hendry and Price, 1993). On the contrary, anthocyanins, which are natural pigments belonging to the flavonoid family and are responsible for the red-blue coloration (Pascual-Teresa and Sanchez-Ballesta, 2008), are known to accumulate in leaves with nutrient

deficiency, especially P and N (Neill, 1994; Hodges and Nozzolillo, 1995). Thus, these pigments may be good indicators of plant stress (Hendry and Price, 1993).

In this work, anthocyanin, chlorophyll and carotenoid concentrations were measured in Bico Branco and Nipponbare cultivars shoots and results are presented in figure 4.2.1.2.

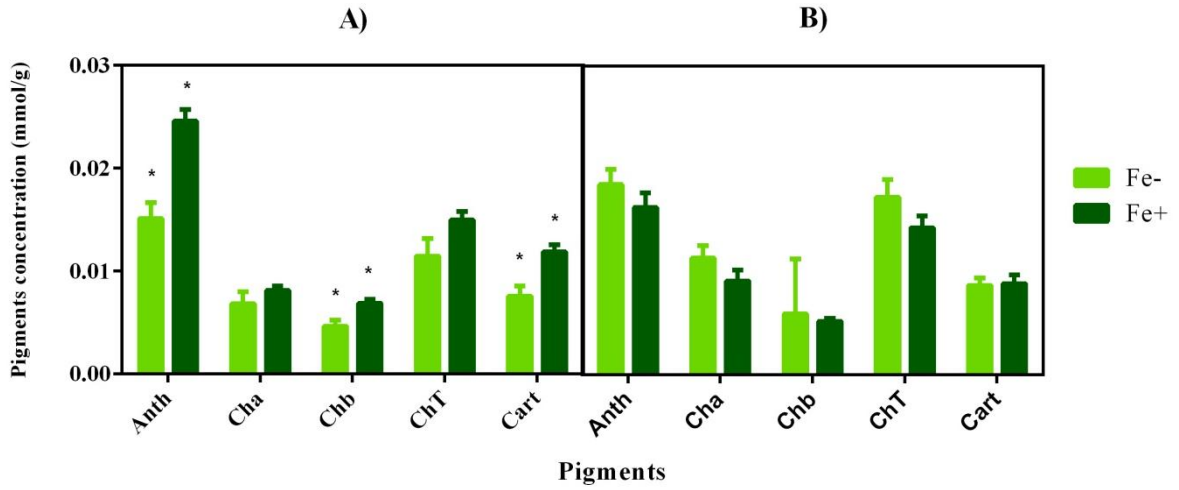

Figure 4.2.1.2 Anthocyanin (Anth), chlorophyll a (Cha) and b (Chb), total chlorophyll (ChT) and carotenoid (Cart) concentrations in shoots of (A) Bico Branco and (B) Nipponbare cultivars. Plants were grown in 0 μM Fe(III)-EDDHA (Fe-) and 20 μM Fe(III)-EDDHA (Fe+) conditions for three weeks. Results show the mean±SEM of five plants. Significant differences are indicated by asterisk (p<0.05).

The results obtained showed that Bico Branco cultivar had lower chlorophyll, carotenoid and anthocyanin levels under Fe deficient conditions than Nipponbare cultivar. Particularly with the anthocyanin, chlorophyll b and carotenoid values, Bico Branco suffered a significant decrease (p<0.05) when under Fe deficiency when compared to the control treatment (Fe+).

In rice, the effects of Fe deficiency in chlorophyll concentration have already been studied. Wu *et al*., (2001) evaluated the chlorophyll content through SPAD-502, a portable meter, in rice (cv. Nipponbare) leaves during 14 days, and found that after five days of Fe deprivation there was a significant decline of chlorophyll concentration, and chlorotic symptoms were induced in newly developed leaves. Zheng *et al*. (2009) also studied the chlorophyll content of rice (cv. Nipponbare) under –Fe+P, +Fe+P, +Fe–P and –Fe–P conditions, and showed that chlorophyll content decreased in Fe deficiency (–Fe) media. They

also observed a chlorotic phenotype, consistent with chlorophyll content and Fe concentrations decreased in both roots and shoots.

Hodges and Nozzolillo (1995) demonstrated that anthocyanins tended to increase under N starvation and to decrease under P and K starvation. In contrast, in bean plants cultured on P deficiency media, the concentrations of anthocyanins were higher in leaves maybe to protect the plant from oxidative stress (Juszczuk *et al*. 2004). It is known that anthocyanins can persist throughout the leaf's entire life span (Gould *et al.,* 2000), or else they are induced and retained only after the plant has experienced stress (Chalker-Scott, 1999).

In the present work, anthocyanins pigment accumulated more in Nipponbare cultivar than in the Bico Branco counterpart under Fe deficiency, and as the Nipponbare cultivar presents less signs of chlorosis than Bico Branco, these results support the idea that this pigment may protect plants from their degradation. Furthermore, both cultivars had higher levels of this pigment than any other parameter (Figure 4.2.1.2), which suggests that anthocyanins are produced in response to Fe deficiency, and not just to P and N deficiency, as it has been described.

In general, the Nipponbare cultivar seems to be less affected by chlorosis since no significant differences were detected between Fe treatments (Figure 4.2.1.1B) ($p>0.05$).

4.2.2 Mineral Accumulation in shoots and roots

When the nutrient supply into roots is compromised, plants trigger a series of mechanisms to resolve these imbalances (Marschner, 1995) and an increase in mineral content in one organ results from the uptake and translocation from the soil, or from remobilization from one organ to another (Sperotto *et al.*, 2012a).

There are several micronutrients considered to be essential for higher plants, such as Fe, Zn, Mn, Cu, Ni, B, Mo, and Cl (Welch and Shuman, 1995). To test the impact in whole plant mineral dynamics by Fe deficiency, Bico Branco and Nipponbare cultivars were grown under Fe deficient and Fe sufficient conditions and nutrient accumulation was determined by ICP-OES. Figure 4.2.2.1 shows the accumulation of micronutrients in the shoots and roots of Bico Branco (Figure 4.2.2.1A) and Nipponbare (Figure 4.2.2.1B) plants.

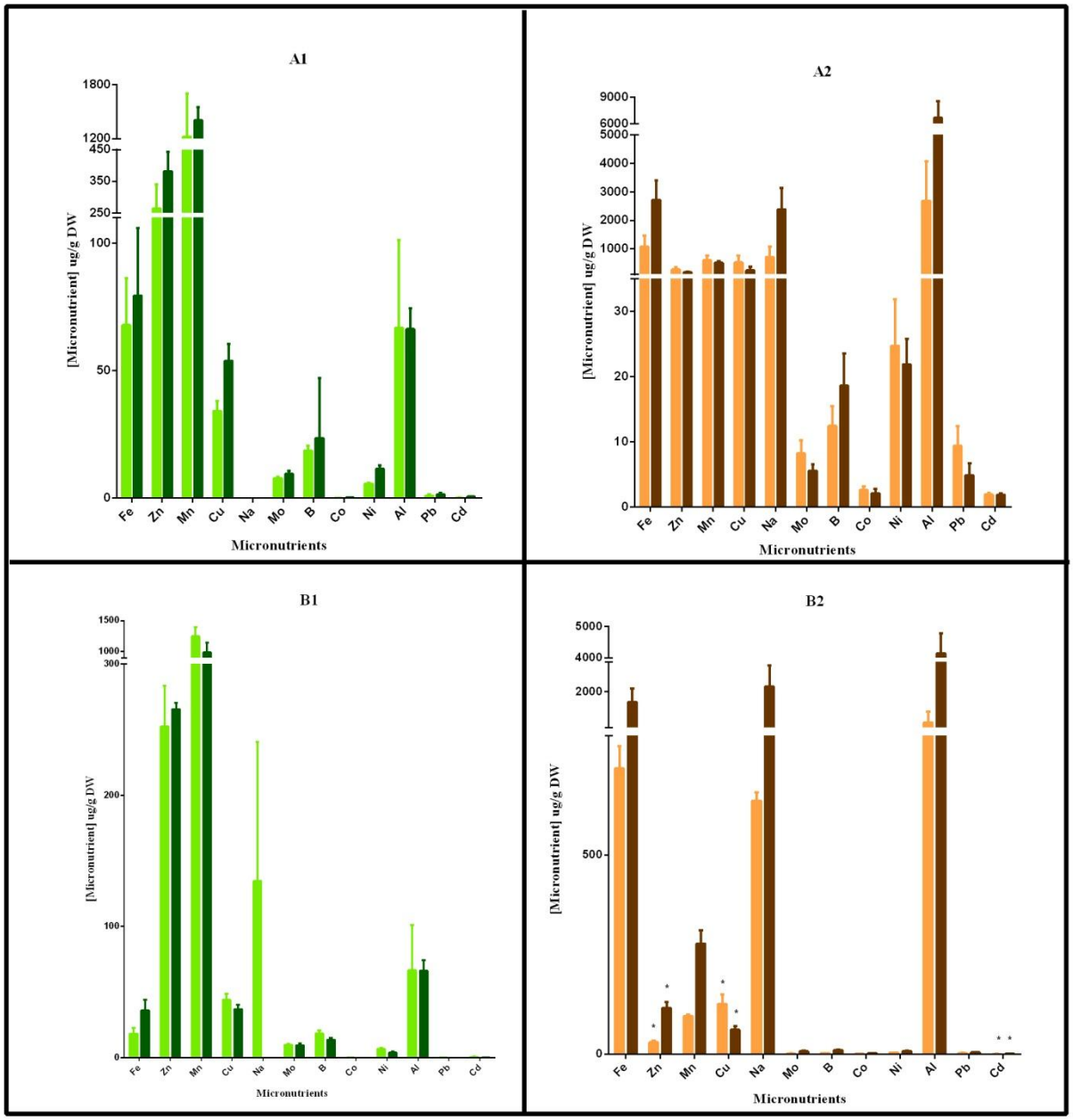

Figure 4.2.2.1 Micronutrient concentrations (µg/g dry weight) of shoots (■, ■) and roots (■, ■) of (A) Bico Branco and (B) Nipponbare cultivars, using ICP-OES. Rice plants were grown for three weeks in Fe deficient (0 µM Fe(III)-EDDHA) (■, ■) and Fe sufficient (20 µM Fe(III)-EDDHA) (■, ■) hydroponic conditions. Results show the mean±SEM of three plants. Significant differences between Fe treatments for each species are indicated by an asterisk (p<0.05).

According to Sperotto *et al.* (2012a), rice accumulates lower Fe concentrations in both shoots and roots of plants grown under Fe deficient conditions than under Fe sufficiency, but accumulates more in roots than in shoots (Silveira *et al*., 2007). In this study, Bico Branco shoots had 67.89 µg/g DW of Fe under Fe deficiency and 79.38 µg/g DW of Fe under Fe sufficiency (Figure 4.2.2.1A1). Also, roots accumulated more Fe than shoots, as previously reported, namely 1078.29 µg/g DW of Fe under Fe deficiency and 2711.40 µg/g DW of Fe under Fe sufficiency (Figure 4.2.2.1A2) (Sperotto *et al.,* 2012a).

Nipponbare cultivar also had less Fe concentration in Fe deficient tissues when compared to Fe sufficient ones, 18.39 µg/g DW and 36.18 µg/g DW in shoots and 718.11 µg/g DW and 1828.03 µg/g DW in roots, respectively (Figure 4.2.2.1B1 and B2), suggesting less accumulation of Fe by this cultivar when compared to Bico Branco cultivar.

Sperotto *et al.* (2012a) characterized mineral accumulation in rice (cv. Kitaake) tissues under different Fe supplies, namely 5, 20 and 200 mM. Under medium Fe supply, Fe concentration ranged from 50 to 70 µg/g DW in shoots, and between 1000 and 2000 µg/g DW in roots, which is consistent with the results obtained here for Fe sufficient conditions.

In what concerns the study of the other micro and macronutrients, Sperotto *et al*. (2012a) showed that Zn, Cu, and Ni were more accumulated in roots and Mn, Ca, Mg and K in leaves, when low Fe concentration is available. They also found that Fe, Mn and Ca were at lower concentrations in roots and Zn and Ni in leaves. Here, under Fe deficiency, Bico Branco cultivar had higher accumulation of Zn, Cu and Mn in roots, but not in shoots. This may have happened because under low Fe supply, Fe transporters such as NAS1 and NAS2, are induced and that could result in increased uptake of other nutrients (Ramani and Kannan, 1987). This is especially important under hydroponic culture conditions, since ions of Zn, Mn and Cu exist abundantly in the culture solution.

Also, when low Fe concentrations were predominant, there was higher accumulation of Ni, Mo, Pb and Cd in Bico Branco roots (Figure 4.2.2.1A2), which was also obtained by Sperotto *et al*. (2012a). Under Fe deficiency, Na accumulated in Bico Branco roots, but no values were detected in shoots.

In Nipponbare cultivar, Cu was the only mineral that had higher accumulation in roots under low Fe supply (Figure 4.2.2.1B2). Since Fe and Cu can share the same transporters, it is understandable that when Fe uptake decreases other minerals uptake, like Cu increases (López-Millán *et al*. 2004).

In contrast to what was seen in Bico Branco cultivar, higher levels of Mn and Cu were detected in Nipponbare shoots, with slightly smaller amounts of Na, Mo, B, Co, Ni, Al, Pb and Cd (Figure 4.2.2.1B1). It was already demonstrated that, besides Fe, other micronutrients are affected by Fe deficiency in rice plants, especially in the early stages of rice development (Silveira *et al*. 2007; Sperotto *et al*., 2012a). Ramani and Kannan (1987) showed an increase of Mn uptake and subsequent translocation to shoots, since Mn moves easily from root to shoot in the xylem-sap transpiration stream. Zhang *et al*. (1991) also described increase of Mn and Cu in rice plants under Fe deficiency, possible because of the presence of PS in the rhizosphere that may increase the availability of these ions both in the rhizosphere itself and in the apoplast. In Nipponbare roots a significantly higher accumulation ($p < 0.05$) of Zn, Co and Cd in roots was detected under Fe sufficiency compared with the plants grown under Fe deficiency (Figure 4.2.2.1B2).

4.2.3 Root Fe reductase activity assay

Monocotyledonous plants such as rice, usually utilize Strategy II for Fe uptake. Dicotyledonous plants, on the other hand, use Strategy I mechanisms for Fe uptake, where the soil pH is acidified by a release of protons and an increase of the activity of a root ferric reductase, which converts the less soluble ferric Fe (Fe^{3+}) to the more soluble ferrous Fe (Fe^{2+}) (Römheld and Marschner, 1986) is observed. Studies looking at root Fe reduction capacity usually report that plants have higher reductase activity under Fe deficiency than under Fe sufficiency (Romera *et al*., 1992; Cinelli *et al*., 1995; Kochian and Lucas, 1991). However, rice plants have been described to not reduce Fe^{3+} actively to Fe^{2+} because their Fe^{3+} chelate reductase activity is very low (Maruiama *et* al., 2005; Ishimaru *et al*., 2006) or absent (Vasconcelos *et al*., 2004).

In the present study, reductase activity was measured in roots of plants grown in Fe deficient and Fe sufficient hydroponic conditions (Figure 4.2.3.1). Furthermore, reductase activity contribution from root soluble reductants release was measured and accounted for in the root reductase activity rates shown in Figure 4.2.3.1.

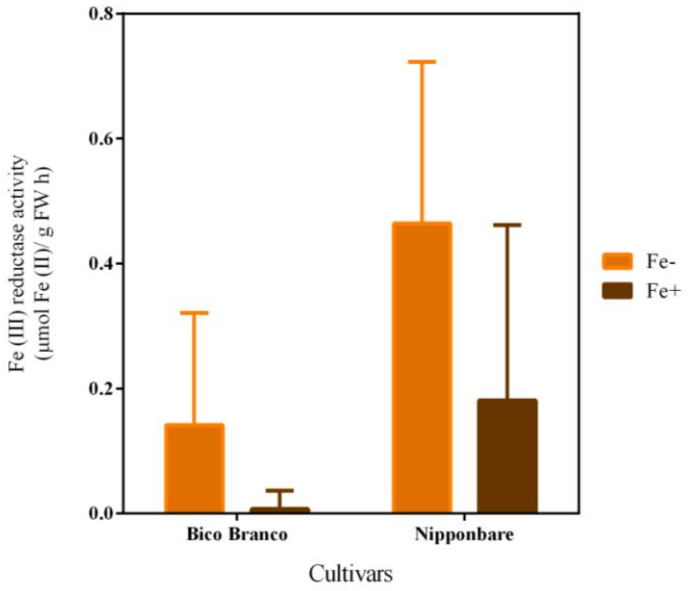

Figure 4.2.3.1 Roots Fe reductase activity of Bico Branco and Nipponbare cultivars, when grown for three weeks under Fe deficient (0 µM Fe(III)-EDDHA) (■) and Fe sufficient (20 µM Fe(III)-EDDHA) (■) hydroponic conditions. Results show mean±SEM of five plants. Significant differences are indicated by asterisk (p<0.05).

The results obtained by Ishimaru and co-workers (2006) demonstrated that reductase activity in Nipponbare plants changes along 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, and reaching 0.025 µmol Fe (II) g^{-1} FW h^{-1} after 14 days under Fe deficiency. After an Fe resupply at day 14 they found a slight increase in Fe^{3+}-chelate reductase activity (0.030 µmol Fe (II) g^{-1} FW h^{-1}). In the current experiment, at three weeks of Fe starvation a maximum values of 0.464 µmol Fe (II) g^{-1} FW h^{-1} for Nipponbare cultivar and 0.141 µmol Fe (II) g^{-1} FW h^{-1} for Bico Branco cultivar were obtained, although without significant differences between treatments (p>0.05) that can possibly be attributed to the low number of biological replicates in the study.

Other studies show that under Fe deficient conditions, some bean populations reduce Fe^{3+} around 0.2 µmol Fe (II) g^{-1} FW h^{-1}, which are values in the range of the ones obtained here.

4.3 Molecular response to Fe deficiency

Iron deficiency is one of the most serious problems in agriculture (Guerinot and Yi, 1994). To cope with Fe deficiency, plants developed sophisticated and tightly regulated mechanisms to mobilize Fe (Puig *et a*l., 2007) that are regulated at a molecular level. To study these mechanisms, Fe sufficient and Fe deficient shoots and roots of Bico Branco and Nipponbare cultivars, were analyzed for the Fe deficiency-inducible and other genes involved in the transport of nutrients through rice using quantitative real-time PCR (qRT-PCR).

4.3.1 Strategy I-related genes

Although rice has been for long described as a Strategy II plant (Mori, 1999), recent studies have demonstrated that rice can adopt a combined mechanism of Strategy I and II (Walker and Connolly, 2008; Ishimaru *et al*., 2006). This cereal is known to grow in a wide range of environments where other crops would fail (IRRI, 2009). In anaerobic soils, where Fe^{2+} is in higher amounts, the capacity of rice to transport reduced Fe into the roots, like plants of Strategy I, has already been described (Walker and Connolly, 2008). On the other hand, under 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), secrets PS at lower amounts compared to other grasses (Mori, 1991), and for this reason, it suffers from severe problems of Fe deficiency, especially in the early stages of rice development. Gross *et al*. (2003) suggests that Fe reductase genes (FRO) from *Arabidopsis thaliana* may be present in the rice genome. Thus, there are evidences that rice may benefit from the capacity to reduce Fe, to compensate the lack of Fe in upland soils.

It has been shown in *Arabidopsis* that under limiting Fe availability the expression of *AtFRO2* in roots is increased (Mukherjee *et al*., 2006). In rice plants (*Oryza sativa* L.), *OsFRO2* are thought to be exclusively expressed in shoots (Ishimaru *et al*., 2006). Figure 4.3.1.1 shows that under Fe deficiency, the expression of *OsFRO2* was very low in roots and shoots of Bico Branco cultivar (Figure 4.3.2.1.1A), whereas in Nipponbare plants, shoots supplied with Fe had a strong induction of this gene expression (Figure 4.3.1.1B).

As referred before, *FRO* genes encode the Fe^{3+}-chelate reductase enzymes and the activity of this enzyme was quantified as presented in figure 4.2.3.1. Rice is known to have low root Fe^{3+}-chelate reductase activity, but it is thought to be higher under Fe deficient conditions (Ishimaru *et al.*, 2006). In the current study, Bico Branco cultivar presented low expression of *OsFRO2* in Fe deficiency (Figure 4.3.1.1A) and, accordingly, low reductase activity (Figure 4.2.3.1). On the other hand, Nipponbare cultivar had higher levels of *OsFRO2* gene expression in roots and, again, reductase activity was also higher (Figure 4.3.1.1B and 4.2.3.1, respectively). These results show that the expression of *OsFRO2* gene in roots, could be important for root Fe uptake, particularly under high Fe availability.

After Fe reduction by FRO, Strategy I plants transport Fe across the plasma membrane of root epidermal cells by IRT1 (Grotz *et al.*, 2006). In graminaceous plants, such as maize and barley, the inducible Fe^{2+} transporter system either is absent or is expressed at very low levels (Zaharieva and Romheld, 2001). In rice, on the other hand, it was described that despite absorbing Fe^{3+}-PS efficiently through OsYSL15 (Lee *et al.*, 2009a), this plant also possesses a ferrous transporter, OsIRT1, and can take up Fe^{2+} (Ishimaru *et al*., 2006).

Walker and Connolly (2008) also showed that *OsIRT1* gene is expressed in roots under Fe deficient conditions. The ability to absorb Fe^{2+} probably evolved in rice as an adaptation to flooded paddies, where Fe^{2+} is frequently more abundant than Fe^{3+} due to the anaerobic conditions that prevail in wet-fields (Cheng *et* al., 2007).

In the current study, as there was more expression of *OsFRO2* in Fe sufficient conditions in both cultivars, the expression of *OsIRT1* was also higher in roots of Fe sufficiency grown plants (Figure 4.3.1.1). However, in Fe deficiency treatment, shoots seemed to up-regulate this gene (Fig. 4.3.1.1), which was also described by Ishimaru *et al*. (2006), suggesting that Fe^{2+} transporters participate in Fe distribution and partitioning in rice plants. They analyzed stems of transgenic plants expressing the OsIRT1 promoter–GUS fusion, which showed that GUS activity was present exclusively in the phloem under Fe sufficient conditions, with 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.

In summary, our data suggest that rice is able to up-regulate the Strategy I genes *FRO* and *IRT1*, but this regulation seems to be cultivar dependent.

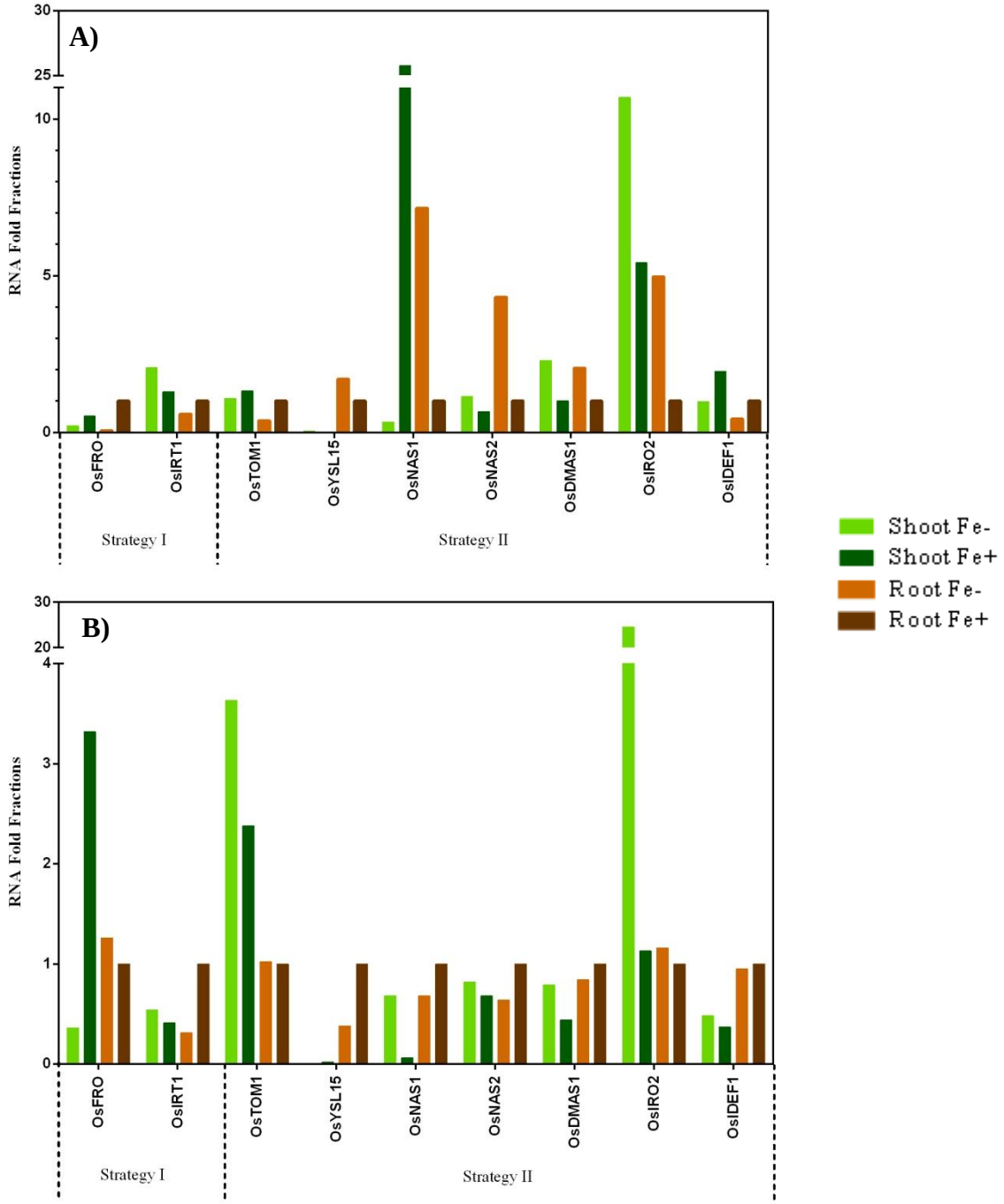

Figure 4.3.1.1 Quantitative RT-PCR analysis of genes related with Strategy I and Strategy II of Fe uptake, in (A) Bico Branco and (B) Nipponbare cultivars. Total RNA was extracted from shoots and roots of plants grown hydroponically for three weeks under Fe deficient (0 μM Fe(III)-EDDHA) and Fe sufficient (20 μM Fe(III)-EDDHA) conditions. A pool of three plants of each condition was used. The results were normalized using the housekeeping gene 18S (-rRNA).

4.3.2 Strategy II-related genes

There are several genes known to be related to Fe uptake in Strategy II in which PSs are released into the rhizosphere (Romheld and Marschner, 1990). In the biosynthesis of PS, MAs are biosynthesized in roots and secreted into the rhizosphere via a specific exporter, the OsTOM1, and under Fe deficient conditions, this transporter is expected to be more induced in rice roots and less in rice shoots (Nozoye *et al*., 2011).

However, the expression of OsTOM1 by Bico Branco cultivar was lower under Fe deficiency than under Fe sufficiency, both in shoots and roots (Figure 4.3.1.1A), and in the Nipponbare cultivar this transporter was 3.5 fold more expressed in shoots than in roots, under Fe deficient conditions (Figure 4.3.1.1B).

As showed by Nozoye *et al*. (2011), the expression of OsTOM1 appears to be similar to the genes involved in DMA biosynthesis, being responsible not only for Fe acquisition from the soil, but also for internal Fe transport of DMA to the phloem and xylem. Accordingly, these results (Figure 4.3.1.1) suggest that OsTOM1 is implicated in internal Fe transport, although it seems that in the current conditions this gene was not particularly involved in Fe acquisition. However, in the work referenced above, rice plants were transferred to Fe deficiency medium four weeks after germination, staying in this condition for only 5-7 days, differently from what was done in the current experiment, where plants were maintained under Fe deficiency for three weeks after germination.

When PS are released into the rhizosphere, they chelate with Fe^{3+} and form a complex, that is transported into root cells by transmembrane proteins of the yellow-stripe like (YSL) family (Inoue *et al*., 2009; Lee *et al.*, 2009a). The first characterized YS1 ortholog from rice was *OsYSL15* (Inoue *et al.,* 2009) and it was described to be up-regulated in roots and in reproductive tissues under Fe deficient conditions (Lee *et* al., 2009a). MAs are biosynthesized from methionine through NA (Romheld and Marschner, 1990) and it is hypothesized that OsYSL15 is involved in the transport of Fe(III)-MAs complexes, as its expression in the roots' phloem cells may function in the long distance transport from roots to shoots via phloem (Lee *et al*., 2009a).

Lee *et al*. (2009a) tested the disruption or overexpression of *OsYSL15* in rice and concluded that only the concentration of Fe was affected, and not of Zn, Mn or Cu, showing

that OsYSL15 is an Fe-specific transporter. Since this gene functions as a transporter of Fe(III)-NA or Fe(II)-NA complexes, the obtained higher expression in root tissue (and null in shoots), was expectable for both cultivars (Figure 4.3.1.1).

Moreover, under Fe deficiency, Bico Branco cultivar had almost double the amount expressed in the control (Fe sufficiency), whilst Nipponbare rice plants presented an inverse pattern (Figure 4.3.1.1A vs. 4.3.1.1A), which could indicate that the first cultivar is more susceptible to Fe deficiency than the latter.

As referred above, NA is a chelator of transition metals and plays an important role in long- and short-distance transport of metal cations, including Fe^{2+} and Fe^{3+}, in higher plants (von Wirén *et al*. 1999). 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 plant species including maize, *Arabidopsis*, barley and rice (Higuchi *et al*., 1999*;* Inoue *et al*., 2003*;* Mizuno *et al*., 2003; Klatte *et al*., 2009). In rice, NA is a biosynthetic precursor of PSs and its increase causes an increase in transport of Fe from root to shoot. NA also serves as a transition metal chelator, and although all plants can synthesize it, only grasses convert NA to PSs (Lee *et al*., 2009b; Conte and Walker, 2011). Interestingly, Bico Branco cultivar overexpressed seven and four times more *OsNAS1* and *OsNAS2*, respectively, in response to Fe deficiency, when compared to the Fe sufficiency plants (Figure 4.3.1.1A). This expression could be augmented in order to increase NA synthesis, to consequently produce and secrete increased amounts of MAs and help in Fe uptake, as seen in Inoue *et al*., (2003). *OsNAS1* and *OsNAS2* were also shown to be expressed in the pericycle cells adjacent to the protoxylem and metaxylem I (Inoue *et al*., 2003). These cells participate in Fe long-distance transport, suggesting that NA synthesis is required for xylem loading and also for loading and unloading to the phloem (Schmidke *et al*., 1999). Hence, it is understandable that when in Fe sufficiency, NAS-related genes expression was increased in shoots of Bico Branco cultivar (Figure 4.3.1.1A). As it has been observed with other genes, Nipponbare cultivar presented a pattern of expression that indicates less susceptibility to Fe deficient conditions (Figure 4.3.1.1B). Under Fe deficiency, *OsNAS1* was up-regulated in shoots, but no drastic changes in root expression were observed, and both shoots and roots expressed similar levels of *OsNAS2*, independently of the Fe treatment.

The tolerance of these plants to low Fe availability is thought to increase with the production and secretion of MAs (Bashir *et al*., 2003). However, the Nipponbare cultivar showed less stress signals when compared with the Bico Branco cultivar, as previously seen in chlorophyll content results, where the former didn't show as acute signs of chlorosis as the latter (Figure 4.2.1.2). This corroborates that the Nipponbare cultivar has less susceptibility to low Fe conditions than the Bico Branco cultivar, reducing the need to synthesize PS synthesis related genes.

Another important PS synthesis-related gene is *OsDMAS1***.** This gene participates in DMA biosynthesis in rice and, like the *OsNAS* genes, its expression is expected to be higher in root tissues under Fe deficient conditions (Inoue *et al*., 2003*;* Bashir and Nishizawa *et al*., 2006*).* The pattern of expression in the Bico Branco cultivar is consistent with these reports (Figure 4.3.1.1B), but the Nipponbare cultivar presented lower expression of *OsDMAS1* in roots under Fe deficient conditions (Figure 4.3.1.1B).

More specifically, shoots under Fe deficiency showed increased expression in both rice cultivars (Figure 4.3.1.1). In Mori *et al*. (1991), DMA was quantified and detected in Fe sufficiency shoots and was increased under Fe deficiency, and they proposed that it is possible that the DMA detected in Fe sufficiency rice shoots was translocated from roots in a complex with Fe (Mori *et al*., 1991). They also found that under Fe deficient conditions *OsDMAS1* gene expression was localized in phloem sap, cells that participate in long-distance transport. Therefore this could explain why the levels of expression were higher in shoots than in roots for both cultivars in this study (Figure 4.3.1.1) (Bashir and Nishizawa *et al*., 2006; Bashir *et al*., 2006).

In rice, histochemical analysis of promoter-glucuronidase (GUS) transformants revealed that *OsNAS1*, *OsNAS2*, *OsDMAS1* and *OsNAAT1* share highly similar expression patterns, with significant expression under Fe deficient conditions (Bashir et al., 2006, Inoue et al., 2003), which is consistent with the results here obtained.

More recently, the genes participating in DMA biosynthesis in rice, including *OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1* and *OsYSL15*, have been found to be under the regulation of an Fe-deficiency-inducible bHLH (basic helix–loop–helix) transcription factor, OsIRO2 (Ogo *et al.*, 2006) - specific to graminaceous plants. Ogo *et al*. (2007) studied rice plants overexpressing *OsIRO2* (IRO2-OX) and *OsIRO2* RNAi knockdown lines, and they suggest

that *OsIRO2* regulates the PS-mediated Fe uptake system of rice, but not the Fe^{2+} uptake mechanism.

OsIRO2 gene is described to be overexpressed in both rice roots and shoots under Fe deficient conditions (Ogo *et al*., 2007). Accordingly, in this study, the Bico Branco cultivar, in particular, presented the double and quintuple of induction in roots and shoots respectively, in Fe deficient compared to Fe sufficient conditions (Figure 4.3.1.1A). The Nipponbare cultivar had the same strong induction in Fe deficiency shoots, but in roots almost no differences were detectable (Figure 4.3.1.1B).

Another transcription factor known to be expressed in the roots and shoots under Fe deficient conditions is *OsIDEF1*, which positively regulates the induction of several known Fe uptake and utilization genes in rice, such as *OsYSL2*, *OsYSL15*, *OsIRT1*, *OsIRO2*, *OsNAS1*, *OsNAS2*, *OsNAS3* and *OsDMAS1* (Kobayashi *et al.*, 2007; Kobayashi *et al.*, 2009).

It has also been described that OsIDEF1 senses the cellular Fe status in the first days of exposure to Fe deficiency, but after a few days, this ability is lost (Kobayashi *et al.*, 2009). Results reported here show that three weeks after exposure to Fe deficiency, *OsIDEF1* was down-regulated in roots and shoots of the Bico Branco cultivar (Figure 4.3.1.1A) whereas it did not seem to be affected by the Fe treatments in the Nipponbare cultivar, having an augmented expression in roots. These results suggest that in Bico Branco plants *OsIDEF1* was not expressed since there was a diminished expression under Fe starvation, as seen in the work of Kobayashi *et al.*, (2012); on the other hand, in the Nipponbare cultivar the expression of this transcription factor was similar in both Fe deficiency and sufficiency showing that, independently of the Fe supply, this plant is able to maintain the cellular Fe status (Figure 4.3.1.1B).

Figure 4.3.2.1 integrates the results obtained in the current study for both rice cultivars.

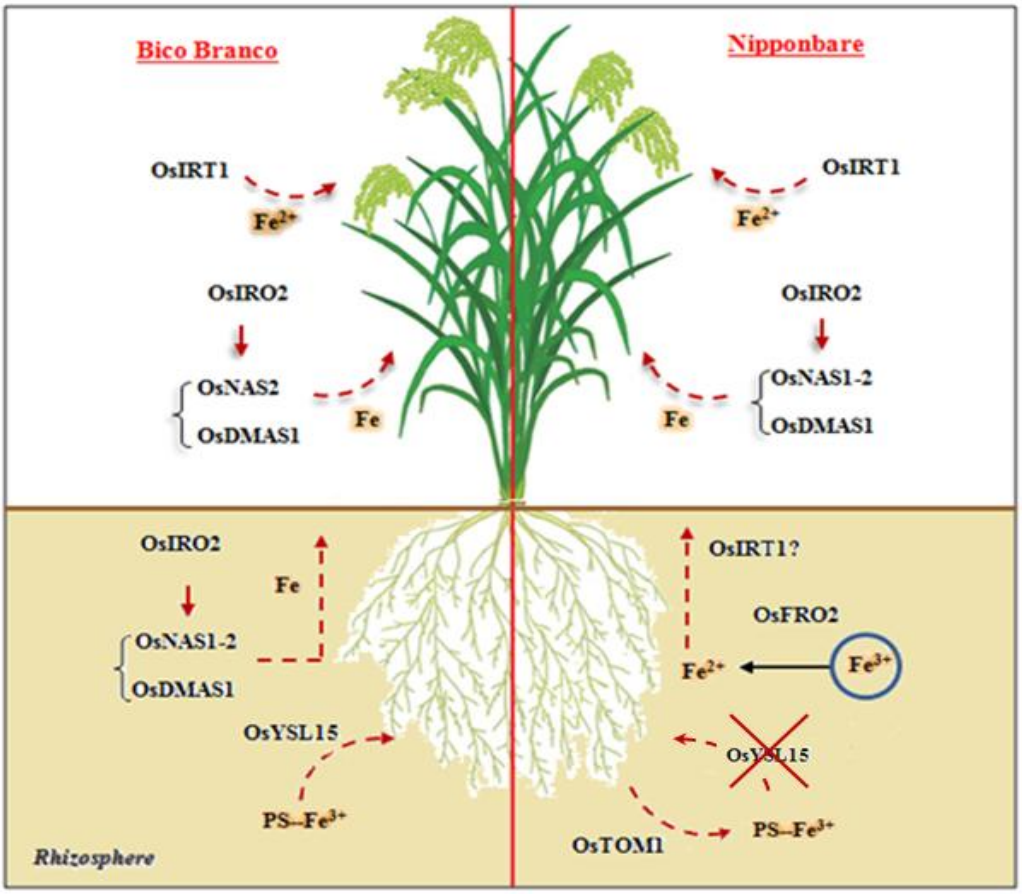

Figure 4.3.2.1 Schematic diagram integrating the expression data of Strategy I and II-related genes in both rice cultivars obtained with quantitative RT-PCR analyses (please see text for further details).

4.3.3 New candidate genes in rice

Although a large number of Fe-deficiency-inducible genes have been isolated, the mechanisms behind direct Fe transport are still little known (Sperotto *et al.*, 2012b). Ferroportin (FPN) is a protein involved in Fe absorption in mammals (Muckenthaler *et al.*, 2008) and, as previously mentioned, has close orthologs in *A. thaliana* - *AtFPN1* and *AtFPN2* (Morrissey *et al.*, 2009). These promising Fe transporters play a role in Fe transport from root

to shoot, and have never been described in rice. Here, we searched for two orthologs from *A. thaliana* in *Oryza sativa* L., *OsFPN1* and *OsFPN2*.

In the Bico Branco cultivar, both genes were up-regulated by Fe deficient roots (Figure 4.3.3.1A). Previous studies have shown that FPN1 is not Fe-regulated but FPN2 is (Colangelo and Guerinot, 2004; Muckenthaler *et al*. 2008). More specifically, AtFPN1 is localized in the plasma membrane and expressed in the stele, effluxing metals from cytoplasm into the vasculature and allowing the movement of metals from root to shoot; AtFPN2 sequesters metals in the outer cell layers of the roots, that are effluxed into the vacuole, especially under Fe deficiency, suggesting that FPN2 could serve to sequester excess free Fe that would otherwise not be chelated or transported out of the cell quickly enough (Schaaf *et al*., 2006). Hence, while both ferroportins likely efflux metal from the cytoplasm, they play different roles in metal homeostasis. The Nipponbare cultivar revealed higher expression in shoots treated in Fe deficient conditions being more pronounced for FPN1 than for FPN2 (Figure 4.3.2.1B), showing that these genes are indeed induced by Fe deficiency.

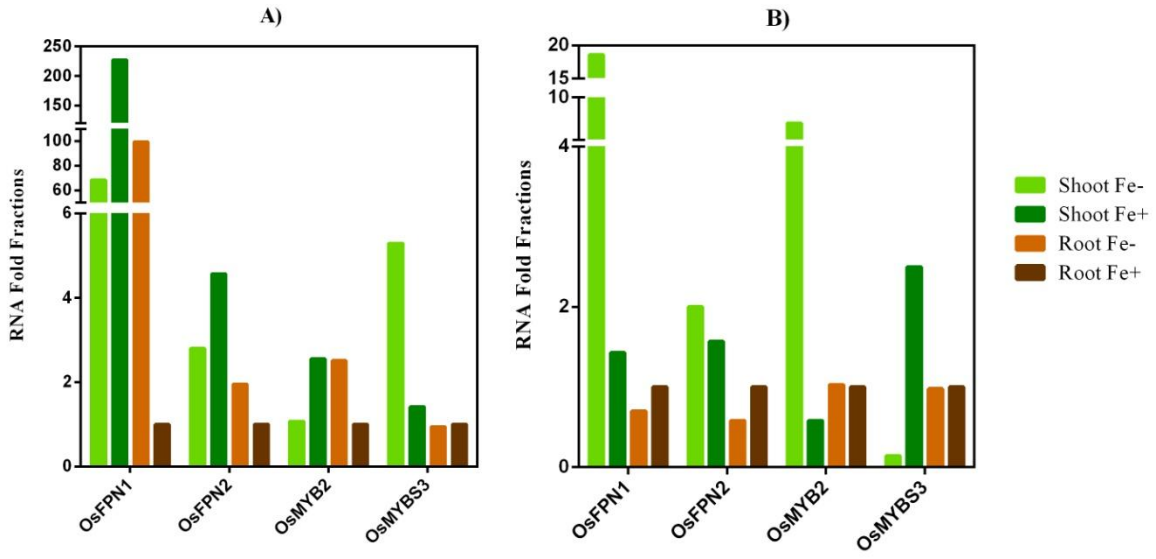

Figure 4.3.3.1. Quantitative RT-PCR analysis of new candidate genes in (A) Bico Branco and (B) Nipponbare cultivars. Total RNA was extracted from shoots and roots of plants grown hydroponically for three weeks under Fe deficient (0 μM Fe(III)-EDDHA) (Fe-) and Fe sufficient (20 μM Fe(III)-EDDHA) (Fe+) conditions. A pool of three plants from each condition was used. The results were normalized using the housekeeping gene 18S (-rRNA).

Morrissey *et al*. (2009) studied chlorophyll and carotenoid levels in *A. thaliana*, and showed that a loss of FPN1 results in chlorosis, also suggesting that FPN1 loads Fe into the vasculature; on the other hand *fpn2* mutant was not chlorotic under Fe sufficiency, but its chlorophyll and carotenoid levels were slightly lower than the wild type on Fe deficient medium. The loss of both ferroportins, however, resulted in a greatly increased Fe deficiency response. In the current work, ferroportins were higher expressed in Nipponbare shoots, which shows less chlorosis and higher photosynthetic pigments concentration under Fe deficiency, which is in accordance with the previous study that suggested a relation between these genes and photosynthetic parameters.

Other genes related with Fe trafficking and allocation in plants but not described in rice yet, are MYB2 and MYBS3 (Chen *et al.*, 2006; Shen *et al.*, 2008). As previously referred in the Introduction section, MYB genes belong to a large family of transcription regulators which have important roles in plants, like regulation of plant development, hormone signaling and metabolism (Lipsick, 1996). Previously, it was shown that the expression of two MYB genes was upregulated by Fe deficiency (Colangelo and Guerinot, 2004), suggesting that MYB genes may play a role in Fe homeostasis. It was reported that *DwMYB2* expression in transgenic *Arabidopsis*, initially isolated from orchid *Dendrobium hybrid Woo Leng*, affected Fe translocation from root and shoot, resulting in Fe accumulation in roots and deficiency in shoots (Chen *et al.*, 2006). Here, we searched for an ortholog of DwMYB2 in *Oryza sativa* L.

As expected, roots under Fe deficient conditions up-regulated *OsMYB2* in the Bico Branco cultivar (Figure 4.3.3.1A). On the other hand, studies show that expression of DwMYB2 in *Arabidopsis* caused development of yellow leaf phenotype, the typical symptom of Fe deficiency, and hypersensitivity to Fe deficiency (Chen *et al.*, 2006). This, alongside with the strong induction of MYB2 under Fe deficient conditions (Figure 4.3.3.1) obtained in this study reveal that MYB2 gene has a role in Fe homeostasis.

Another transcription factor belonging to the MYB genes family is OsMYBS3, homologs of MxMYB1. Although OsMYBS3 was described to acts as a repressor of α-amylase gene expression in sugar starvation (Lu *et al*., 2002), MxMYB1 possibly acts as a negative regulator of Fe uptake in plants (Shen *et al.*, 2008).

Studies with *M. xiaojinensis* could not find the function of MxMYB1 in the regulation of Fe uptake, but evidences from this gene's expression in *A. thaliana* suggest that MYB1 might cooperate with other proteins to modulate Fe homeostasis under Fe deficient conditions (Shen *et al.,* 2008). As MxMYB1 homologs exist in a wide range of monocotyledonous and dicotyledonous plants, OsMYBS3 may also be related with Fe deficiency.

Previous studies showed that OsMYBS3 was expressed in all tissues, with the highest expression in senescent leaves, when sugars are depleted, preventing α-amylase genes from being induced by the low sugar levels, which would be a wasteful process in dying cells (Lu *et al*., 2002). In the current experiment, the higher levels of expression of OsMYBS3 were found in Bico Branco cultivar, especially in shoots under Fe deficiency (Figure 4.3.3.1A), which, as can be seen in Figure 4.2.1.2 showed signs of severe chlorosis and initial senescence.

5. CONCLUSIONS

In the current study, mineral content analysis suggests that Fe supply results in higher Fe concentration in roots and shoots, especially in the Bico Branco cultivar. Overall, under Fe deficiency, Bico Branco cultivar accumulated more minerals in roots than Nipponbare, while Nipponbare counterpart accumulated more nutrients in shoots. The results obtained here support that there is an interaction between minerals, namely, when Fe uptake is decreased the uptake of some other minerals is increased, as showed by Sperotto *et al*. (2012a).

Furthermore, photosynthetic pigments, which can be used as indicators of crop stress under Fe deficiency, were quantified and the results showed that the Bico Branco cultivar is more susceptible to Fe deficiency than the Nipponbare cultivar. However, both cultivars had Fe chlorosis and growth disturbance when grown under Fe deficient conditions, as also shown by Maruyamaa *et al.* (2005).

In what concerns Fe reductase activity, a typical process of Strategy I plants, Nipponbare cultivar revealed the highest reductase activity under Fe deficient conditions. When looking at qRT-PCR results, this cultivar had higher expression of *OsFRO2*, responsible for Fe^{3+} reduction, evidencing the agreement between these results. This is a new, revealing result that shows that rice may have a capacity to reduce Fe. This could be an adaptation of rice when grown under upland rice (aerobic soils), where although Fe^{3+} is abundant, rice does not produce PS in sufficient amounts to cope with its Fe needs. Thus, Bico Branco, which did not show evidences of Strategy I transport system, showed more activity of genes involved in Strategy II for Fe uptake, suggesting that the induction of Strategy I and II genes is cultivar-dependent.

On the other hand, although *OsIRT1* was not expressed in roots of both cultivars under Fe deficiency, there are evidences of Fe^{2+} transport in shoots, suggesting a role in Fe long-distance transport in both rice cultivars, as also shown by Ishimaru *et al*. (2006).

Relatively to the new candidate genes, this study showed that *OsFPN1*, *OsFPN2*, *OsMYB2* and *OsMYBS3* are Fe-regulated, especially in shoots of Nipponbare cultivar and in roots of Bico Branco cultivar. Under Fe deficient conditions, *OsMYBS3* seems to play a role in shoots of Bico Branco cultivar.

These data provide novel insights for the regulation of genes involved in Fe transport in rice and identified potential candidate genes for further investigation. These findings also

showed that there are different responses to Fe deficiency between the studied cultivars that are from different climates. Between Bico Branco and Nipponbare cultivars, the former one (never studied until now) revealed to be an interesting cultivar both for sensing Fe deficiency, and for Fe uptake and transport through the plant.

6. FUTURE WORK

As referred in the Introduction section, rice is a very diverse specie with more than 1500 rice cultivars. Although the Nipponbare genome has already been sequenced, few studies have selected other cultivars to compare the responses. For that reason, in this work we looked at several rice varieties before choosing the most suitable ones for our study.

Although significant progress has been made in recent years in our understanding of how metals are obtained from the soil and distributed throughout the rice plant, there is still some controversy about which Strategy rice uses for Fe uptake. Attempts to insert the *FRO2* gene from *A. thaliana* (*AtFRO2*) into rice (*Oryza sativa* L.) have already been described, although in a different rice cultivar (ssp. *indica* cv. IR68144) (Vasconcelos *et al*., 2004). It would be important to do the same in different rice cultivars, such as Bico Branco cultivar, and also analyze Fe-reductase activity in these plants grown under Fe deficient conditions.

It is not known yet if each rice cultivar uses a different Strategy, or if they have a capacity to choose which strategy is better according with the conditions of the environment in which they grow. Thus, it would be interesting to grow rice cultivars in aerobic or flooded soil conditions, to compare the response of genes involved in Strategy I and II under these conditions. Since the amount of NA excreted by the rhizosphere will dictate, to a large extent, the capacity of the rice plant to survive in Fe limiting soils, it would be interesting to quantify the amount of NA excreted by our two cultivars, and see if that could explain their contrasting efficiencies to IDC.

In what concerns the new candidate genes, it is important to investigate the localization of their expression in rice organs, maybe using the promoter-GUS analysis, to gain a more detailed insight into the physiological roles of each new gene referred here. Also, the *MYB2* and *MYBS3*, as transcription factors, had the capacity to regulate other genes, so it is interesting to know if they have any role in the regulation of genes related with Fe homeostasis in rice.

7. Annexes

Table 3.7.1 Accession orthologs used in qRT-PCR.

Gene	Original Accession Number	Accession orthologs	Maxim score	Total score	Query cover	E value	Identity
OsTOM1	AB016925.1	NM_001186569.1	374	374	47 %	$2e^{-101}$	73 %
OsFPN1	NM_129402.5	NM_001064402.1	96.9	148	35 %	$1e^{-17}$	65 %
OsFPN2	NM_001203288.1	NM_001064403.1	84.2	84.2	23 %	$6e^{-14}$	65 %
OsMYB2	AF485893	FJ940216.1	403	403	22 %	$5e^{-110}$	83 %

Table 3.7.2 Accession numbers and primer sequences used in qRT-PCR analysis.

Gene (Accession Number)	Forward Primer	Reverse Primer
OsFRO2 (AB126085)	ACTTTGGCAAACAAGGGACG	AGGCCGCCATTCTCGTACA
OsIRT1 (AB070226)	TCGAGATAGGCATCGTGGTG	AAGAAGACGAGCACCGACCT
OsYSL15 (AB190923)	TCCCCTAAGAAAGGCTTTGG	GCCTCCCGTGTAGAACCATT
OsNAS1 (AB021746.2)	GCTGCATTTGCGAAGCTAAG	ACAGATGGCATGTTCCTCGT
OsNAS2 (AB023818.1)	TAATCCTGGCTGTGTCTCGC	ACTCGTCGTTGTCCCCTAGA
OsDMAS1 (AB269906)	TCAGGCAGACGCTATGGAAC	GAAGTTGCAGACGCCGATG
OsIRO2 (BR000688)	TCCCCTCCTACCCAGCTAAC	AGAAGATGTCCGCCTCAAGC
OsIDEF1 (BR000654)	GGCCATGACAGTCGTGCTA	CATGTCACTGGGAGCACCAT
OsMYBS3 (AY151044)	TGTCAAGCCTGTTCCAGTTC	TGTGCCCTTGTTGGATT
OsTOM1 (NM_001186569.1)	ATGAGGAAGCTGGTCCCCTC	AATTGAACCAGCGCGACG
OsFPN1 (NM_001064402.1)	CATGTTCGACCTGCTCACCT	TCCAGTTCATCTTGGGCAGG
OsFPN2 (NM_001064403.1)	GCCACTCTTTTCGGTCCCAT	CAACACGACCAATGTTGCGA
OsMYB2 (FJ940216.1)	CCAGCCGTGCGAATTTCAAG	TCTTTGGAGCCCTGCAAGTT
18S	TTAGGCCATGGAGGTTTGAG	GAGTTGATGACACGCGCTTA

8. REFERENCES

- Abadía, J., Morales, F., Abadía, A. 1999. Photosystem II efficiency in low chlorophyll, iron-deficient leaves. *Plant and Soil* **215:** 183-192.
- Abadía, J., Lopez-Millan, A., Rombola, A., Abadía, A. 2002. Organic acids and Fe deficiency: a review, *Plant and Soil* **241**: 75–86.
- Abbott, A.J. 1967. Physiological effects of micronutrient deficiencies in isolated roots of *Lycopersicum esculentum. New Phytologist* **66**: 419-437.
- Agricultural Engineering Unit, IRRI (International Rice Research Institute). 2013. "Rice milling." Available: http://www.knowledgebank.irri.org/ericeproduction/PDF_&_Docs/Teaching_Manual_Rice_Milling.pdf **[**date visited: 10/10/2013].
- ANIA (Associação Nacional dos Industriais de Arroz). 2006. "Balanço 1997/2006." Available: www.ania.pt. [date visited: 03/12/2013].
- Arulanantham, A.R., Rao, IM., Terry, N. 1990. Limiting factors in photosynthesis. VI. Regeneration of ribulose 1,5-bisphosphate limits photosynthesis at low photochemical capacity. *Plant Physiology* **93**: 1466-1475.
- Bashir, K., Nishizawa, N. K. 2006. DMA, a Gene Important for Fe-Acquisition and Homeostasis. *The Journal of Biological Chemistry* **1**(6): 290–292.
- Bashir, K., Inoue, H., Nagasaka, S., Takahash, M., Nakanishi, H., Mori, S., Nishizawa, Naoko K. 2006. Cloning and Characterization of Deoxymugineic Acid Synthase Genes from Graminaceous Plants". *The Journal of Biological Chemistry*. **281**(43): 32395–32402.
- Barry, G. 2013. "Golden Rice and vitamin A deficiency." International Rice Research Institute. Available: http://irri.org/images/stories/Golden_Rice/Golden%20Rice%20Project%20Brief.pdf [date visited: 13/11/2013].
- Belkhodja, R., Morales, F., Quílez, F., López-Millán, A.F., Abadía, A., Abadía, J. 1998. Iron deficiency causes changes in chlorophyll fluorescence due to the reduction in the dark of the Photosystem II acceptor side. *Photosynthesis Research* **56**(3): 265-276).

- Caballero, B. 2002. Global Patterns of Child Health: The Role of Nutrition. *Annals of Nutrition and Metabolism* **46**: 3–7.
- Chalker-Scott L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology*. **70**(1):1–9.
- Chaney, R.L., Chen, Y., Green, C.E., Holden, M.J., Bell, P.F., Luster, D.G., Angle, J.S. 1992. Root hairs on chlorotic tomatoes are an effect of chlorosis rather than part of the adaptive Fe-stress response. *Journal of Plant Nutrition* **15**: 1857-1875.
- Chen, Y., Wu, X., Ling, H., and Yang, W. 2006. Transgenic Expression of DwMYB2 Impairs Iron Transport from Root to Shoot in *Arabidopsis Thaliana*. *Cell Research* **16**(10): 830–40.
- Cheng, L., Wang, F., Shou, H., Huang, F., Zheng, L., He, F., Li, J. *et al*. 2007. Mutation in Nicotianamine Aminotransferase Stimulated the Fe(II) Acquisition System and Led to Iron Accumulation in Rice. *Plant Physiology* **145**(4): 1647–57.
- Cinelli, F., Viti, R., Byrne, D.H., Reed, D.W. 1995. Physiological characterization of two peach seedling rootstocks in bicarbonate nutrient solution. I. Root iron reduction and iron uptake. *Iron Nutrition in Soils and Plants* **59**: 323–328.
- Clark, R.B. 1983. Plant genotype differences in the uptake, translocation, accumulation, and use of mineral elements required for plant growth. *Genetic Aspects of Plant Nutrition* **8**: 49-70.
- Colangelo, E. P., Guerinot, M. L. 2004. The Essential Basic Helix-Loop-Helix Protein FIT1 Is Required for the Iron Deficiency Response. *The Plant Cell* **16**: 3400–3412.
- Colangelo, E.P., Guerinot, M.L. 2006. Put the metal to the petal: metal uptake and transport throughout plants. *Current Opinion in Plant Biology* **9**:322-330.
- Comission of the European Communities. 2002. "The Agricultural Situation in the European Union. Report 2000." Report in conjunction with the General Report on the Activities of the European Union. Available: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2002:0067:FIN:EN:PDF [date visited: 03/12/2013].
- Conte, S. S., Walker E.L. 2011. Transporters Contributing to Iron Trafficking in Plants. *Molecular Plant* 1-13.
- Curie, C., and Briat, J-F. 2003. Iron Transport and Signaling in Plants. *Annual Review of Plant Biology* **54**: 183–206.

- Decree-Law nr. 62/2000. "D.R. I Série A" 93 (19/04/2000) 1689.
- Duan, M., Sun, S.S.M. 2005. Profiling the expression of genes controlling rice grain quality. *Plant Molecular Biology* **59**: 165-178.
- Durrett, T.P., Gassmann, W., Rogers, E.E. 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation, *Plant Physiology* **144**: 197–205.
- Escribano-Bailón, M.T., Santos-Buelga, C., Rivas-Gonzalo, J.C. 2004. Anthocyanins in cereals. *Journal of Cromatografy A* **1054**: 129–141.
- FAO. 2004. "Rice and Human Nutrition." Available: http://www.fao.org/rice2004/en/f-sheet/factsheet3.pdf [date visited: 02/09/2013].
- FAO. 2012. "FAO Statistics." Available: http://faostat3.fao.org/faostat-gateway/go/to/browse/Q/*/E [date visited: 12/10/2013].
- FAO/WHO. 2001. "Human Vitamin and Mineral Requirements." Food and Nutrition Division FAO Rome. Available: http://www.fao.org/docrep/004/y2809e/y2809e00.htm#Contents [date visited: 17/10/2013].
- Fox, T.C., Guerinot, M.L. 1998. Molecular biology of cation transport in plants. *Annual review of plant biology* **49**: 669-696.
- Garris, Amanda J., Tai, Thomas H., Coburn, J., Kresovich, S., McCouch, S. 2005. Genetic Structure and Diversity in Oryza Sativa L. *Genetics* **169**(3): 1631–8.
- Ge, S., Sang, T., Lu, B.R., Hong, D.Y. 1999. Phylogeny of rice genomes with emphasis on origins of allotetraploid species. *Proceedings of the National Academy of Sciences of the United States of Americ* **96**: 14400–14405.
- Gillespie, S., Haddad, L. 2001. Effective food and nutrition policy responses to HIV/AIDS: what we know and what we need to know. *Journal of International Development* **13**(4): 487-511.
- Gómez-Galera, S., Rojas, E., Sudhakar, D., Changfu, Zhu, Pelacho A.M., *et al.* 2010. Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic Research* **19**: 165-180.
- Gould, K.S. 2004. Nature's Swiss Army Knife: The Diverse Protective Roles of Anthocyanins in Leaves. *Journal of Biomedicine and Biotechnology* **5**: 314–320.

- Gould, K.S., Markham, K.R., Smith, R.H., Goris, J.J. 2000. Functional role of anthocyanins in the leaves of *Quintinia serrata* A. Cunn. *Journal of Experimental Botany*. **51**(347):1107–1115.
- Gregorio, G.B., Senadhira, D., Htut, H., Graham, R.D. 2000. Breeding for Trace Mineral Density in Rice. *Food and Nutrition Bulletin* **21**(4): 382–386.
- Gross, J., Stein, R.J., Fett-neto, A.G., Fett, J.P. 2003. Iron Homeostasis Related Genes in Rice. *Genetics and Molecular Biology* **497**: 477–497.
- Grotz, N., and Guerinot, M.L. 2006. Molecular Aspects of Cu, Fe and Zn Homeostasis in Plants. *Biochimica Et Biophysica Acta (BBA – Moelcular Cell Research)* **1763**(7): 595–608.
- Grusak, M.A, Dellapenna, D. 1999. Improving the Nutrient Composition of Plamts to Enhance Human Nutrition and Health. *Annal Review Plant Physiology and Plant Molecular Biology* **50**: 133-61
- Grusak, M.A., Welch, R.M., Kochian, L.V. 1990. Does iron deficiency in *Pisum sativum* enhance the activity of the root plasmalemma iron transport protein? *Plant Physiology*. **94**: 1353–1357.
- Guerinot, M.L., Yi, Y. 1994. Iron: Nutritious, Noxious, and Not Readily Available. *Plant Physiology* **104**(3): 815–820.
- Harborne, J.B. 1993. The flavonoids; Advances in research since 1986. (ed) Chapman and Hall, London.
- Hendry, G.A.F., Price, A.H., 1993. Stress indicators: chlorophylls and carotenoids. In: Hendry, G.A.F., Grime, J.P. (Eds.), Methods in Comparative Plant Ecology. Chapman and Hall, London, pp. 148–152.
- Higuchi, K., Suzuki, K., Nakanishi, H., Yamaguchi, H., Nishizawa, N-K., Mori, S. 1999. Cloning of Nicotianamine Synthase Genes, Novel Genes Involved in the Biosynthesis of Phytosiderophores. *Plant Physiology* **119**(2): 471-480
- Hocking, P.J., Pate, J.S. 1977. Mobilization of minerals to developing seeds of legumes. *Annals of Botany* **41**:1259–1278.
- Hodges, D.M., Nozzolillo, C. 1995. Anthocyanin and Anthocyanoplast Content of Cruciferous Seedlings Subjected to Mineral Nutrient Deficiencies. *Journal of Plant Physiology* **147**(6): 749–754.

- Hurrell, R.F. 1992. Prospects for improving the iron fortification of foods. *Nestle nutrition workshop series* **30**: 193–208.
- Hurrell, R.F., Juillerat, M., Reddy, M.B., Lynch S.R., Dassenko, S.A., Cook, J.D. 1992. Soy Protein, and Iron Absorption in Humans. *The Amercian Journal Society of Chlinical Nutrition* **56**: 573-8.
- Inoue, H., Higuchi, K., Takahashi, M., Nakanishi, H., Mori, S., Nishizawa, Naoko K.. 2003. Three Rice Nicotianamine Synthase Genes, OsNAS1, OsNAS2, and OsNAS3 Are Expressed in Cells Involved in Long-distance Transport of Iron and Differentially Regulated by Iron. *The Plant Journal* **36**(3): 366–381.
- Inoue, H., Kobayashi, T., Nozoye, T., Takahashi, M., Kakei, Y., Suzuki, K., Nakazono, M., Nakanishi, H., Mori, S., Nishizawa, N.K. 2009. Rice OsYSL15 Is an Iron-regulated Iron(III)-deoxymugineic Acid Transporter Expressed in the Roots and Is Essential for Iron Uptake in Early Growth of the Seedlings. *The Journal of Biological Chemistry* **284**(6): 3470–9.
- IRRIa (International Rice Research Institute). 2009. "Rice growing environments." Available: http://www.knowledgebank.irri.org/submergedsoils/rice-growing-environments/lesson-1.html [date visited: 5/10/2013].
- IRRIb (International Rice Research Institute). 2009. "Rice basics". Available: http://irri.org/index.php?option=com_k2&view=item&layout=item&id=9081&lang=en [date visited: 5/10/2013].
- IRRIa (International Rice Research Institute). 2013. "Rice production and processing." Available: http://irri.org/index.php?option=com_k2&view=item&id=9151&Itemid=100480&lang=en [date visited: 11/09/2013].
- IRRIb (International Rice Research Institute). 2013. "What is Indica rice?" Available: http://www.irri.org/index.php?option=com_k2&view=item&id=12091&lang=en [date visited: 12/10/2013].
- Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., Wada, Y., *et al*. 2006. Rice Plants Take up Iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *The Plant Journal* **45**(3): 335–46.
- Jeong, J., Connolly, E.L. 2009. Iron Uptake Mechanisms in Plants: Functions of the FRO Family of Ferric Reductases. *Plant Science* **176**(6): 709–714.

- Jeong, J., Guerinot, M.L. 2009. Homing in on Iron Homeostasis in Plants. *Trends in Plant Science* **14**(5): 280–5.
- Juszczuk, I.M., Wiktorowska, A., Malusa, E. et al. 2004. Changes in the concentration of phenolic compounds and exudation induced by phosphate deficiency in bean plants (*Phaseolus vulgaris L*.). *Plant and Soil* **267**:41–49.
- Kato, S., Kosaka, H., Hara, S. 1928. On the affinity of rice varieties as shown by the fertility of rice plants. *Central Agricultural Institute of Kyushu Imperial University* **2**: 241-276.
- Khush, G.S. 1997. Origin, dispersal, cultivation and variation of rice. *Plant Molecular Biology* **35**: 25-34.
- Klatte, M., Schuler, M., Wirtz, M., Fink-Straube, C., Hell, R., Bauer, P. 2009. The Analysis of Arabidopsis Nicotianamine Synthase Mutants Reveals Functions for Nicotianamine in Seed Iron Loading and Iron Deficiency Responses. *Plant Physiology* **150**(1): 257-271.
- Kobayashi, T., Itai, R.N., Aung, M.S., Senoura, T., Nakanishi, H., Nishizawa, Naoko K. 2012. The Rice Transcription Factor IDEF1 Directly Binds to Iron and Other Divalent Metals for Sensing Cellular Iron Status. *The Plant Journal : for Cell and Molecular Biology* **69**(1): 81–91.
- Kobayashi, T., Itai, R.K., Ogo, Y., Kakei, Y., Nakanishi, H., Takahashi, M. and Nishizawa, Naoko K. 2009. The Rice Transcription Factor IDEF1 Is Essential for the Early Response to Iron Deficiency, and Induces Vegetative Expression of Late Embryogenesis Abundant Genes. *The Plant Journal* **60**(6): 948–61.
- Kobayashi, T., Ogo, Y., Itai, R.N., Nakanishi, H., Takahashi, M., Mori, S. and Nishizawa, N.K. 2007. The novel transcription factor IDEF1 regulates the response to and tolerance of iron deficiency in plants. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 19150–19155.
- Kochian, U., Lucas, W.J. 1991. Do plasmalemma oxidoreductases play a role in plant mineral ion transport? In: Crane FU, Morre DJ, Low HE, eds. Oxidoreduction at the plasma membrane: relation to growth and transport. Madison, Wiconsin, USA: Soil Science Society of America, 189–205.
- Krämer, Ute, Talke, Ina N., Hanikenne, Marc. 2007. Transition Metal Transport. *Federation of European Biochemical Societies (FEBS) Letters* **581**(12): 2263–72.

- Lains, P. 2003. "Portugal's growth paradox, 1870-1950." Universidade do Porto, Faculdade de Economia do Porto. *Working Paper* no. 135.
- Lee, S., Chiecko, J.C., Kim, S.A., Walker, E.L., Lee, Y., Guerinot, M.L., An, G. 2009a. Disruption of OsYSL15 Leads to Iron Inefficiency in Rice Plants. *Plant Physiology* **150**: 786–800.
- Lee, S., Jeon, U.S., Lee, S.J., Kim, Yoon-Keun, Persson, D.P., Husted, S., Schjørring, J.K. *et al*. 2009b. Iron Fortification of Rice Seeds Through Activation of the Nicotianamine Synthase Gene. *Proceedings of the National Academy of Sciences of the United States of America* **106**(51): 22014–9.
- Lipsick, J.S. 1996. One billion years of MYB. *Oncogene* **13**: 223–235.
- Livak, K.J., Schmittgen, T.B. 2001 Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ Method. *Methods* **25**: 402–408-
- López-Millán, A-F., Ellis, D.R., Grusak, M.A. 2004. Identification and characterization of several new members of the ZIP family of metal transporters in Medicago truncatula. *Plant Molecular Biology* **54**:583–596.
- Londo, J.P., Chiang, Y., Hung, K., Chiang, T., Schaal. B.A. 2006. Phylogeography of Asian Wild Rice, Oryza Rufipogon, Reveals Multiple Independent Domestications of Cultivated Rice, Oryza Sativa. *Proceedings of the National Academy of Sciences of the United States of America* **103**(25): 9578–83.
- Lu, C., Ho, T.D., Ho, S., Yu. S. 2002. Three Novel MYB Proteins with One DNA Binding Repeat Mediate Sugar and Hormone Regulation of α-Amylase Gene Expression. *The Plant Cell* **14**: 1963–1980.
- Maclean, J.L., Dawe, D.C., Hardy, B, Hettel, G.P. 2002. Importance of rice. Third Edition. In: Rice Almanac. CABI Publishing, Philippines. pp. 1-9. Available: http://books.irri.org/9789712201721_content.pdf [date visited: 22/11/2013].
- Marschner, H. 1995. Mineral Nutrition of Higher Plants, 2nd edn. Academic, London.
- Maruyamaa, T., Higuchia, K., Yoshidaa, M., Tadano, T. 2005. Comparison of Iron Availability in Leaves of Barley and Rice. *Soil Science and Plant Nutrition* **51**(7): 1035-1042.
- Masuda, H., Ishimaru, Y., Aung, M.S., Kobayashi, T., Kakei, Y., Takahashi, M., Higuchi, K., Nakanishi, H., Nishizawa, Naoko K. 2012. Iron Biofortification in Rice

by the Introduction of Multiple Genes Involved in Iron Nutrition. *Scientific Reports* **2**: 543.

- MADRP (Ministério da Agricultura do desenvolvimento rural e das pescas). 2002. Cereais em Portugal no Século XX (Instrumentos de Pesquisa para a História dos Organismos Responsáveis pela Política Cerealífera em Portugal no Século XX). Lisboa.
- Mizuno, D., Higuchi, K., Sakamoto, T., Nakanishi, H., Mori S., Nishizawa, N.K. 2003. Three Nicotianamine Synthase Genes Isolated from Maize Are Differentially Regulated by Iron Nutritional Status. *Plant Physiology* **132**(4): 1989-1997.
- Mori, S. 1999. Iron acquisition by plants. *Curr. Opin. Plant Biol.* **2**: 250–253.
- Mori, S., Nishizawa, N.K., Hayashi, H., Chino, M., Yoshimura, E., Ishihara, J. 1991. Why are young rice plants highly susceptible to iron deficiency? *Iron Nutrition and Interactions in Plants* **130**: 143–156.
- Morrissey, J., Baxter, I. R., Lee, J., Li, L., Lahner, B., Grotz, N., Kaplan, J., Salt, D.E., and Guerinot, M.L. 2009. The Ferroportin Metal Efflux Proteins Function in Iron and Cobalt Homeostasis in *Arabidopsis*. *The Plant Cell* **21**(10): 3326–38.
- Muckenthaler, M.U., Galy, B., Hentzem M.W. 2008. Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network. *Annual Review of Nutrition* **28**: 197-213.
- Mukherjee, I., Campbell, N. H., Ash, J.S., Connolly, E.L.. 2006. Expression Profiling of the Arabidopsis Ferric Chelate Reductase (FRO) Gene Family Reveals Differential Regulation by Iron and Copper. *Planta* **223**(6): 1178–90.
- Neill, S.O.. 2002. The Functional Role of Anthocyanins in Leaves [PhD of Biological Sciences]. New Zeland, 196pp. University of Auckland. Available: https://researchspace.auckland.ac.nz/handle/2292/497 [date visited: 12/11/2013].
- Nishio, J.N., Taylor, S.E., Terry, N. 1985. Changes in thylakoid galactolipids and proteins during iron nutrition-mediated chloroplast development. *Plant Physiology* **77**: 705-711.
- Nozoye, T., Nagasaka, S., Kobayashi, T., Takahashi, M., Sato, Y., Sato, Y., Uozumi, N., Nakanishi, H., Nishizawa, Naoko K. 2011. Phytosiderophore Efflux Transporters Are Crucial for Iron Acquisition in Graminaceous Plants. *The Journal of Biological Chemistry* **286**(7): 5446–54.

- Ogo, Y., Itai, R.N., Nakanishi, H., Inoue, H., Kobayashi, T., Suzuki, M., Takahashi, M., Mori, S., Nishizawa, Naoko K. 2006. Isolation and Characterization of IRO2, a Novel Iron-regulated bHLH Transcription Factor in Graminaceous Plants. *Journal of Experimental Botany* **57**(11) : 2867–78.
- Ogo, Y., Itai, R.N., Nakanishi, H., Kobayashi, T., Takahashi, M., Mori, S., Nishizawa, Naoko K. 2007. The Rice bHLH Protein OsIRO2 Is an Essential Regulator of the Genes Involved in Fe Uptake Under Fe-deficient Conditions. *The Plant Journal : for Cell and Molecular Biology* **51**(3): 366–77.
- Ohnishi, T., Yoshino, M., Yamakawa, H., Kinoshita. T., 2011. The Biotron Breeding System: a Rapid and Reliable Procedure for Genetic Studies and Breeding in Rice. *Plant & Cell Physiology* **52(**7): 1249–57.
- Pacheco Dias, M.F., Nunes da Rocha, A.M.; Nunes da Rocha. 2012. "Evolução das estratégias de investigação do arroz no INIA - Portugal. *Revista De Ciências Agrárias* **35**: 69–87.
- Palmer, C.M., Guerinot, Mary Lou. 2009. Facing the Challenges of Cu, Fe and Zn Homeostasis in Plants. *Nature Chemical Biology* **5(**5): 333–40.
- Panzone, L., Malta, M., Simões, O., Baldi, L. 2009. Country of origin bias and firm's international procurement: A case study on the rice sector of Portugal. *Economia Global e Gestão* **14**(1): 81-95.
- Pascual-Teresa, S.,Sanchez-Ballesta, M.T. 2008. Anthocyanins: from plant to health. *Photochemistry Reviews*. **7**: 281–299.
- Puig, S., Andrés-Colás, N., García-Molina, A., Peñarrubia, L. 2007. Copper and Iron Homeostasis in Arabidopsis: Responses to Metal Deficiencies, Interactions and Biotechnological Applications. *Plant, Cell & Environment* **30**(3): 271–90.
- Pushnika, J.C., Miller, G.W., Manwaringa, J.H. 1984. The role of iron in higher plant chlorophyll biosynthesis, maintenance and chloroplast biogenesis *Journal of Plant Nutrition* **7:** 733-758.
- Ramani, S., Kannan, S. 1987. Manganese absorption and transport in rice. *Physiologia Plantarum* **33**:133–137.
- Robinson, N.J., Procter, C.M., Connolly, E.L., Guerinot M.L. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature* **397**: 694-697.

- Romera, F.J., Alcántara, E., De la Guardia, M.D. 1992. The role of roots and shots in the regulation of the Fe deficiency responses in sunflower and cucumber. P*hysiologia Plantarum* **85**:141–146.
- Romheld, V. 1987. Different strategies for iron acquisition in higher plants. *Physiologia Plantarum* **70**:231-234.
- Römheld, V., Marschner, H. 1986. Mobilization of iron in the rhizosphere of different plant species. *Advances in Plant Nutrition*. **2**:155–204.
- Romheld V., Marschner, H. 1990. Genotypical Differences Among Graminaceous Species in Release of Phytosiderophores and Uptake of Iron Phytosiderophores. *Plant and Soil* **123**: 147–153.
- Romheld, V., Marschner, H. 1981. Rhythmic iron stress reactions in sunflower at suboptimal iron supply. *Physiologia Plantarum* **53**: 347-353.
- SACN (Scientific Advisory Committee on Nutrition). 2010. *Iron and Health 2010*. London: The Stationery Office.
- Sautter, C., Poletti, S., Zhang, P., Gruissem, W.. 2007. Biofortification of Essential Nutritional Compounds and Trace Elements in Rice and Cassava. *Proceedings of the Nutrition Society* **65**(02): 153–159.
- Schaaf, G., Honsbein, A., Meda, A.R., Kirchner, S., Wipf, D., and vonWiren, N. 2006. AtIREG2 encodes a tonoplast transport protein involved in iron-dependent nickel detoxification in *Arabidopsis thaliana* roots. *Journal of Biological Chemistry* **281**: 25532–25540.
- Shen, J., Xu, X., Li, T., Cao, D., Han. Z., 2008. An MYB Transcription Factor from Malus Xiaojinensis Has a Potential Role in Iron Nutrition. *Journal of Integrative Plant Biology* **50**(10): 1300–6.
- Silveira, V.C., Oliveira, A.P., Sperotto, R.A., Espindola, L.S., Amaral, L., Dias, J.F., Cunha, J.B., Fett, J.P. 2007. Influence of iron on mineral status of two rice (*Oryza sativa L.*) cultivars. *Brazilian Journal of Plant Physiology* **19**:127–139.
- Sims, D.A., Gamon, J.A. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment* **81**: 337– 354.

- Sperotto, R.A., Vasconcelos, M.W., Grusak, M.A., Fett, J.P. 2012a. Effects of Different Fe Supplies on Mineral Partitioning and Remobilization During the Reproductive Development of Rice (*Oryza Sativa* L.). *Rice* **5**(1): 27.
- Sperotto, R.A., Ricachenevsky, F.K., Waldow,V.A., Fett, J.P. 2012b. Iron Biofortification in Rice: It's a Long Way to the Top. *Plant Science : an International Journal of Experimental Plant Biology* **190**: 24–39.
- Spiller, S., Terry, N. 1980. Limiting factors in photosynthesis. 11. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. *Plant Physiology* **65**: 121-125.
- Stracke, R., Werber, M., Weisshaar, B. 2001. The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **4**(5): 447–456.
- Terry, N. 1980. Limiting factors in photosynthesis. I. Use of iron stress to control photochemical capacity in vivo. *Plant Physiology* **65**: 114-120.
- Theil, E.C. 2004. Iron, ferritin, and nutrition. *Annual Review of Nutrition*. **24**: 327-343.
- Vasconcelos, M.W., Musetti, V., Li, C-H., Datta, S.K., Grusak, S.K. 2004. Functional analysis of transgenic rice (*Oryza sativa* L.) transformed with an *Arabidopsis thaliana* ferric reductase (AtFRO2). *Soil Science and Plant Nutrition* **50**(7): 1151-1157.
- Vasconcelos, M.V., Ekert, H., Arahana, V., Graef, G., Grusak, M.A., Clemente, T. 2006. Molecular and phenotypic characterization of transgenic soybean expressing in the *Arabidopsis thaliana* ferric chelate reductase gene, *FRO2*. *Planta* **224**: 1116–1128.
- Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M.L., Briat, J., Curie, C. 2002. IRT1, an Arabidopsis Transporter Essential for Iron Uptake from the Soil and for Plant Growth. *The Plant Cell* **14**: 1223–1233.
- Vianna e Silva, M. 1975. "A Cultura do Arroz." Clássica Editora, Lisboa
- von Wirén, N., Klair, S., Bansal, S., Briat, J-F., Khodr, H., Shioiri, T.,. Leigh, R.A. and Hider, R.C. 1999. Nicotianamine Chelates Both Fe^{III} and Fe^{II}. Implications for Metal Transport in Plants. *Plant Physiology* **119**: 1107–1114.
- Walker, E.L., Connolly, E.L. 2008. Time to Pump Iron: Iron-deficiency-signaling Mechanisms of Higher Plants. *Current Opinion in Plant Biology* **11**(5): 530–5.

- Walker, E.L., Waters, B.M. 2011. The Role of Transition Metal Homeostasis in Plant Seed Development. *Current Opinion in Plant Biology* **14**(3): 318–24.
- Welch, R.M., Graham, D. 2004. Breeding for Micronutrients in Staple Food Crops from a Human Nutrition Perspective. *Journal of Experimental Botany* **55** (396): 353–64.
- Welch, R. M., Shuman, L. 1995. Nutrition of Plants. *Plant Sciences* **14**(1): 49-82.
- White, P.J., Broadley, M.R. 2005. Biofortifying crops with essential mineral elements. *Trends in Plant Science* **10**: 586–593.
- White, P.J., Broadley, M.R. 2009. Biofortification of Crops with Seven Mineral Elements Often Lacking in Human Diets--iron, Zinc, Copper, Calcium, Magnesium, Selenium and Iodine. *The New Phytologist* **182**(1): 49–84.
- WHO (World Health Organization). 2005. "Worldwide Prevalence of Anaemia." Available: http://whqlibdoc.who.int/publications/2008/9789241596657_eng.pdf [date visited:18/10/2013].
- WHO (World Health Organization). 2013. "Micronutrient deficiencies." Available: http://www.who.int/nutrition/topics/ida/en/index.html [date visited: 05/09/2013].
- Wu, H., Li, L., Du, J., Yuan, Y., Cheng, X., Ling, H. 2005. Molecular and Biochemical Characterization of the Fe(III) Chelate Reductase Gene Family in *Arabidopsis thaliana*. *Plant and Cell Physiology* **46**:(9): 1505–14.
- Wu, J., Wang, C., Zheng, L., Wang, L., Chen, Y., *et al*., 2001. Ethylene is involved in the regulation of iron homeostasis by regulating the expression of iron-acquisition-related genes in *Oryza sativa*. *Journal of Experimental Botany* **6**(2): 667–674.
- Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiqiang, L., *et al*. 2006. The MYB Transcription Factor Superfamily of *Arabidopsis*: Expression Analysis and Phylogenetic Comparison with the Rice MYB Family. *Plant Molecular Biology* **60**(1): 107–24.
- Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L. 2012 Primer-BLAST a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**:134.
- Yi, Y., Guerinot, M.L. 1996. Genetic Evidence That Induction of Root Fe(III). *The Plant Journal* **10**(5): 835–844.

- Zaharieva, T., Romheld, V. 2001. Specific Fe2+ uptake system in strategy I plants inducible under Fe deficiency. *Journal of Plant Nutrition* **23**: 1733–1744.
- Zhang, F.S., Römheld, V., Marschner, H. 1991. Diurnal rhythm of release of phytosiderophores and uptake rate of zinc in iron-deficient wheat. *Soil Science and Plant Nutrition* **37**:671–678.
- Zhao, F., Shewry, P.R. 2011. Recent developments in modifying crops and agronomic practice to improve human health. *Food Policy* **36**: S94–S101.
- Zheng, L., Narsai, R., Wu, J., Giraud, E., He, F., *et* al. 2009. Physiological and Transcriptome Analysis of Iron and Phosphorus Interaction in Rice Seedlings. *Plant Physiology* **151**: 262-274.
- Zimmermann, M.B., Hurrell, R.F. 2007. Nutritional Iron Deficiency. *The Lancet* **370:** 511-520.